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Original Article

Adverse effects of farnesyltransferase inhibitors on insulin actions

Sun-Ju Yi[1], Byung Hak Jhun[2*], Kyunghwan Kim[1*]

[1]*School of Biological Sciences, College of Natural Sciences, Chungbuk National University, Cheongju 28644, Korea*
[2]*Department of Cogno-Mechatronics Engineering, Pusan National University, Busan 46241, Korea*

Ras activates a series of downstream effectors, including the mitogen-activated protein kinase pathway and the Rac/Rho pathway after insulin stimulation. Mutations in *Ras* are found in approximately 30% of all human cancers and are critical factors in tumor initiation and maintenance. There are four Ras proteins with 80-90% amino acid sequence homology with major differences in the carboxyl termini. Ras proteins undergo farnesylation on their carboxyl termini catalyzed by the enzyme protein farnesyltransferase (FTase), which facilitates localization of Ras proteins to the inner surface of the plasma membrane. Because inhibition of FTase would prevent Ras from processing into its active form, FTase is viewed as a potential therapeutic target. A variety of FTase inhibitors have showed great potency against tumor cells in preclinical studies. Although many farnesyltransferase inhibitors have been developed, their adverse effects on the mitogenic and metabolic actions of insulin are not completely understood. Here we show that YH3096, a farnesyltransferase inhibitor, inhibits insulin-mediated DNA synthesis in HIRc-B cells without affecting c-Jun expression and membrane ruffling in HIRc-B cells. Moreover, YH3096 and its derivatives did not affect insulin-induced glucose uptake in 3T3-L1 adipocytes. Our results provide a laboratory evaluation of the effects of Ras inhibitors on insulin functions.

Key words: Ras, farnesyltransferase inhibitor, YH3096, insulin, DNA synthesis

Introduction

Mutations in *Ras* were the first specific genetic alterations identified in human cancer [1]. Intensive studies have reported that gain-of-function mutations in *Ras* are found in approximately 30% of all human cancers. Ras proteins are critical regulators that link diverse extracellular stimuli, including peptide growth factors, cytokines, and hormones, with a diverse range of biological responses [2, 3]. Ras proteins cycle between guanosine-5′-triphosphate (GTP)-bound active forms and guanosine diphosphate-bound inactive forms. This exchange is mediated by guanine nucleotide exchange factors or GTPase-activating proteins (GAPs) [4]. Ninety-eight percent of *Ras* mutations occur at one of the amino acid residues G12, G13, or Q61. The single amino acid substitution at 12, 13, or 61 creates mutant proteins that are insensitive to GAP action [5], resulting in uncontrolled cell proliferation.

The four Ras proteins—H-Ras, N-Ras, K-Ras 4A, and K-Ras 4B—are localized to the cytoplasmic face of the plasma membrane through a series of posttranslational processing [6]. Farnesyl transferase catalyzes the addition of a C15 farnesyl isoprenoid lipid onto cysteine in the Ras C-terminal CAAX motif (where C is cysteine, A is an aliphatic compound, and X is typically methionine or serine). Inhibition of farnesylation using farnesyl transferase inhibitors (FTIs) disrupts the association with the Ras membrane and aberrant Ras activity to transform the cells [7, 8]. Although FTIs have been developed to inhibit oncogenic Ras-transformed cells and suppress the growth of carcinoma xenografts, their cytotoxicity against normal cells has been controversial. For example, manumycin, an analog of farnesyl diphosphate, inhibits the proliferation of Chinese hamster ovary (CHO) cells that express insulin receptors [9]. By contrast, James et al. (1994) demonstrated that BZA-5B, another FTI, ap-

***Corresponding author:** Kyunghwan Kim
School of Biological Sciences, College of Natural Sciences, Chungbuk National University, Cheongju 28644, Korea
Tel: +82-43-261-2292, Fax: +82-43-260-2298, E-mail: kyungkim@cbnu.ac.kr
Byung Hak Jhun
Department of Cogno-Mechatronics Engineering, Pusan National University, Busan 46241, Korea
Tel: +82-55-350-5291, Fax: +82-55-350-5653, E-mail: bjhun@pusan.ac.kr

pears to be relatively nontoxic because it does not inhibit the proliferation of untransformed cells [10]. These results are explained by a bypass mechanism through which K-Ras and N-Ras can act as a substrate for geranylgeranyl transferase-1 in the presence of FTIs [11].

Insulin controls many aspects of metabolism, growth, and survival. The major insulin signaling pathways regulate metabolism and gene expression, with central roles for phosphatidylinositol 3-kinase/protein kinase B and Ras/Raf/MEK [12-15]. Ras protein plays a central role in the insulin and insulin-like growth factor-1 signaling pathways and is required for gene expression and DNA synthesis [16]. Recently, we found that lonafarnib partially inhibits DNA synthesis stimulated by insulin, but not glucose uptake in 3T3-L1 adipocytes [17]. On the contrary, manumycin suppresses the antiapoptotic action of insulin in untransformed CHO cells [9]; therefore, it is important to determine whether FTIs affect normal cellular functions, such as insulin actions.

In this study, we investigated the effects of farnesyl transferase inhibitor YH3096 and its derivatives YH3938 and YH3945 on the mitogenic and metabolic actions of insulin. We demonstrated that YH3096 blocked farnesylation of H-Ras but did not affect processing of K-Ras and N-Ras. YH3096 inhibited insulin-mediated DNA synthesis but did not affect c-Jun expression or membrane ruffling induced by insulin. In addition, YH3096 and its derivatives did not block insulin-induced glucose uptake.

Materials and Methods

Materials

The farnesyl transferase inhibitors —YH3096, YH3938 and YH3945—were kindly provided by Yuhan Corporation (Seoul, South Korea). Cys-Ile-Ile-Met motif was utilized as a template peptide for synthesis of YH3096 and its derivatives [18]. Pan-Ras monoclonal antibodies were purchased from Merck Millipore (Billerica, USA) and anti-H-Ras antibody and anti-c-Jun antibody were from BD biosciences (San Diego, USA). Methyl-[^{3}H]-thymidine, and 2-deoxy-D-1-[^{3}H] glucose were obtained from GE healthcare (Pittsburg, USA). TRITC-conjugated phalloidin was purchased from Jackson Immunoresearch Laboratories (West Grove, USA). All culture media were from Gibco (Waltham, USA). All other reagents were purchased from Sigma (St. Louis, USA).

Cell culture

HIRc-B cells, which are rat-1 fibroblasts overexpressing the human insulin receptors, were maintained as previously described [16]. To investigate insulin actions, HIRc-B cells were starved in DMEM supplemented with 4 mM L-glutamine and 100 unit/mL penicillin-100 μg/mL streptomycin for 24 h. 3T3-L1 preadipocytes were maintained as fibroblasts and differentiated as described [19].

Immunoblot analysis

HIRc-B cells were grown in 12-well plates and treated with the indicated doses of YH3096 for 24 h. Cells were washed and lysed, and cell lysates were analyzed on 12.5% SDS-PAGE followed by immunoblotting with anti-Ras antibodies.

DNA synthesis

HIRc-B cells were grown in 24-well plates and serum-starved in the presence of dimethyl sulfoxide (DMSO) or 0.1, 0.5, 1 μM YH3096 for 24 h. Then cells were stimulated with insulin of the indicated concentrations for 16 h and then pulsed with [^{3}H]-thymidine, 0.5 μCi/mL, for 4 h at 37°C. The cells were washed, and the incorporated thymidine was counted [20].

Membrane ruffling and c-Jun expression

To investigate the effect of YH3096 on membrane ruffling [16], HIRc-B cells were grown on 12-mm glass coverslips and incubated with serum-free DMEM in the presence of YH3096 (0.5 or 1 μM) for 24 h. Then cells were stimulated with insulin (100 ng/mL) for 10 min. The cells were fixed, permeabilized, and incubated with TRITC-conjugated phalloidin (0.1 mg/mL) for 1 h. Results were analyzed on the fluorescence microscopy. The results represent the mean of at least three independent experiments in which at least 300 cells were counted. HIRc-B cells were grown on 12-well plates, and the medium was replaced with serum-free DMEM in the presence of YH3096 for 24 h. Then cells were stimulated with insulin (100 ng/mL) for 4 h and immunoblotted with anti-c-Jun antibody.

2-Deoxyglucose uptake in 3T3-L1 adipocytes

The differentiated 3T3-L1 adipocytes were incubated with the indicated doses of YH3096 for 4 d, and then glucose uptake was examined as previously reported [19].

Results and Discussion

Inhibitory effect of YH3096 on prenylation of Ras in HIRc-B cells

The farnesyl transferase inhibitors —YH3096, YH3938 and YH3945—are developed by Yuhan Corporation (Seoul, South Korea) by utilizing Ras CAAX motif as a template peptide [18]. It was shown that YH3096 and its derivatives blocked the cell growth by inhibiting FTase, leading to G2/M enrichment in human tumor cells harboring *ras* mutation [18]. To examine the effects of

YH3096 on insulin actions, we used insulin-responsive HIRc-B cells derived from normal rat-1 fibroblasts and engineered to express 100,000 human insulin receptors per cell [21]. Because all four Ras proteins are expressed in HIRc-B cells and well-known substrates for FTase, we first attempted to check the effect of YH3096 on Ras prenylation in HIRc-B cells. To do this, HIRc-B cells were treated with the indicated amounts of YH3096 for 24 h. Because a prenylated Ras protein migrates faster on sodium dodecyl sulfate polyacrylamide gel electrophoresis than its unprenylated counterpart, we performed an immunoblot using antibodies against pan-Ras and H-Ras to differentiate the prenylated Ras protein from the unprenylated form. As shown in Fig. 1, immunoblot analysis with anti-Pan-Ras antibody showed that the endogenous H-, K-, and N-Ras proteins in DMSO-treated HIRc-B cells are normally prenylated. In contrast, treatment with 1.0 μM YH3096 rendered approximately 30% of endogenous Ras unprenylated. Subsequently, an immunoblot using H-Ras antibody revealed that >95.0% H-Ras was unprenylated by 1.0 μM of YH3096. Consistent with previous reports [8], our results demonstrated that prenylation of exclusively farnesylated H-Ras is completely

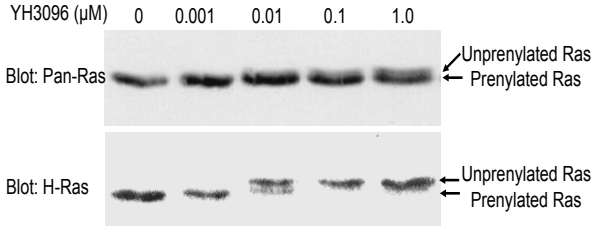

Fig. 1. *Effect of YH3096 on prenylation of endogenous Ras in HIRc-B cells.* HIRc-B cells were grown in 12-well plates and treated with the indicated concentration of YH3096 for 24 h. Cells were lysed with RIPA buffer, and total cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-Pan Ras antibody and anti-H-Ras antibody.

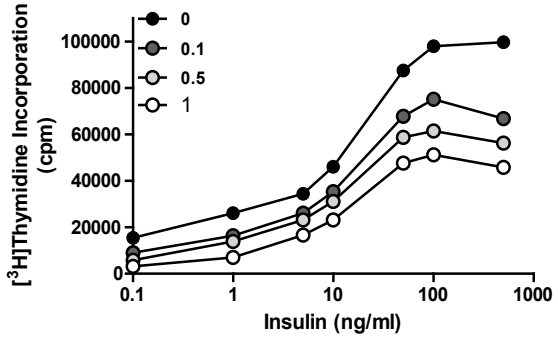

Fig. 2. *Effect of YH3096 on DNA synthesis induced by insulin.* HIRc-B cells were grown in 24-well plates and starved with serum-free DMEM in the presence or absence of YH3096 (0.1, 0.5, and 1.0 μM) for 24 h.The cells were then stimulated with the indicated concentration of insulin for 16 h. [^{3}H]-thymidine (0.5 μCi/mL) was added to each well, and the cells were further incubated for 4 h. Cells were washed, lysed, and subjected to liquid scintillation counting. The data represent mean ± SD of three independent observations.

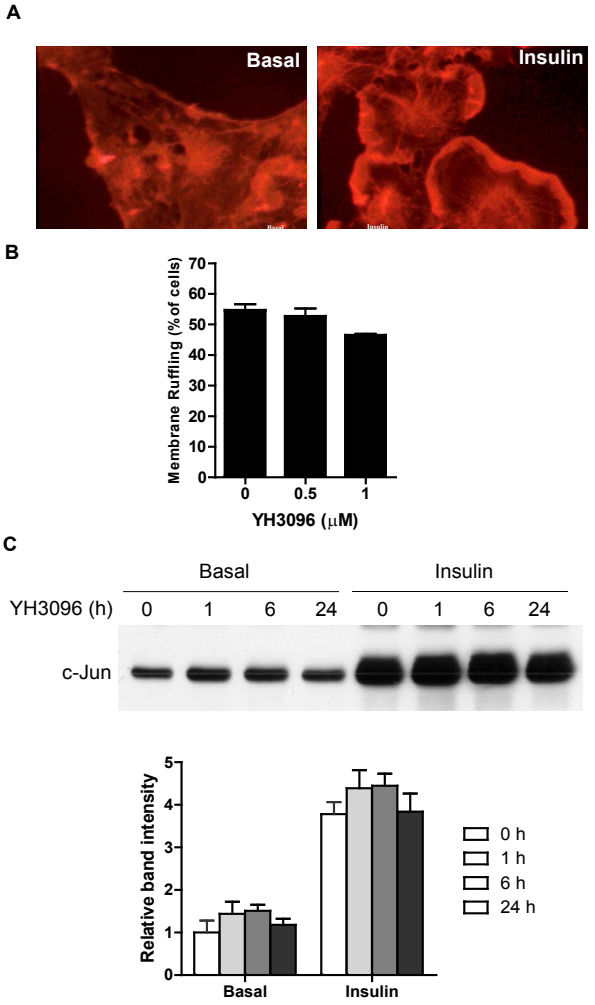

Fig. 3. *Effect of YH3096 on insulin-induced membrane ruffling and c-Jun expression.* (A) Serum-starved HIRc-Bcells were grown on 12-mm glass coverslips and stimulated with or without insulin (100 ng/mL) for 10 min. Cells were fixed and immunostained. (B) HIRc-Bcells as in (A) were incubated in serum-free DMEM containing 0, 0.5 or 1 μM of YH3096 for 24 h. Then cells were stimulated with insulin (100 ng/mL) for 10 min. Cells were fixed and immunostained. Results are expressed as the percent of total cells. Bars, the mean ± SD of three independent experiments. (C) HIRc-B cells were serum-starved for 24 h in serum-free DMEM containing 1 μM of YH3096 and then stimulated with insulin (100 ng/mL) for 4 h. Whole cell lysates were subjected to 10% SDS-PAGE followed by immunoblotting with anti-c-Jun antibody (upper panel). Data were quantitated by using ImageJ software, and the relative band intensity of three independent experiments are presented as mean ± SD (lower panel). Values from cells in the absence of YH3096 and insulin treatment (Basal 0 h) are set to 1.

inhibited by YH3096, but that K-Ras and N-Ras become alternatively genranylgeranylated in the presence of YH3096.

Effect of YH3096 on insulin-induced DNA synthesis

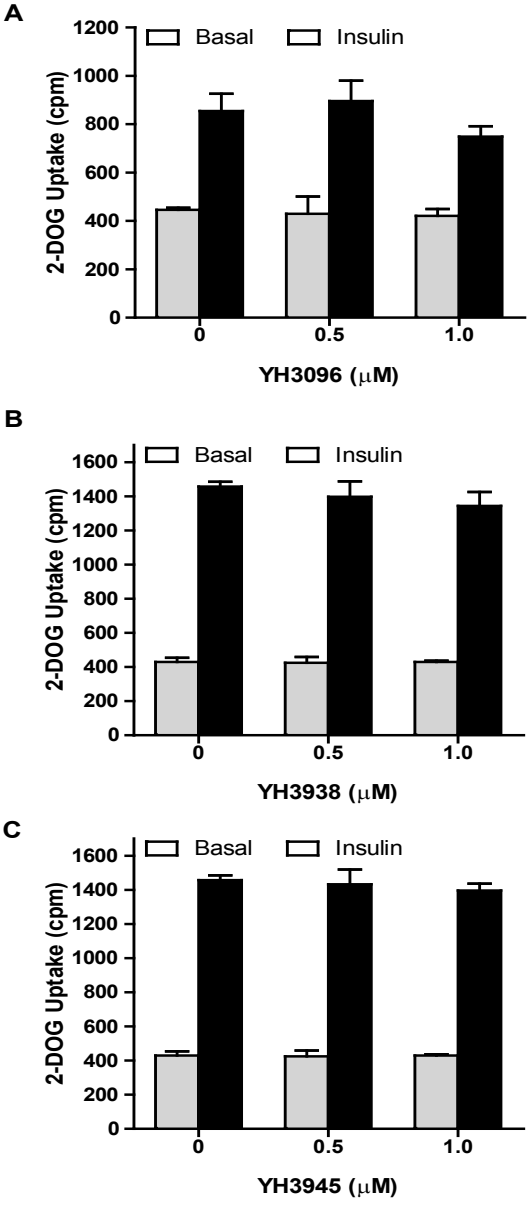

Fig. 4. *Effects of YH3096 and its derivatives on insulin-induced 2-Deoxyglucose uptake*. 3T3-L1 preadipocytes were differentiated in 12-well plates for 7 d. More than 90% of the cells were fully differentiated into adipocytes. The indicated concentration of YH3096 (A), YH3938 (B), or YH3945 (C) was further treated for 4 d. Then 3T3-L1 adipocytes were starved for 4 h, stimulated with insulin (100 ng/mL) for 15 min and incubated with [^{3}H]-2-DOG (0.2 μCi/mL) for 15 min. The incorporation of [^{3}H]-2-DOG was determined. Bars, mean ± SD of three independent experiments.

Knowing that Ras protein is important for insulin-induced DNA synthesis [16], we next investigated the effects of YH3096 on DNA synthesis after insulin stimulation. HIRc-B cells were starved in the presence or absence of 0.1, 0.5, or 1.0 μM YH3096 for 24 h. We then measured the amount of radiolabeled thymidine incorporated into the DNA in response to the increasing concentrations of insulin. As shown in Fig. 2, insulin treatment stimulates DNA synthesis up to fivefold in a dose-dependent manner; however, treatment with YH3096 gradually inhibits insulin-induced DNA synthesis. This result suggests that farnesylation of Ras protein is largely implicated in DNA synthesis, although geranylgeranylation of K-Ras and N-Ras might compensate for the loss of farnesylated Ras function to a lesser extent.

Effect of YH3096 on insulin-mediated membrane ruffling, c-Jun expression, and glucose uptake

To determine whether YH3096 affects other insulin actions, including membrane ruffling, c-Jun expression, and glucose uptake, serum-starved HIRc-B cells were treated with 0.5 or 1.0 μM YH3096 for 24 h and then stimulated with 100 ng/mL insulin. We first determined the effect of YH3096 on insulin-mediated membrane ruffling. Insulin stimulation of HIRc-B cells rapidly induced membrane ruffling (Fig. 3A), but treatment with YH3096 had no effect on it (Fig. 3B). Similarly, YH3096 had no effect on insulin-mediated c-Jun expression (Fig. 3C).

To determine whether YH3096 modulates insulin-mediated glucose uptake, differentiated 3T3-L1 adipocytes were treated with either 0.5 or 1.0 μM YH3096 for 4 d and subjected to an assay of 2-deoxyglucose uptake. As shown in Fig. 4A, insulin similarly increases glucose uptake in both DMSO- and YH3096-treated 3T3-L1 adipocytes. In addition, YH3096 derivatives YH3938 and YH3945 had no effect on insulin- induced 2-deoxyglucose uptake by insulin (Figs. 4B and 4C).

Interestingly, insulin's metabolic pathway, unlike the mitogenic pathway, was not affected by YH3096. These results indicate that geranylgeranylation of K-Ras and N-Ras proteins is mainly involved in membrane ruffling, c-Jun expression, and glucose uptake. It will be important to elucidate the differential functions of farnesylated and geranylgeranylated Ras. Our study demonstrates that YH3096 has an inhibitory effect on insulin-induced DNA synthesis by blocking Ras farnesylation.

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ORCID

Kyunghwan Kim, http://orcid.org/0000-0001-5622-2452

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