Vol. 212, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 6, 1995 Pages 263-269

Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells

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Received June 5, 1995

Summary: The effect of wortmannin, which inhibits phosphatidylinositol 3-kinase (PI 3-kinase), on adipocytic differentiation of 3T3-L1 cells was examined. The extent of differentiation was evaluated by the staining of adipocytes with Oil Red O and measurement of glycerol 3-phosphate dehydrogenase (GPDH) activity. Wortmannin, at over 100 nM, significantly inhibited adipogenesis of cells treated with isobutyl-methylxanthine, dexamethasone, and insulin with an IC50 value about 50 nM. These results suggest that PI 3-kinase plays a role in adipocytic differentiation of 3T3-L1 cells.

Mouse fibroblast 3T3-L1 cells are known to differentiate into adipocytes under appropriate conditions (1). Of these, insulin or insulin-like growth factor-I plays an essential role (2). Ras proteins, one of the members of low molecular weight GTP-binding proteins, are activated by insulin and mediate the effect of insulin in inducing adipocytic differentiation of 3T3-L1 cells (3). It is known that insulin also stimulates PI 3-kinase and PI 3-kinase is involved in cell growth signaling pathways (4), but it is not understood well about the role of PI 3-kinase in adipocytic differentiation of 3T3-L1 cells. Recently wortmannin, a fungal metabolite that inhibits chemotactic peptide-induced superoxide production in human neutrophils, was shown to inhibit PI 3-kinase

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0006-291X/95 $12.00

activity (5). In this study we examined the effect of wortmannin, a specific PI 3-kinase inhibitor, on adipocytic differentiation of 3T3-L1 cells.

Materials and Methods

Cell culture and differentiation: 3T3-L1 cells, provided from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in 60 mm culture dishes with 3.5 ml of Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % fetal calf serum (FCS, JRH Biosciences). Two days after the cells became confluence (day 0), adipocytic differentiation was induced as follows. Cells were cultured with DMEM containing 10 % FCS, 0.5 mM isobutyl-methylxanthine (Wako, Japan), 1 µM dexamethasone (Wako) and 1.7 µM insulin (Wako) for 48 hours (control). Cells that were not differentiated were maintained in DMEM containing 10 % FCS during this period. Then the medium was changed to DMEM supplemented with 10 % FCS and was changed every 48 hours. Wortmannin (Wako) was dissolved in dimethyl sulfoxide at 10 mM and diluted with medium just before use. It was added at indicated time at indicated concentrations.

Oil Red O staining: Cell morphology was examined of the staining with Oil Red O (Nacalai Tesque, Japan). For this, culture dishes were fixed with 10 % formaldehyde and stained with a saturated solution of Oil Red O in 60 % isopropanol / 40 % water. After removal of the staining solution, the cultures were photographed.

GPDH activity: GPDH activity was determined spectrophotometrically in the sonicated cell extracts (6). The reaction buffer contained 100 mM triethanolamine-HCl (pH 7.5), 2.5 mM EDTA, 0.1 mM 2-mercaptoethanol, 0.12 mM NADH (Wako) and, to start reaction, 0.2 mM dihydroxyacetone phosphate (Sigma). The change in absorbance at 340 nm was followed at 25 °C with a Shimazu UV2000 spectrophotometer. The results were expressed as milliunits per mg protein, where 1 mU is the activity for oxidation of 1 nmol NADH / min. Protein concentration was measured with a Pierce BCA protein assay kit (Pierce). BSA was used as a standard.

PI 3-kinase and phosphatidylinositol 4-kinase (PI 4-kinase) assay: Two days after confluence, cells were serum starved overnight, and treated with wortmannin at indicated concentration for 30 minutes. Then cells were stimulated with 1.7 µM insulin for 10 minutes. The cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine antibody PY20 (Leinco) and anti-mouse IgG agarose (American Qurlex). Then the immunoprecipitates were subjected to the PI 3-kinase assay as previously described (7). PI 3-kinase activity on PI-4-P was measured essentially in the same way, but wortmannin was treated after immunoprecipitates were prepared. PI 4-kinase assay using PI as substrates was done in the same way as PI 3-kinase assay, except that 0.5 % Nonidet P-40 (NP-40) was added into the reaction mixture to inhibit PI 3-kinase. The radioactivity was determined by Fuji BAS2000 Bioimaging analyzer, and visualized by autoradiography.

Results and Discussion

The effect of wortmannin on differentiation of 3T3-L1 cells: To examine the effect of wortmannin on adipocytic differentiation of 3T3-L1 cells, it was added 30 minutes before the initiation of differentiation and at the induction with other drugs. By this procedure, adipogenesis was inhibited by some extents although not significant (data not shown). Because wortmannin's effect does not last long (8), we added wortmannin 30 minutes before the beginning of differentiation and every 6 hours for 96 hours after the initiation of differentiation (Fig.1-A). On day 8, the extent of differentiation was evaluated morphologically and biochemically. First, for morphological evaluation, culture dishes were stained with Oil Red O and photographed. In control (as described in Materials and Methods), about 90 % cells were stained. But with wortmannin, lipid accumulation was inhibited in a dose-dependent manner (Fig.1-B). Next GPDH activities were measured as described in Materials and Methods to evaluate the extent of differentiation biochemically. Wortmannin treatment was carried out in the same way as described above. Wortmannin, at over 100 nM, significantly reduced GPDH activity and IC50 value was estimated to be about 50 nM from this result (Fig.2). These results show that wortmannin inhibited adipocytic differentiation of 3T3-L1 cells morphologically and biochemically. We next determined PI 3-kinase activity in this experiment.

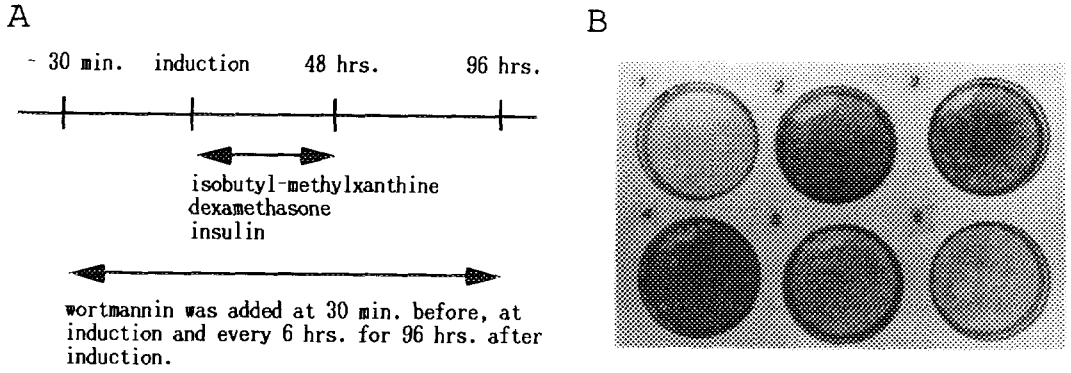

Fig.1. Differentiation was inhibited in a dose-dependent manner by wortmannin. A)Time course of experiment. B)Photograph of stained dishes with Oil Red O (representative of three independent experiments). 8 days after the induction, culture dishes were stained with Oil Red O as described in Materials and Methods and photographed. Cells that contained neutral lipids appears to be black. 1.no additions: differentiation was not induced. 2.control 3-6.differentiation was induced with wortmannin at 1 nM, 10 nM, 100 nM and 1 μM.

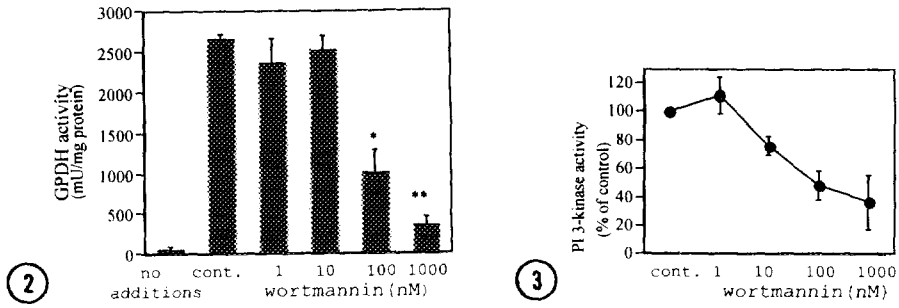

Fig.2. The effect of wortmannin on GPDH activity. 8 days after the induction, GPDH activities were measured as described in Materials and Methods. Wortmannin was added as described in Fig.1-A. GPDH activity was expressed as mU/mg protein, where 1 mU is the activity for oxidation of 1 nM NADH/min. no additions: differentiation was not induced. cont.: control. wortmannin: wortmannin was added at indicated concentration. Values are expressed as mean ± S.D.($n = 3$). *, $p<0.05$; **, $p<0.01$ (compared to control).

Fig.3. Dose dependent effect of wortmannin on PI 3-kinase activity. After 30 minutes treatment with wortmannin, cells were stimulated with 1.7 μM insulin for 10 min. Then cells were lysed and PI 3-kinase activity was measured using PI as substrates as described in Materials and Methods. PI 3-kinase activity was shown as percent of the activity of control. cont.: control. Values are expressed as mean ± S.E.($n = 3$).

Inhibition of insulin-stimulated PI 3-kinase activity by wortmannin: PI 3-kinase activity was measured as described in Materials and Methods. By treating cells with wortmannin, insulin-stimulated PI 3-kinase activity was inhibited dose-dependently although the inhibition was not complete even at 1 μM (Fig.3). For this reason, we can think of some possibilities. One is that wortmannin inhibited novel type of PI 3-kinase (10). And we immunoprecipitated phosphorylated PI 3-kinase in doing kinase assay, phosphorylated PI 4-kinase could be also immunoprecipitated. Wortmannin's inhibitory effect on PI 4-kinase was weak even at 1 μM (9), so it was speculated that considerable amounts of phosphatidylinositol-4-phosphate (PI-4-P) were produced by PI 4-kinase, and they were separated together with phosphatidylinositol-3-phosphate (PI-3-P) on TLC plate because it is hard to distinguish PI-3-P from PI-4-P by chromatography. Furthermore, it was reported that wortmannin exerts its effect by binding the 110 kDa subunit of PI 3-kinase and this binding may be untied by washing the immunoprecipitates extensively before the kinase assay (7). In order to exclude the effects of extensive washings, we next added wortmannin after washing the

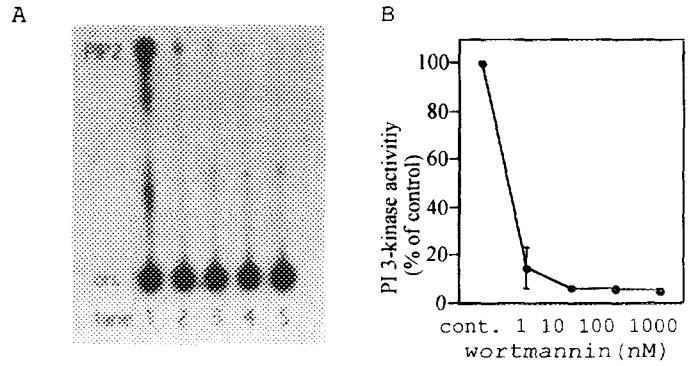

Fig.4. Wortmannin's inhibitory effect on PI 3-kinase activity on PI-4-P. Cells were stimulated with 1.7 μM insulin for 10 minutes. Then cells were lysed and immunoprecipitated. After washing the precipitates, wortmannin at indicated concentration was added into reaction mixture. PI 3-kinase activity was measured using PI-4-P as substrates as described in Materials and Methods. A) Autoradiogram of the TLC plate was shown (representative of three independent experiments). lane 1: control. lane 2~5: wortmannin was added at 1, 10, 100, 1000 nM. ori: origin, PIP2: phosphatidylinositol-3,4-bisphosphate. B) Dose dependent effect of wortmannin was shown. PI 3-kinase activity on PI-4-P was shown as percent of the activity of control. Values were expressed as mean ± S.E. (n = 3).

precipitates and, to exclude the effect of PI 4-kinase, we measured kinase activity with PI-4-P as substrates instead of phosphatidylinositol (PI). By these procedure, PI 3-kinase (PIP 3-kinase) activity on PI-4-P was inhibited almost completely at 10 nM (Fig.4A-lane3). IC_{50} value was estimated to be bellow 1 nM (Fig.4B) and this is accordance with IC_{50} value of previously identified PI 3-kinase (5). This result suggests that wortmannin inhibited previously identified PI 3-kinase not novel one.

The effect of wortmannin on PI 4-kinase: To further confirm that PI 4-kinase was not inhibited by wortmannin at higher concentration and PI-4-P were also separated on TLC plate, we determined PI 4-kinase activity. PI 4-kinase activity was measured in the same way as PI 3-kinase assay was done except that 0.5 % NP-40 was added into the reaction mixture to inhibit PI 3-kinase. PI 4-kinase activity was not suppressed as to inhibit adipocytic differentiation at 100 nM and 1 μM (Fig.5A-lane 2 and 3, Fig.5B), so wortmannin's effect on differentiation was not due to the inhibition of PI 4-kinase. It was also suggested that PI 3-kinase was not suppressed well even at higher concentration of wortmannin due to the contamination of PI-4-P

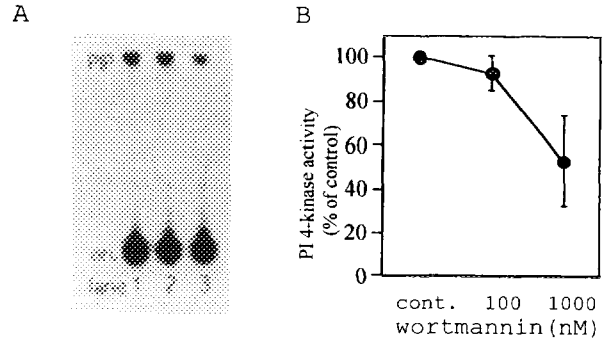

Fig.5. Wortmannin's effect on PI 4-kinase. Cells were stimulated with 1.7 μM insulin, then cells were lysed and immunoprecipitated. After washing the precipitates, wortmannin at indicated concentration was added into assay mixture. PI 4-kinase activity using PI as substrates was measured as described in Materials and Methods. A) Autoradiogram of the TLC plate was shown (representative of four independent experiments). lane 1: control. lane 2,3: wortmannin was added at 100 and 1000 nM. ori: origin, PIP: phosphatidylinositol-4-phosphate. B) Dose dependent effect of wortmannin was shown. PI 4-kinase activity was shown as percent of the activity of control. Values were expressed as mean ± S.E. (n = 3).

produced by PI 4-kinase when we assayed PI 3-kinase activity with PI as substrates.

In this study, wortmannin inhibited adipogenesis of 3T3-L1 cells with an IC_{50} value about 50 nM. In the previous report, wortmannin inhibited PI 3-kinase with an IC_{50} value bellow 5 nM (8). Wortmannin also inhibits phosphatidylinositol 4-kinase and myosin light chain kinase but IC_{50} values for the inhibition of these kinases are much higher than IC_{50} value that was obtained in this study (9). Furthermore, wortmannin at 100 nM inhibited adipogenesis significantly and wortmannin's effect on other kinases at this concentration is less apparent. Indeed, PI 4-kinase was not inhibited well at higher concentration of wortmannin (Fig.5). Wortmannin's effect was attenuated in serum (8), so this may be the reason that we needed higher concentration of wortmannin to inhibit adipocytic differentiation of 3T3-L1 cells. In fact, in the study about the role of PI 3-kinase in preventing apoptosis, they required 10 times higher concentration of wortmannin in serum to prevent apoptosis of PC-12 cells (11), and dose-dependency obtained in that report was very similar to our result. By all the results described above, it was suggested that wortmannin inhibited adipogenesis of 3T3-L1 cells by inhibiting PI 3-kinase.

Acknowledgments: This study was supported in part by a research grant from the Kanehara Ichiro Foundation to Y.W.. We thank Dr. M. Ui (Institute of Physical and Chemical Research), Dr. O. Hazeki, Dr. T. Katada (Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo) and Dr. S. Kawamoto (Second Department of Internal Medicine, Faculty of Medicine, Osaka University) for advice.

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