Title	GENISTEIN, A TYROSINE KINASE INHIBITOR, BLOCKS THE CELL CYCLE PROGRESSION BUT NOT Ca2+ INFLUX INDUCED BY BAY K8644 IN FRTL-5 CELLS
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GENISTEIN, A TYROSINE KINASE INHIBITOR, BLOCKS THE CELL CYCLE
PROGRESSION BUT NOT Ca^{2+} INFLUX INDUCED BY BAY K8644 IN FRTL-5 CELLS

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SUMMARY: IGF-I, when added to TSH-primed FRTL-5 cells, induces a long lasting Ca^{2+} influx and then DNA synthesis. Ca^{2+} channel agonist, BAYK 8644, is capable to mimic these effects on cell proliferation. We studied the effect of genistein, a specific tyrosine kinase inhibitor, on BAY K8644- or IGF-I- induced cell cycle progression in FRTL-5 cells. Genitein inhibited DNA synthesis induced by BAY K8644 and by IGF-I. In contrast, Ca^{2+} influx stimulated by BAY K8644 was not inhibited. These data demonstrate that the signal transduction pathway induced by BAY K8644 or IGF-I may possibly involve genistein-sensitive process at the downstream step of Ca^{2+} entry. © 1993 Academic Press, Inc.

Proliferation of cells can be induced by combined addition of a competence factor and a progression factor. In rat thyroid cells(FRTL-5), thyroid stimulating hormone(TSH) can function as a competence factor to make cells to respond to a progression factor such as IGF-I(1). Our previous studies(2) showed IGF-I causes sustained Ca^{2+} influx by stimulating G_i protein in TSH-primed FRTL-5 cells and this long lasting Ca^{2+} entry is necessary for the signal transduction for the DNA synthesis. Also in TSH-primed cells, BAY K8644, Ca^{2+} channel agonist, can induce DNA synthesis by causing Ca^{2+} influx which is similar to that induced by IGF-I. Recently there are many reports that show proto-oncogene products which have tyrosine kinase activity such as src, fps, fes, and ros, may play an important role in cell proliferation(3,4). Furthemore, the pre-

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vious studies show TSH or IGF-I can induce the tyrosine phosphorylation of the 175 kD protein in FRTL-5 cells(5,6).

Based on these data,we studied the effects of genistein, known as a specific tyrosine kinase inhibitor(7,8), on the DNA synthesis induced by BAY K8644 or by IGF-I, and on Ca^{2+} influx induced by BAY K8644 so that we might be able to determine the position of the process in which such products may take part.

We show that genistein inhibits DNA synthesis induced by BAY K8644 or IGF-I without inhibiting Ca^{2+} influx, and that genistein has little effect on priming action of TSH. Additionally,we confirm that genistein can work, at least, as a tyrosine kinase inhibitor by showing the inhibitory effect on tyrosine phosphorylation of the 175 kD substrate in FRTL-5 cells.

Matrials and Methods

  Materials:Genistein and TSH were purchased from Sigma, BAY K8644 from Calbiochem, IGF-I and ECL detection kit from Amersham, Anti-phosphotyrosine antibody(PTO1L) from Oncogene Science, $(^{45}Ca)Cl_2$ and [metyl-^{3}H] thymidine from DuPont. Other materials and chemicals were obtained from commercial sources.
  Cell culture and assay for DNA synthesis and Ca^{2+} influx:The rat thyroid cell line (FRTL-5) were seeded into a 24 well Costar dish in Ham's F-12 mediun containing 5% new born calf serum, TSH(250μU/ml), transferrin(5μg/ml), insulin(10μg/ml), somatostatin(10ng/ml), cortisone(10nM), and glycyl-L-histidyl-L-lysin acetate(10ng/ml)(6H medium), and incubated to be subconfluent, then the cells were incubated in serum and hormone free F-12 medium (G$_0$ medium) for 2 days to be growth-arrested(7). For the priming treatment, quiscent FRTL-5 cells were stimulated with TSH(100μU/ml) for 9 hours. This treatment makes the arrested FRTL-5 cells to respond the progression factors to induce Ca^{2+}entry and growth. For measurement of DNA synthesis, primed cells on Costar 24 well culture plates were incubated in Ham F-12 medium containing 0.25%BSA and (^{3}H) thymidine(37kBq/ml) for 48 hours with or without progression factors in each experiment. The reaction was stopped by addidion of 10% trichloroacetic acid and the radio activity in acid insoluble materials was counted in a liquid scintillation spectrometer. For measurement of Ca^{2+} influx, TSH-primed cells on a ϕ3.5 cm dish were stimulated by BAY K8644 for 30 minutes, then put in F-12 medium containing 185kBq/ml $(^{45}Ca)Cl_2$. After 60, 90 or120 seconds medium was removed, washed 5 times with ice-cold PBS containing 25mM MgCl$_2$, then cells were lysed with 3N NaOH and radio activity of ^{45}Ca was counted(8,9). Each assay were performed in triplicate.
  Immunoblots for phosphotyrosine:FRTL-5 cells(1.0x10^6 cells) were seeded into ϕ6 cm dishes in 6H medium. Five days later the cell were washed with PBS, and cultures were continued for additional 48 hours in G$_0$ medium untill the cells became quiescent. The medium was then replaced with G$_0$ medium with or without TSH or genistein and cells were incubated for 8 more hours. Then the cells were treated with IGF-I or BAY K8644 with or without genistein. After 30 minutes, the cells were lysed at 4℃ in 100μl of radioimmune precipitation buffer solution as described(6). The monolayer was scraped into a sample tube. 30μl of concentrated SDS-PAGE sample buffer solution(3xLaemmlis'sample buffer solution containing

9% SDS and 6% 2-mercaptoethanol) was added to 60μl of each sample, and
the mixture was incubated for 5 minutes at 100°C. Each sample was run on
7.5% SDS-PAGE, and immunoblots with anti-phosphotyrsine antibody were
performed as previously described(12), except first antibodies were de-
tected by using ECL western blotting system.

Results

  In TSH-primed cells, genistein(2.0mg/l) markedly inhibited DNA synthe-
sis induced by BAY K8644(Fig.1).

  We next investigated whether genistein modulates the DNA synthesis in-
duced by IGF-I. Genistein(1.0mg/l) markedly inhibited IGF-I-stimulated
DNA synthesis. In contrast, genistein(1.0mg/l) did not affect the prim-
ing action of TSH(Table 1). Furthermore, 2.0mg/l genistein also did not
inhibit the priming action of TSH(data not shown).

  We studied whether genistein acts as a tyrosine kinase inhibitor in
FRTL-5 cells. As shown in Fig.2, the tyrosine phosphorylation of the 175
kD substrate was observed in TSH-primed cells but not in quiescent cells.
BAY K8644 had no effect on the phosphotyrosine content of the 175 kD
substrate both in quiescent cells and in TSH-primed cells. By con-
trast, IGF-I induced the tyrosine phosphorylation of the 175 kD band in
quiescent cells and increased the phosphotyrosine content of the same
substrate in TSH-primed cells. Genistein blocked the tyrosine phosphory-
lation of the 175 kD band increased by TSH and/or IGF-I.

  We further examined whether BAY K8644-stimulated Ca^{2+} entry can be
blocked by genistein in TSH-primed cells. Genistein(2.0mg/l), when added
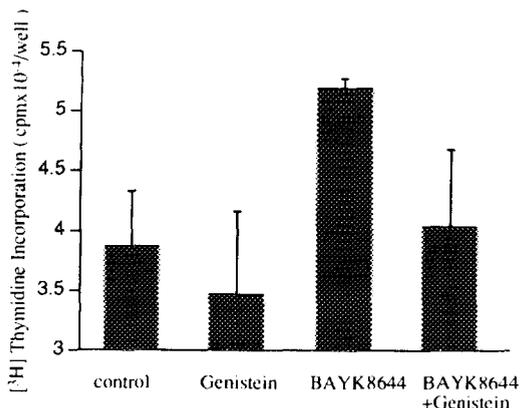
Fig.1. Inhibition of BAY K8644 induced DNA synthesis by genistein. The
primed cells were incubated with or without BAY K8644(0.2μM) with or
without the same time addition of genistein(2.0mg/l). The results are
shown with mean ±SD for triplicate determinations.

Table 1. Effects of genistein on the priming action of TSH

primed with \ treated with	[^{3}H] Thymidine Incorporation (cpm/dish)	
	IGF-I	IGF-I+Genistein
TSH	48559.3 ± 6998.6	28374.0 ± 1583.0
TSH +Genistein	42338.0 ± 3971.3	24383.3 ± 1594.9

The quiescent cells were incubated with TSH(100μU/ml) for 9 hours with or without genistein (1.0mg/l). The cells were washed twice with PBS(-) and incubated with IGF-I(20ng/ml) with or without the same time addition of genistein(1.0mg/l). The results are shown with mean ±SD for triplicate determinations.

with BAY K8644, did not show any inhibitory effect on Ca^{2+} influx(Fig.3). Genistein showed little effect on DNA synthesis induced by a combination of TSH and IGF-I in the arrested cells when it was added together for 48 hours(Fig.4). In addition, the tyrosine phosphorylation of the 175 kD substrate reappeared after 8 hours incubation with a combination of TSH and genistein(Fig.5). Thus, it seems likely that the actions of genistein may only last short period of time in FRTL-5 cells. Therefore, the inhibitory action of genistein on the DNA synthesis induced by BAY K 8644 or IGF-I may not be due to non-specific toxic effect.

Discussion

Our present study shows that genistein blocks cell cycle progression induced by BAY K8644 without inhibiting Ca^{2+} entry. Thus, the mitogenic signal transduction pathway stimulated by the sustained Ca^{2+} entry may

200kD →
175kD →
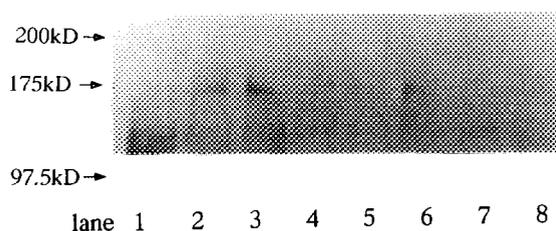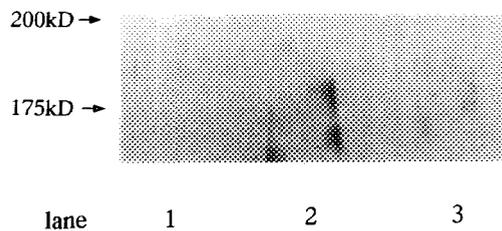97.5kD →
lane 1   2   3   4   5   6   7   8

Fig. 2. Inhibitory action of genistein on tyrosine phosphorylation in FRTL -5 cells. Quiescent cells were incubated with(lane 2,3,4,6,7) or without (lane 1,5,8) TSH(100μU/ml) for 8 hours, then added IGF-I(20ng/ml)(lane 3,4,5), BAY K8644(0.2μM)(lane 6,7,8), and genistein(2.0mg/l)(lane 4,7). After 30 minutes cells were lysed and analyzed by immunoblots with anti-phosphotyrosine antibody.
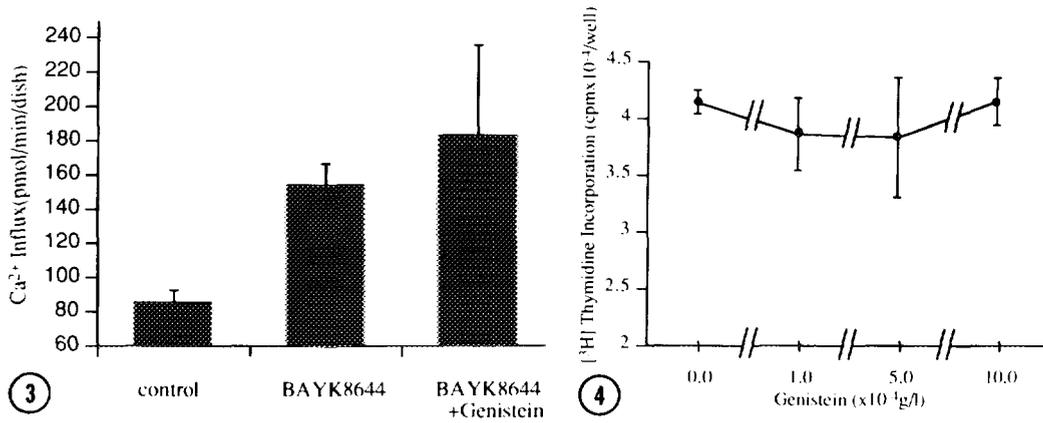
Fig.3.Effect of genistein on BAY K8644-induced Ca^{2+} influx. The TSH-primed cells were stimulated with BAY K8644(0.2μM) in the absence or presence of genistein(2.0mg/l). The results are shown with mean ±SD for triplicate determinations.

Fig. 4. Effect of genistein on DNA synthesis by TSH plus IGF-1 in quiescent cells. The quiescent cells were incubated with TSH(100μU/ml), IGF-1 (20ng/ml) and genistein(0,0.1,0.5 or 1.0mg/l), and [^{3}H]thymidine incorporation was counted after 48 hours. The results are shown with mean ±SD for triplicate determinations.

involve genistein-sensitive process. In contrast, genistein does not inhibit priming action of TSH. Therefore, there is little possibility that any genistein-sensitive proteins exist on the signal transduction step activated by TSH to make the arrested FRTL-5 cells respond to progression factors. However, the possibility that the genistein-sensitive pathway might exist on the late phase of the priming process by TSH remains to be clarified, since the actions of genistein may last a short period.

Fig.5.Duration of genistein action on tyrosine phosphorylation in FRTL-5 cells. The quiescent cells were incubated for 8 hours with(lane 2,3) or without(lane 1) TSH(100μU/ml). Genistein(2.0mg/l) was added at the same time(lane 3).

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The IGF-I receptor is known to have tyrosine kinase in the intra-cellular domain(13,14). Genistien, however, does not inhibit the tyrosine kinase activity of IGF-I receptor(15). On the other hand, IGF-I is capable of inducing a long lasting Ca^{2+} entry in TSH-primed cells. More-ever, like BAY K8644, IGF-I-induced Ca^{2+} entry is the mitogenic signal. Therefore, it seems possible that the signal transduction pathway evoked by IGF-I might involve genistein-sensitive process, at least in a part, at the downstream step of Ca^{2+} entry.

Genistein-sensitive proteins activated on the progression process are not identified at present. However, genistein is known as a specific tyrosine kinase inhibitor. In fact, genistein can act as a tyrosine kinase inhibitor in FRTL-5 cells. So, we might be able to say that there is high possibility that proto-oncogene products, which have tyrosine kinase activity such as src or abl, exist on the mitogenic pathway at the downstream of Ca^{2+} entry. Also recently, there appeared some inter-esting data that such tyrosine kinases can be activated by Ca^{2+} iono-phore treatment(16).

Further, our previous studies showed that there exists a signal trans-duction pathway which is sensitive to a HMG-CoA reductase inhibitor at the downstream of Ca^{2+} entry(17). This fact means that small G protein such as p21 ras may possibly exist at the close position of genistein-sensitive step.

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