On the Mechanism for Neomycin Reversal of Wortmannin Inhibition of Insulin Stimulation of Glucose Uptake*

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Although a number of studies and approaches have indicated that activation of the Ser/Thr kinase called Akt/protein kinase B is critical for the insulin-stimulated increase of glucose uptake in adipocytes, other studies have indicated that this enzyme may play an ancillary role. For example, a recent study indicated that neomycin would allow insulin-stimulated Glut4 translocation and glucose transport in the presence of the phosphatidylinositol (PI) 3-kinase inhibitor, wortmannin, a known inhibitor of Akt activation (James, D. J., Salaün, C., Brandic, F. M., Connell, J. M. C., and Chamberlain, L. H. (2004) J. Biol. Chem. 279, 20567–20570). To better understand this observation, we examined a number of downstream targets of Akt. As previously reported, treatment of 3T3-L1 adipocytes with neomycin prevented the wortmannin inhibition of insulin-stimulated glucose transport. However, in the presence of neomycin, wortmannin did not inhibit the insulin-stimulated phosphorylation of several downstream targets of Akt including a proline-rich Akt substrate of 40 kDa, ribosomal protein S6, and glycogen synthase kinase-3. In addition, neomycin did not prevent the ability of a structurally unrelated PI 3-kinase inhibitor, LY294002, to inhibit the insulin-stimulated activation of glucose uptake. Moreover, neomycin reversed the inhibitory effect of wortmannin but not LY294002 on insulin stimulation of Akt kinase activity. Finally, neomycin was found to inactivate in vitro the PI 3-kinase inhibitory actions of wortmannin but not LY294002. These results indicate that the effects of neomycin in adipocytes are not mediated via its ability to sequester phosphatidylinositol 4,5-bisphosphate but are instead caused by the ability of neomycin to inactivate wortmannin.

The first step in insulin action is ligand stimulation of the insulin receptor tyrosine kinase leading to the subsequent tyrosine phosphorylation of various endogenous substrates including insulin receptor substrates IRS-1 and IRS-2 (1). Tyrosine phosphorylated IRS-1 and IRS-2 serve as docking proteins for Src-homology 2 domain containing proteins including the p85 regulatory subunit of phosphatidylinositol (PI)3 3-kinase. This binding results in the membrane localization and activation of the p110 catalytic subunit of PI 3-kinase, leading to the generation of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate. These lipid products induce the activation of a number of signaling kinases including the Ser/Thr kinase Akt (2), which is phosphorylated and activated by the upstream phosphoinositide-dependent kinase 1 (3). Activation of Akt and its downstream signals were shown to stimulate the metabolic actions of insulin such as GLUT4 translocation and glucose transport (4, 5) leading to the hypothesis that this enzyme may play a critical role in mediating this biological response. A number of subsequent studies have supported this hypothesis; knock-out mice and humans lacking Akt2 exhibit a decrease in insulin responsiveness (6, 7), and knockdown of Akt2 in adipocytes by either siRNA or genetically (8, 9) suppressed the ability of insulin to stimulate glucose uptake. However, other data indicate that Akt activation is not required for insulin stimulation of glucose transport and instead indicate that distinct signal cascades stimulated by insulin may be responsible for eliciting this response (10, 11). Most recently a study indicated that neomycin treatment of 3T3-L1 adipocytes would allow insulin-stimulated glucose transport in the presence of wortmannin, an inhibitor of PI 3-kinase and Akt activation (12). Because neomycin binds and sequesters PI 4,5-P2 (13), it was proposed that insulin-stimulated glucose transport may occur in part via the “masking” of PI 4,5-P2 (12).

To better understand the mechanism whereby neomycin reverses wortmannin inhibition of insulin-stimulated glucose transport, we first investigated downstream targets of Akt in insulin-treated 3T3-L1 adipocytes. In the presence of wortmannin, neomycin allowed the insulin-stimulated uptake of glucose as reported previously (12). However, it also allowed the insulin-stimulated increase in phosphorylation of several downstream targets of Akt including the direct substrate PRAS40 (14), two downstream targets of Akt, ribosomal protein S6 and GSK3β (15, 16), as well as allowing insulin-stimulated activation of Akt itself. In addition, neomycin was found incapable of preventing the inhibition of a structurally unrelated PI 3-kinase inhibitor, LY294002 (17). Finally, neomycin was found to inactivate in vitro the inhibitory actions of wortmannin. These results indicate that the effects of neomycin in adipocytes are not mediated via its ability to sequester PI 4,5-P2, but is instead caused by the ability of neomycin to inactivate wortmannin.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to pS6 and pGSK3β were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies to Akt1 and phosphotyrosine (PY20) were from Transduction Laboratories (San

PRAS40; proline-rich Akt substrate of 40 kDa; GSK, glycogen synthase kinase.
Diego, CA). Polyclonal anti-pAkt (pSer473) antibodies were from Covance (Princeton, NJ). Polyclonal antibodies to pPRAS40 were from BIOSOURCE (Beverly, MA), and polyclonal anti-Akt antibodies were as described (18). Goat anti-mouse and anti-rabbit peroxidase conjugated antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Enhanced chemiluminescence detection reagents were from Pierce. Human insulin was from Roche Applied Science. LY294002 and wortmannin were from Calbiochem. Protein A-agarose was from RepliGen Corporation (Waltham, MA). Phosphatidylinositol was from Avanti Polar-Lipid (Alabaster, AL). Neomycin and other chemicals were from Sigma.

Cell Culture—3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C and 5% CO2, and then the indicated concentrations of either wortmannin (A) or LY294002 (B) were added to the media. Ten min later, 100 nM insulin was added as indicated. After an additional 20 min, 2-[3H]deoxyglucose uptake was measured as described under “Experimental Procedures.” Filled bars represent data from neomycin-treated cells, and the open bars represent data of vehicle-treated cells. The results shown are means ± S.E. (n = 3).

**FIG. 1.** Neomycin reverses the wortmannin but not the LY294002 inhibition of insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were incubated in Krebs-Ringer-Hepes buffer containing either 10 mM neomycin or 2 h at 37 °C, and then the indicated concentrations of either wortmannin (A) or LY294002 (B) were added to the media. Ten min later, 100 nM insulin was added as indicated. After an additional 20 min, 2-[3H]deoxyglucose uptake was measured as described under “Experimental Procedures.” Filled bars represent data from neomycin-treated cells, and the open bars represent data of vehicle-treated cells. The results shown are means ± S.E. (n = 3).

Glucose Uptake—Serum-starved 3T3-L1 adipocytes were incubated in serum-free media containing either 10 mM neomycin or vehicle for 2 h at 37 °C, and then 100 nM wortmannin (W) was added to the media as indicated. Twenty min later, 100 nM insulin (I) was added, and after an additional 10 min the cells were lysed and the lysates were analyzed by SDS-PAGE and immunoblotting with either antibodies to pPRAS (A and B) or pS6 (C and D). Results shown are either a representative blot (A and C) or the quantitation of the results from three experiments ± S.E. (B and D). C, control.

**FIG. 2.** Neomycin reverses the wortmannin inhibition of insulin-stimulated phosphorylation of PRAS40 and S6 ribosomal protein in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were incubated in serum-free media containing either 10 mM neomycin or vehicle for 2 h at 37 °C, and then 100 nM insulin (I) was added, and after an additional 10 min the cells were lysed and the lysates were analyzed by SDS-PAGE and immunoblotting with either antibodies to pPRAS (A and B) or pS6 (C and D). Results shown are either a representative blot (A and C) or the quantitation of the results from three experiments ± S.E. (B and D). C, control.
then after an additional 10 min cells were lysed in a lysis buffer containing 25 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 14,000 rpm for 10 min, electrophoresed on an 8% SDS-PAGE, and transferred to nitrocellulose membranes. After blocking in 5% nonfat dry milk in Tris-buffered saline, membranes were incubated with specific antibodies and appropriate secondary antibodies. Bands were visualized by enhanced chemiluminescence and quantified using a Kodak Work Station and Kodak 1D v.3.5.3 software.

Akt Kinase Activity Assay—3T3-L1 adipocytes and CHO-T cells were treated as described above for immunoblotting. After centrifugation, the lysates were incubated with anti-Akt antibodies prebound to protein A-agarose. Akt kinase activity was assessed essentially as described previously (5) using GSK3 peptide (GRPRSSPAEG) as substrate (16). Following the kinase reaction, the phosphorylated peptide was separated from free [γ-32P]ATP on 40% polyacrylamide gels containing 6M urea. The phosphopeptide bands were visualized and quantified using a PhosphorImager and the ImageQuant v1.2 software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Treatment of Wortmannin and LY294002 with Neomycin—Twenty µl of wortmannin or LY294002 (in Krebs-Ringer-Hepes buffer) were incubated in the presence or absence of 1 mM neomycin for 20 min at 37 °C. The samples were then diluted 1:5 and added to immunoprecipitated PI 3-kinase and assayed for enzymatic activity essentially as described previously (20). The radioactive spots on the TLC plates were visualized and quantified using a PhosphorImager and the ImageQuant v1.2 software.

RESULTS

Neomycin Restores the Insulin Stimulation of Glucose Uptake and Downstream Targets of Akt in Wortmannin-treated 3T3-L1 Adipocytes—As reported previously (12), inclusion of 10 mM neomycin in the media of 3T3-L1 adipocytes partially restored (by ~60%) the ability of insulin to stimulate glucose uptake in cells treated with 100 nM wortmannin (Fig. 1A). Interestingly, the ability of 1 µM wortmannin to inhibit insulin stimulation of glucose uptake was not reversed by the presence
of neomycin in the media (Fig. 1A).

To determine under the same conditions whether the ability of insulin to stimulate Akt was effected, PRAS40 phosphorylation was first examined because this protein is likely to be directly phosphorylated by activated Akt (14). As expected, the insulin-stimulated phosphorylation of PRAS40 was almost completely inhibited by prior treatment of the cells with 100 nM wortmannin (Fig. 2, A and B). However in the presence of 10 mM neomycin, insulin-stimulated phosphorylation of PRAS40 was almost unaffected by this concentration of wortmannin (Fig. 2, A and B).

After activation of Akt, mTOR and S6 kinase are stimulated, resulting in increased phosphorylation of ribosomal protein S6 (5, 15). Thus, as expected, the insulin-stimulated phosphorylation of PRAS40 was almost completely inhibited by prior treatment of the cells with 100 nM wortmannin (Fig. 2, A and B). However in the presence of 10 mM neomycin, insulin-stimulated phosphorylation of PRAS40 was almost unaffected by this concentration of wortmannin (Fig. 2, A and B).

Finally, we looked at another downstream target of Akt, GSK3β (16). Again, the inhibition of the insulin-stimulated phosphorylation of GSK3β observed with 100 nM wortmannin was partially reversed by prior treatment of the cells with 10 mM neomycin (Fig. 3, A and B).

These studies suggested that in the presence of neomycin and wortmannin, Akt may still be activated. To directly test this, we measured both the phosphorylation of Akt (because its phosphorylation is the primary mechanism for regulating its activity) as well as its enzymatic activity after immunoprecipitation. As expected, 100 nM wortmannin completely inhibited the insulin-stimulated phosphorylation of Akt on Ser-473 as well as its enzymatic activity after immunoprecipitation (Fig. 3, C and D). However, inclusion of 10 mM neomycin in the media of the cells partially restored (by ~60%) the ability of insulin to stimulate Akt kinase activity in cells treated with 100 nM wortmannin.

Neomycin Restores the Insulin Stimulation of Akt in CHO-T Cells Treated with Wortmannin but Not Those Treated with LY294002—To investigate whether the ability of neomycin to restore insulin signaling after wortmannin treatment was limited to 3T3-L1 adipocytes, we examined the ability of neomycin to restore insulin activation of Akt in CHO cells overexpressing the insulin receptor (CHO-T cells) (19). As previously reported, 100 nM wortmannin almost completely inhibits insulin activation of Akt in these cells (2). Inclusion of 10 mM neomycin in the media of the cells partially restored (by ~60%) the ability of insulin to stimulate Akt activity in cells treated with 100 nM wortmannin.

Neomycin Restores the Insulin Stimulation of Akt in CHO-T Cells Treated with Wortmannin but Not Those Treated with LY294002—To determine whether the ability of neomycin to restore insulin response was unique to wortmannin, we examined whether this drug could also restore the response in CHO-T cells treated with a structurally unrelated...
PI 3-kinase inhibitor, LY294002 (17). In contrast to the results with wortmannin, the LY294002 inhibition of insulin stimulation of Akt activity in CHO-T cells was not reversed by inclusion of neomycin in the media of these cells (Fig. 4B). Moreover, the ability of either 10 or 30 μM LY294002 to inhibit the insulin stimulation of glucose uptake in 3T3-L1 adipocytes was not reversed by inclusion of 10 nM neomycin in the media of these cells (Fig. 1B).

**Neomycin Inactivates Wortmannin but Not LY294002 in Vitro**—Because neomycin blocked the ability of wortmannin but not LY294002 to inhibit insulin-stimulated signaling in cells, we examined whether in a cell-free system neomycin would affect the PI 3-kinase inhibitory activities of wortmannin and LY294002. A 20-min incubation at 37 °C of 1 mM neomycin with either 60 or 200 nM wortmannin (conditions designed to mimic those in the whole cell experiments) resulted in an 80% decrease in the PI 3-kinase inhibitory activity of the wortmannin (Fig. 5). At 600 nM wortmannin, neomycin treatment caused a 60% decrease in its ability to inhibit PI 3-kinase activity. In contrast, incubation of neomycin with several concentrations of LY294002 did not result in any decrease in its PI 3-kinase inhibitory activity (Fig. 5). Interestingly, addition of neomycin alone to the PI 3-kinase reaction mixture had a biphasic effect, stimulating PI 3-kinase activity by 200% at low concentrations (50 μM) and inhibiting the reaction at higher concentrations (1 mM) (Fig. 5 and data not shown).

**DISCUSSION**

Although aminoglycosides like neomycin and gentamicin are bactericidal because of their interaction with the prokaryotic 16S ribosomal RNA of the small ribosomal subunit (21), they have been known for more than 25 years to also bind with high affinity to PI 4,5-P_2 (22). It has been proposed that some of the side effects (including nephrotoxicity and ototoxicity) of these still commonly prescribed antibiotics may be caused by their interactions with these lipids (23). For example, neomycin may bind to PI 4,5-P_2 and inhibit inositol phosphate turnover in response to various agonists, thereby decreasing cell survival (24).

The ability of neomycin to bind PI 4,5-P_2 has also been extensively utilized by many investigators to probe the role of this lipid in various cellular responses (13). In one such recent study, neomycin was found to reverse the ability of wortmannin to inhibit insulin stimulation of glucose transport in 3T3-L1 adipocytes (12). In this report, neomycin was not found to affect the wortmannin inhibition of insulin stimulation of Akt activity. However, insulin stimulation of Akt activity was observed in this study in the presence of 100 nM wortmannin (see Fig. 2 of this prior study). The interpretation of these studies was that neomycin was masking the PI 4,5-P_2 and that this contributed to the insulin-stimulated increase in glucose transport activity.

In the present work, we found that as previously reported the inclusion of 10 nM neomycin in the media was able to restore the insulin-stimulated increase in glucose transport in 3T3-L1 adipocytes treated with 100 nM wortmannin. However, neomycin could not restore this response to insulin when the cells were treated with 1 μM wortmannin. Moreover, we found that neomycin also restored the ability of insulin to stimulate the downstream phosphorylation of three targets of Akt including PRAS40 and ribosomal protein S6 as well as GSK3β. In contrast to the prior study, we also observed that neomycin restored the ability of insulin to stimulate the activity and phosphorylation of Akt in both wortmannin-treated 3T3-L1 adipocytes as well as in another cell type, CHO cells overexpressing the insulin receptor. Although we cannot explain the lack of insulin-stimulation of Akt kinase activity in this prior study when the 3T3-L1 adipocytes were treated with 100 nM wortmannin in the presence of neomycin, it can be observed in Fig. 2 of Ref. 12 that the kinase activity of Akt was elevated after insulin-treatment in the presence of 100 nM wortmannin. This observation is inconsistent with the large body of evidence that 100 nM wortmannin completely inhibits the insulin-induced activation of Akt (2).

If the recovery of insulin-stimulated glucose transport with neomycin treatment was the result of the masking of PI 4,5-P_2, it should also be observed with a structurally unrelated PI 3-kinase inhibitor. Therefore we tested whether neomycin could also restore insulin responsiveness to cells treated with a structurally unrelated PI 3-kinase inhibitor, LY294002 (17). In contrast to the results with wortmannin, we found that neomycin had no effect on the ability of LY294002 to inhibit the insulin stimulated increase in glucose transport in 3T3-L1 adipocytes and Akt activation in CHO-T cells. These results suggested that the neomycin might be blocking the inhibitory actions of wortmannin.

To directly test this hypothesis, we incubated neomycin together with different concentrations of wortmannin under conditions designed to mimic what would occur when wortmannin was added to cells containing neomycin in the media. The wortmannin was then tested for its ability to inhibit PI 3-kinase activity in an *in vitro* assay. Neomycin at 1 mM was found to inhibit 80% of the PI 3-kinase inhibitory activity of 60 or 200 nM wortmannin after a 20-min incubation at 37 °C. In contrast, a comparable incubation of LY294002 with neomycin had no effect on the inhibitory activity of this compound. These results indicate that it is likely that the effects of neomycin in adipocytes are not mediated via its ability to sequester PI 4,5-P_2 but are instead caused by the ability of neomycin to inactivate wortmannin.

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