The Journal of Applied Pharmacology, **12**, 74-78(2004)

Inhibition of GLUT-1 Expressed in *Xenopus laevis* Oocytes by Acetoxyscirpendiol of *Paecilomyces tenuipes*

Dong-Hee LEE and Ha Won KIM

Department of Life Science, The University of Seoul, 90 Jeonnong-Dong, Seoul 130-743, Korea

(Received June 7, 2004; Accepted June 25, 2004)

Abstract – *Paecilomyces tenuipes,* a caterpillar fungus, contains many health-promoting ingredients. Recent reports indicate that consumption of *P. tenuipes* helps reducing blood sugar content for diabetes. Mechanism for reduction in the circulatory sugar content, however, still remains least understood. Methanolic extraction of *P. tenuipes* (MPT) was prepared and acetoxyscirpendiol (ASD) was subsequently purified from MPT. Glucose transporter-1 (GLUT-1) was expressed in the *Xenopus* oocytes and the effect of MPT or ASD on the expressed GLUT-1 was analyzed according to the uptake of 2-dideoxy-D-glucose (2-DOG). MPT was shown to inhibit GLUT-1 activity significantly compared to the non-treated control. In the presence of ASD and its derivatives, GLUT-1 activity was greatly inhibited in a dose-dependent manner. Among ASD and its derivatives, AS-1 showed most significant inhibition. Taken together, these results strongly indicate that ASD in *P. tenuipes* may serve as a functional substance in lowering blood sugar in the circulatory system. ASD and its derivatives can be utilized as inhibitors of GLUT-1.

Keywords □ GLUT-1, Acetoxyscirpendiol, *Xenopus* oocytes, Cordyceps, Diabetes

INTRODUCTION

Cordyceps, or vegetative worm, is believed to contain many health-promoting ingredients. Among numerous effects exerted by cordyceps, reducing the blood sugar is considered as one of the most prominent efficacies (Kikuchi *et al.*, 2004). No significant understanding, however, has been accomplished about the physiological mechanism leading to lowering blood sugar in the circulation. The key to understanding the potential mechanism, in lowering blood sugar, requires analysis of functional glucose transport.

Glucose is transported by two classes of specific transporting machinery (Baldwin, 1993). Glucose transporters are integral membrane proteins that mediate the transport of glucose and structurally-related substances across cellular membranes (Doege *et al.*, 2000). At least seven different glucose transporters have been identified: the facilitated-diffusion glucose transporter family (GLUT family), also known as 'uniporters', and the sodium-dependent glucose transporter family (SGLT family), also known as 'cotransporters' or 'symporters' (Kirwan *et al.*, 2003). The GLUT gene encodes a protein that is involved in the active transport of glucose and galactose into eukaryotic and some prokaryotic cells. GLUT-1 work to drive sugar across the membrane of erythrocytes, blood tissue barrier along the concentration gradient (Rumsey *et al.*, 2003). Between the two glucose transport systems, blood glucose level largely depends on GLUT, since GLUT helps glucose rapidly diffuse along the concentration gradients although final glucose concentration is governed by SGLTs (Bell *et al.*, 1993).

Methanolic extraction was prepared from the fruit bodies of a class of cordyceps called *P. tenuipes* and the extract was named MPT in this study. Candidate components were prepared from *Paecilomycetes tenuipes* by methanol extraction method as described in Nam *et al.* (2002). Among many candidates, acetoxyscirpendiol (ASD) was subsequently purified from MPT. Based upon its chemical structure (4 beta-acetoxyscirpene-3 alpha, 15-diol), ASD belongs to a class of trichothecene. This study investigates the effect of ASD on the activity of GLUT since inhibition on GLUT may help alleviating a surge in blood glucose concentration. Following the heterologous expression of human GLUT-1, oocytes were subjected to influx studies in the presence of MPT or ASD. MPT was shown to contain functional components in inhibiting GLUT-1 expressed in the oocytes. Subsequently, ASD was recognized

*Corresponding author
Tel: 02-2210-2480, Fax: 02-2210-2888
E-mail: hwkim@uos.ac.kr

as one of the actual ingredients with the inhibitory effect. Other chemical analogues of ASD were also confirmed to have similar inhibitory effects on GLUT activity.

MATERIALS AND METHODS

Microinjection of GLUT-1 cRNA into oocytes

The cRNAs of GLUT-1 were synthesized from pSP6-GLUT-1 harboring cloned respective human cDNA by T7 polymerase according to the manufacturer's protocol (Promega, WI, USA). The cRNA was microinjected into *Xenopus* oocytes as described in Lee *et al.* (1995, 1998). An ovary was manually excised from an adult *Xenopus*. Microinjection was performed as follows: The ovary was copiously washed in the Barth solution [5 mM KOH, 100 mM NaOH, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 100 mM methanesulfonic acid, and 10 mM HEPES (pH 7.4)] and incubated at 14°C until injection. Stage V-VI oocytes were manually defolliculated and injected with 50 nl of injection mixture containing cRNA and incubated in the Barth's solution at 14°C for 24 h before uptake assays. ASD analogs were purchased from Sigma Chemicals. 15-Acetoxyscirpendiol (Natural Mycotoxin Registry Number: 2623-22-5) was named AS-1 while diacetoxyscirpenol (Natural Mycotoxin Registry Number: 2270-40-8) refers to AS-2.

Assay of expressed GLUT-1

GLUT-1 was expressed in *Xenopus* oocytes. Following expression, both proteins were extracted in phosphate buffered saline (PBS) with 0.2% mercaptoethanol using a Dounce homogenizer. The transmembranal feature of the expressed GLUT-1 was assayed using surface biotinylation. Injected oocytes were subjected to surface biotinylation and subsequently to precipitation by avidin-conjugated agarose. GLUT-1 was detected by Western Blotting using antisera against each protein. The precipitated proteins were also immunoprecipitated by antisera against GLUT-1.

^{3}H-dideoxy-D-glucose (2-DOG) uptake comparison and inhibition by ASD

GLUT-1's transport was measured using tritium-labeled 2-dideoxy-D-glucose (2-DOG), a non-metabolized model substrate. ASD inhibition of 2-DOG uptake was assayed by incubating 5 oocytes in 2 mM ^{3}H-2-DOG with ASD concentrations ranging 0 to 50 mM in 1 ml of OR2 solution. After 10 minute incubation, oocytes were thoroughly washed with cold OR2 solution. Glucose entry was analyzed using ^{3}H -2-DOG (2 μCi/0.5 ml) under 30 min influx period. The influx of 2-DOG was initiated by incubating five oocytes in 1 ml of Barth's solution containing 2.5 mCi of ^{3}H 2-DOG and cold 2-DOG at concentrations of 1 to 50 mM and at a constant osmolarity of 179.1 mOsm/L achieved by adding 1 M sucrose solution. Oocytes were removed to a scintillation vial containing 0.5 ml of Barth's solution. After 2 minutes, oocytes were transferred to another scintillation vial. Five hundred microliter of 0.1% SDS was added to both vials and mixed by vortex. NEN scintillation cocktail (DuPont NEN, Boston, MA, 5 ml) was added before counting.

Kinetic analysis and inhibition by ASD and its derivatives

To obtain Michaelis-Menten kinetics, oocytes were incubated with five different 2-DOG concentrations (5, 15, 30, 60, 120 mM) for 10 h. Oocytes were then subjected to zero trans and equilibrium assays (Due *et al.*, 1995). Zero trans influx was analyzed using ^{3}H-2-DOG (2 mCi/0.5 ml) under 30 min influx period. The influx of 2-DOG was initiated by incubating five oocytes in 1 ml of Barth's solution containing 2.5 mCi of ^{3}H-2-DOG and cold 2-DOG at concentrations of 1 to 50 mM, at a constant osmolarity of 179.1 mOsm/L achieved by adding 1 M sucrose solution. Oocytes were removed to a scintillation vial containing 0.5 ml of Barth's solution. Control experiments were undertaken on oocytes injected with water under respective conditions and the control transport rates were subtracted from transport rates by oocyte-expressing GLUT-1. Equilibrium exchange influx of 3-OMG into *X. laevis* oocytes was performed after overnight incubation at 18°C in 1 ml of Barth's solution containing cold 2-DOG at concentrations of 1 to 50 mM. Osmolarity was also maintained at 179.1 mOsm/L by adding 1 M sucrose. *Km* and *Vmax* values were calculated using the GRAPHPAD PRISM™ software (GraphPad Software, San Diego, USA).

RESULTS

This study assayed whether ASD, purified from *P. tenuipes*, and its derivatives serve as the functional ingredient in lessening blood sugar surge. The extent of inhibition by ASD and its derivatives were measured in the *X. laevis* oocyte system expressing GLUT-1.

Significant amounts of the GLUT-1 *Xenopus* oocyte membranes expressed. Following injection of cRNA for human GLUT-1, expressed proteins were detected by immunoblotting (Fig. 1). The detected molecular mass corresponds to the

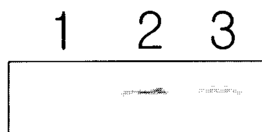

Fig. 1. Expression of GLUT-1 in *Xenopus* oocytes. Membrane fractions were prepared from oocytes injected with cRNA for human GLUT-1. Proteins were isolated and detected by immunoblotting. Lane 1 refers to water injected oocyte. Lane 2 refers to the proteins that are detected by anti-GLUT antisera while lane 3 refers to the biotinylated protein that are further detected by anti-GLUT antisera.

known sizes for GLUT-1. The two proteins were neither detected in oocytes injected with water. As expected, GLUT-1 was detected in the pool of biotinylated proteins.

Fig. 2 shows that microinjection of *in vitro* transcribed human GLUT-1 mRNA into *Xenopus* oocytes resulted in a substantial increase, compared to water injected oocytes in the rate of 2-DOG transport at a substrate concentration of 100 mM. Saponin is known as a polyphenol causing nonspecific membrane-perturbation and caused significant influx of 2-DOG at high concentration. Similar as saponin, the oocyte-expressed GLUT-1 showed significant increase in uptake of 2-DOG. In terms of mRNA amount of injection, difference was evident in the degree of uptake. Indeed oocytes injected with 10 ng show higher uptake activity in comparison to those with 5 ng. These results are consistent with those of previous studies

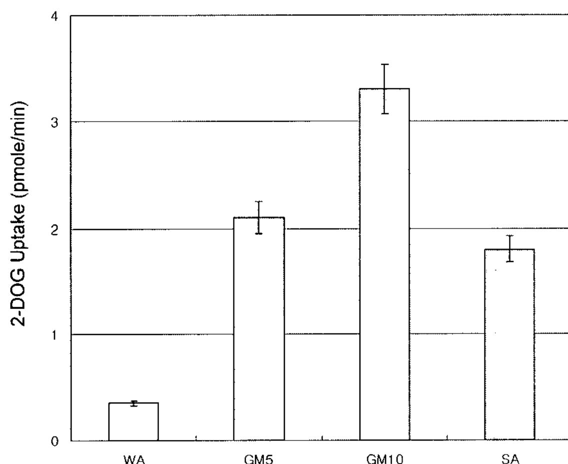

Fig. 2. Comparison of 2-DOG uptake by oocytes. 2-DOG uptake efficiency was compared among oocytes that were injected with water (WA), GLUT-1 message (GM), and saponin (SA). GM5 refers to oocyte injected with 5 ng of GLUT-1 cRNA while GM10 with 10 ng. Following injection, oocytes were incubated with 5 mM 2-DOG for 6 hours. Entry of 2-DOG within oocytes was measured following copious washing.

that the amount of mRNA injected into oocytes correlates to glucose transporter protein expression and affect transport rates of GLUTs (Due *et al.*, 1995).

The effect of ASD on GLUT-1 was measured in the presence of 0.5 mM 2-DOG (Fig. 3). At the fixed concentration of 2-DOG, GLUT-1 activity was significantly reduced. When a fractional uptake rate (v/v_0 = inhibited/non-inhibited uptake rate) was calculated, the value decreased in a dose dependent manner. In this case, non-inhibited control used GLUT-1 expressing oocytes that are not treated with ASD. Transport of 2-DOG into oocytes expressing GLUT-1 was significantly reduced to levels measured for water injected. This is consistent with cytochalasin B, a specific inhibitor of facilitative glucose transport.

Inhibitory effect by ASD on GLUT-1 was further analyzed kinetic analysis. The functional characteristics of ASD inhibition on GLUT-1 activity was studied by analyzing the kinetics affected by ASD under two conditions. Fig. 4A shows that the apparent half-saturation rate constant and the maximal velocity of zero-trans 2-DOG uptake decreased in the presence of ASD. Reduction in the half-saturation rate constant by ASD was further supported in the equilibrium exchange experiment that uses 3-OMG as a glucose analog (Fig. 4B). Different from 2-DOG, 3-OMG is transported through GLUT-1 but not phosphorylated following influx into the cell. Due to severe Inhibition of the catalytic activity by ASD, accumulation of 3-OMG at 100 mM 3-OMG equilibrium concentration become significantly retarded compared to ASD-free control. Considering the

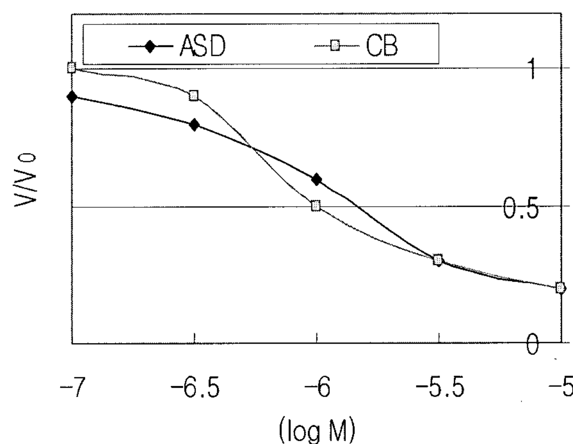

Fig. 3. Effect of ASD on GLUT-1. The effects of ASD on GLUT-1 were measured in the presence of 0.5 mM 2-DOG. *Xenopus* oocyte expressing GLUT-1 were incubated with ASD or CB (cytochalasin B). The measurements (V/V_0) were calculated as fractional value of inhibited per non-inhibited uptake rates.

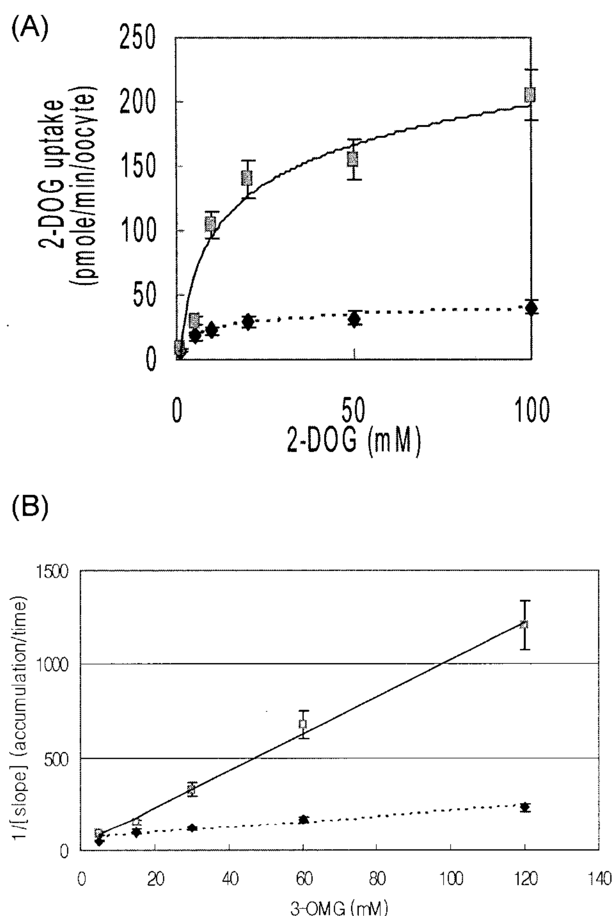

Fig. 4. Kinetic analysis on ASD inhibition characteristics. (A) Zero-trans influx of 2-DOG at the indicated concentrations was determined in the oocytes expressing GLUT-1 in the absence or presence of 0.5 mM ASD (Michaelis-Menten graphs). Closed squares (■) refer to the ASD-free treatment while closed diamonds (◆) to the ASD treatment. Values were standardized by subtracting those for non-injected oocytes. Each value is derived from the mean of data from 10 oocytes and is corrected for 2-DOG uptake in non-injected *Xenopus* oocytes (ASD treatment: *Km*=9.1 mM *Vmax*=288 pmole/min/oocyte; ASD-free treatment: *Km*=3.8mM *Vmax*=36 pmole/min/oocyte). (B) Equilibrium exchange influx kinetics was determined at the 3-OMG equilibrium concentrations indicated. As in Fig. 4A, closed squares (■) refer to the ASD-free treatment while closed diamonds (◆) to the ASD treatment. Accumulation of 3-OMG was investigated for 1 h and expressed as modified by logarithmic transformation. For each time point, 10 oocytes per group were employed in the assay. The negative reciprocals of the slopes were used to plot against 3-OMG concentrations according to the Hanes plotting application. On the y-axis, 1/[slope] refers to the reciprocal value of each absolute value (ASD-free treatment: *Km*=25.0 mM *Vmax*=257±13; ASD-treatment: *Km*= 4.5±0.9 *Vmax*=37±5).

Michaelis-Menten constants of GLUT-1 is consistent with previous reports (Murata *et al.*, 2002), values from equilibrium exchange and zero-trans influx experiments appear genuine.

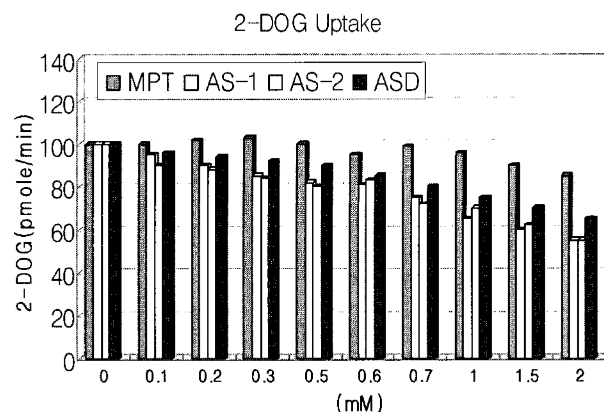

Fig. 5. Effect of ASD and derivatives on GLUT-1. Effect of ASDs on GLUT was assayed similarly as in Fig. 3. The effects of ASD on GLUT-1 was measured in the presence of 0.5 mM glucose. The measurements were expressed as relative values considering ASD-free value as 100%.

Whether ASD analogs affect GLUT-1 activity similarly as ASD was assayed (Fig. 5). The inhibitory effect was still observed for the analogs. GLUT-1 appears significantly affected in the presence of all the ASD analogs tested. In comparison to ASD, AS-1 exerts the most significant level of inhibition. This result bears a ramification that the inhibitory effect can be modulated by chemical library screening.

DISCUSSION

Cordyceps have been consumed to prevent and cure diabetes in the Eastern Asia. No functional ingredient, however, has been identified to date. In the current study, however, we have utilized the *Xenopus laevis* oocyte expression system to assay transport of the glucose analog 2-deoxy-D-glucose and characterize the glucose transport properties of GLUT-1 in the presence of ASD. Our results demonstrate that GLUT-1 facilitated transport of glucose is significantly inhibited in the presence of ASD. ASD likely possesses the features affecting the pathway critical to facilitative transport of glucose governed by GLUT-1. The isolation of these compounds indicated that *P. tenuipes* is a promising source for producing various biologically active substances including trichothecenes.

GLUT-1 expressing *Xenopus laevis* oocytes demonstrated that ASD significantly decreases the transport of 2-DOS in a dose dependent manner. The results are significant to understanding the potential role and importance of ASD and its derivatives as GLUT inhibitors. In treating diabetes, important

alternative routes of glucose disposal are GLUT inhibitors. *Xenopus laevis* oocytes demonstrated significant decreases in the transport of *Xenopus* oocyte membranes expressed high amounts of GLUT-1.

Kinetic analysis indicated that ASD resulted in a lower *Km* and *Vmax*. These studies demonstrate the inhibition mode can be comparable with that by cytochalasin B, a known inhibitor of GLUTs. In the kinetic analysis, the normalized *Vmax* with ASD GLUT-1 was significantly lower than that of GLUT-1 under ASD-free conditions. This reduction in transport activity most likely due to decreased affinity of the transported substrate, because the *Km* values were significantly affected. Thus, it is likely that the ASD affects one or more steps in the transport mechanism at the immediate stage of substrate binding. ASD inhibition of 3-OMG uptake showed more than fivefold reduction in the apparent affinity. This lowered affinity was reflected in a *Km* change for 3-OMG. Taken together, results from the kinetic study indicate that ASD is involved even in equilibrium binding of glucose to the glucose transporter. This result strongly indicates that inhibition of GLUT by ASD is directly targeted at GLUT rather than signaling pathways leading to glucose uptake and metabolism.

ASD analogs inhibit glucose influx as comparable to ASD. Among ASD analogs, AS-1 shows the highest degree of inhibition. These compounds already exist in the list of chemicals and easily synthesized in quantity. Taken together, the analogs can be used as potential drug to treat especially postprandial glucose surge. These chemical compounds can be used to treat insulin-sensitive tissues and also cells with high glucose utilization such as cancer cells.

In conclusion, we have shown that ASD can prevent insulin-stimulated glucose uptake in *Xenopus* oocyte expressing GLUT-1. This inhibitory effect is not due to a nonspecific effect on insulin signaling pathways; rather, direct inhibition on glucose transporters. In addition, ASD does not lie at the level of glucose metabolism. Interference with the translocation of glucose transporters is the basis for the inhibitory effect of ASD. We propose that ASD, trichothecene mycotoxin, is present in *Paecilomyces tenuipes*, which can be utilized in treating diabetes.

ACKNOWLEDGEMENTS

This research was supported in part by University of Seoul Sabbatical Grant (2003.2.-2004.1, HWK). Also supported by the 2000 High Tech Facility Grant to DHL from University of Seoul. We thank for the supports.

REFERENCES

Baldwin, S. A. (1993). Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta* **1154**, 17-49.

Bell, G. I., Burant, C.F., Takeda, J., and Gould, G.W. (1993). Structure and function of mammalian facilitative sugar transporters. *J. Biol. Chem.* **268**, 19161-19164.

Bell, G.I., Kayano, T., and Buse, J.B., (1990). Molecular biology of mammalian glucose transporters. *Diabetes Care* **13**, 198-208.

Doege, H., Bocianski, A., Joost, H.G., and Schurmann, A. (2000). Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leukocytes. *Biochem. J.* **350**, 771-776.

Due, A. D., Qu, Z. C., and Thomas, J. M. (1995). Role of the C-terminal tail of the GLUT-1 glucose transporter in its expression and function in *Xenopus laevis* oocytes. *Biochemistry* **34**, 5462-5471.

Jones, K. (1997). *Cordyceps, Tonic Food of Ancient China*, Sylvan Press, Seattle, Washington, pp. 52-61.

Kikuchi, H., Miyagawa, Y., Sahashi, Y., Inatomi, S., Haganuma, A., Nakahata, N., and Oshima, Y. (2004). Novel Spirocyclic Trichothecanes, Spirotenuipesine A and B, Isolated from Entomopathogenic Fungus, *Paecilomyces tenuipes*. *J. Org. Chem.* **69**(2), 352-356.

Kirwan, J. P., and del Aguila, L. F. (2003). Insulin signaling, exercise and cellular integrity. *Biochem. Soc. Trans.* **31**, 1281-1285.

Lee, D.H. (1998). Characterization of 27K zein as a transmembrane protein. *J. Biochem. Mol. Biol.* **31**(2), 196-200.

Lee, D.H., Selester, B., and Pedersen, K. (1995). Free movement of 27K zein in the endoplasmic reticulum. *Protein Eng.* **9**, 91-96.

Murata, H., Hruz, P.W., and Mueckler, M. (2002). Indinavir inhibits the glucose transporter isoform Glut4 at physiologic concentrations. *AIDS* **12**, 925-926.

Nam, K. S., Jo, Y. S., Kim, Y. H., Hyun, J. W., and Kim, H.W. (2001). Cytotoxic activities of acetoxyscirpenediol and ergosterol peroxide from *Paecilomyces tenuipes*. *Life Sci.* **69**(2), 229-237.

Rumsey, S. C., Daruwala, R., Al-Hasani, H., Zarnowski, M. J., Simpson, I. A., and Levine, M. (2003). Dehydroascorbic acid transport by GLUT4 in *Xenopus* oocytes and isolated rat adipocytes. *J. Biol. Chem.* **275**, 28246-28253.