Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 17-21

PRAVASTATIN, A HMG-CoA REDUCTASE INHIBITOR, BLOCKS THE CELL CYCLE PROGRESSION BUT NOT Ca^{2+} INFLUX INDUCED BY IGF-I IN FRTL-5 CELLS

Hisato Tada, Kaoru Takada†, Nobuyuki Amino and Kiyoshi Miyai

Department of Laboratory Medicine, Osaka University School of Medicine, 1-1-50 Fukushima, Fukushima-ku, Osaka 553, Japan

Received April 12, 1991

SUMMARY: IGF-I, when added to the TSH-primed FRTL-5 cells, induces a long lasting Ca^{2+} influx, and then, DNA synthesis. Moreover, Ca^{2+} channel agonist, BAY K8644 can mimic these effects on cell proliferation. We studied the effect of HMG-CoA reductase inhibitor, Pravastatin on IGF-I-induced cell cycle progression in FRTL-5 cells. Pravastatin inhibited DNA synthesis induced both by IGF-I and by BAY K8644. In contrast, Ca^{2+} influx stimulated by IGF-I was unaffected. These data demonstrate that the signal transduction pathway evoked by IGF-I may possibly involve pravastatin-sensitive process at the downstream step of Ca^{2+} entry. HMG-CoA reductase inhibitors are known to modulate some cellular signal transduction systems by blocking the membrane attachment of low molecular weight GTP binding proteins such as $p21^{ras}$. Therefore, pravastatin-sensitive process that we have shown here might possibly involve some of such small G protein.

Insulin like growth factor-I(IGF-I) is known to induce cell cycle progression in many cell lines. However, the mechanism by which a progression factor, IGF-I, stimulates DNA synthesis is little known. IGF-I can stimulate the sustained Ca^{2+} influx in TSH-primed FRTL-5 cells but not in quiescent cells. Possibly, this long lasting Ca^{2+} entry may be a cell cycle-dependent mitogenic signal, because reduction of IGF-I-stimulated Ca^{2+} influx by Ca^{2+} antagonists such as Co^{2+} and La^{3+}, or in Ca^{2+}-free medium, attenuates IGF-I induced DNA synthesis, and because Ca^{2+} agonist, BAY K8644 also can induce DNA synthesis (1). Further, the importance of the sustained Ca^{2+} influx on the cell cycle progression is shown also in Balb/c 3T3 fibroblasts (2). Here, we studied the effect of pravastatin, HMG-CoA reductase inhibitor, on DNA synthesis and Ca^{2+} influx by IGF-I in FRTL-5 cells. We show that pravastatin inhibits DNA synthesis induced both by IGF-I

† To whom correspondence should be addressed.

Abbreviations: Bt2cAMP;N^6-2'-o-dibutyryladenosine 3'-5'-cyclicmonophosphate, G protein; GTP binding protein, HMG-CoA;3-hydroxy-3-methylglutaryl coenzyme A, IGF-I;Insulin like growth factor-I, TSH;Thyroid stimulating hormone.

0006-291X/91 $1.50

and by BAY K8644 without affecting Ca^{2+} influx. We also show that pravastatin has no effect on priming action of TSH or Bt2cAMP that can activate growth-arrested FRTL-5 cells to respond progression factors. Furthermore, we discuss the possibilities of the involvement of some low molecular G proteins on the signal transduction pathway because HMG-CoA reductase inhibitors are known to inhibit the membrane attachment of several low molecular G proteins and so inactivate them (3) and because several lines of papers suggest that the involvement of low molecular weight GTP binding proteins in the signal transduction pathway by IGF-I (4).

Materials and Methods

Materials: Pravastatin (Mevalotin) is the kind gift of Sankyo co.ltd. [*methyl*-^{3}H]Thymidine and [^{45}Ca]Cl were purchased from DuPont; TSH, forskolin, Bt2cAMP, insulin, and mevalonate from sigma; IGF-I from Mallinckrodt; BAY K8644 from Calbiochem. 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 24-well Costar tray or Ø3.5cm dish in Ham F-12 medium containing 5% newborn calf serum,TSH (10^2μU/ml), transferrin (5μg/ml), insulin (10μg/ml), somatostatin (10ng/ml), cortisone (10nM), and glycyl-L-histidyl-L-lysine acetate (10ng/ml) with some modification from the previous description (5), and were incubated to be subconfluent, then the cells were incubated in serum-and hormones-free F-12 medium for 2 days to be growth-arrested (6). For the priming treatment, quiescent FRTL-5 cells were stimulated with TSH (10^2μU/ml) or Bt2cAMP (1μM) for 6 hours. This treatment makes the arrested FRTL-5 cells respond the progression factors to induce Ca^{2+} entry and growth. For measurement of DNA synthesis, primed cells on Costar's 24-well culture plate 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 addition of 10% trichloroacetic acid and the radioactivity in acid-insoluble materials was counted in a liquid scintillation spectrometer. For measurement of Ca^{2+} influx, TSH-primed cells on Ø3.5cm dish were stimulated by IGF-I for 30 minutes, then put in F-12 medium containing 185kBq/ml [^{45}Ca]Cl. After 30, 60, or 90 seconds, medium was removed,washed 5 times with ice-cold PBS containing 25mM $MgCl_2$. Then cells were lysed with 1N NaOH and radioactivity of [^{45}Ca] was counted (7,8). Each assays were performed in triplicate.

Results

In TSH-primed FRTL-5 cells, pravastatin markedly inhibited IGF-I induced DNA synthesis in a dose-dependent manner (Fig.1). The inhibitory action of pravastatin may not be due to a non-specific toxic effect since the effect of pravastatin was attenuated when mevalonate, the product of the reaction catalyzed by HMG-CoA reductase, was added. These data show that IGF-I stimulates the cell cycle progression via a pravastatin-sensitive pathway in TSH-primed cells.

We next investigated whether the priming action of TSH or Bt2cAMP was modulated by the treatment of the quiescent cells with pravastatin. As shown in Table 1, pravastatin didn't affect the priming effect of TSH or Bt2cAMP.

It has been shown recently that IGF-I can stimulate a long lasting Ca^{2+} influx in TSH-primed FRTL-5 cells (1). We further examined whether the action of IGF-I on Ca^{2+} entry was inhibited by the addition of pravastatin in TSH-primed cells. Pravastatin had no effect on Ca^{2+}

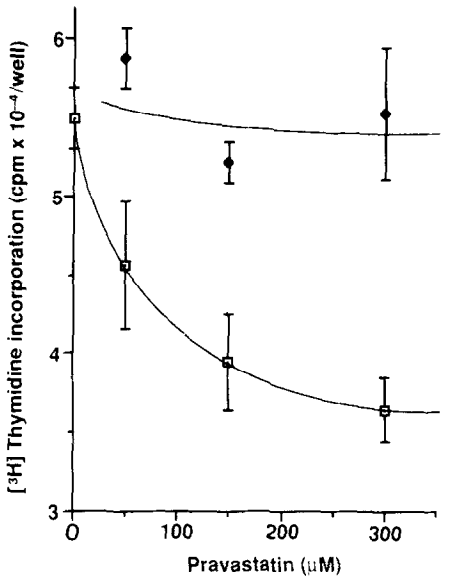


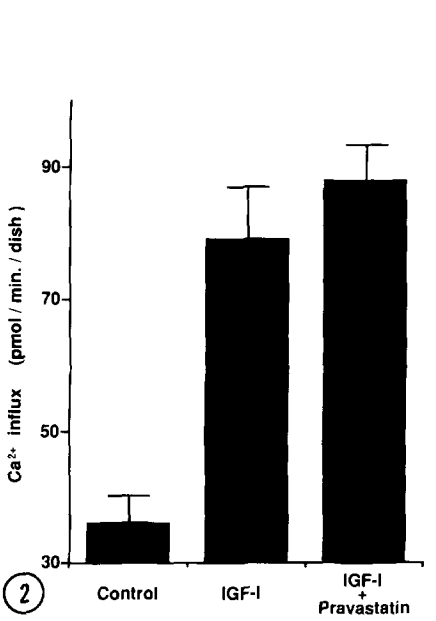
Fig.1. Dose-dependent inhibition by pravastatin on IGF-I-induced DNA synthesis. The primed cells were stimulated by IGF-I (20ng/ml) with the same time addition of the indicated concentrations of pravastatin in the presence (◆) or absence (□) of mevalonate (1.2mM). The results are shown with mean±SD for triplicate determinations.

influx by IGF-I (Fig.2). Thus, the activation of Ca^{2+}-channel by IGF-I may involve a mechanism independent from the pravastatin-sensitive system. In contrast, pravastatin markedly inhibited the cell cycle progression stimulated by BAY K8644, an agonist of voltage-dependent Ca^{2+} channel, in a dose-dependent manner that was similar to that for inhibition of IGF-I-induced DNA synthesis (Fig.3). Therefore, our results demonstrate that the inhibitory effect of pravastatin on cell cycle progression stimulated by IGF-I may result from modulation, at least in a part, at the downstream sites of Ca^{2+} entry on the signal transduction system.

Table 1. No inhibition by pravastatin on the priming effect of TSH or Bt2cAMP

treated with \ primed with	[3H]Thymidine incorporation (c.p.m./ dish)	
	TSH (10^2μU/ml)	Bt2cAMP (1μM)
None	11477 ± 521	15254 ±923
Pravastatin(300μM)	11869 ±1088	14001 ±715
Pravastatin+Mevalonate(1.2mM)	12172 ± 218	13477 ±333

The quiescent FRTL-5 cells were treated with or without above factors for 16 hours. In the last 6 hours of this treatment, TSH or Bt2cAMP was added to prime the cells. Then, the cells were washed twice with phosphate-buffered saline to remove TSH, Bt2cAMP, pravastatin and/or mevalonate, then were stimulated with IGF-I (20ng/ml). The counts without IGF-I stimulation were 5051 ±311 for TSH primed cells, 6085±271 for Bt2cAMP primed cells, respectively, which showed little increase to that of the quiescent cells. Results are shown with mean±SD for triplicate determinations.



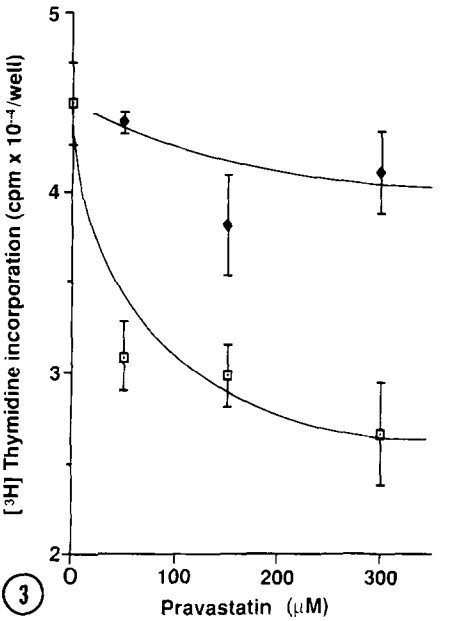


Fig.2. Effect of pravastatin on the Ca^{2+} influx induced by IGF-I. The TSH-primed cells were incubated with or without pravastatin (300μM) for 6 hours, then stimulated by IGF-I (20ng/ml) for 30 min. The results are shown with mean±SD for triplicate determinations.

Fig.3. Dose-dependent inhibition by pravastatin on the DNA synthesis induced by BAY K8644. The primed cells were stimulated by BAY K8644 (0.1μM) with the same time addition of the indicated concentrations of pravastatin in the presence (◆) or absence (□) of mevalonate (1.2mM). The results are shown with mean±SE for triplicate determinations.

Discussion

Our present study showed that pravastatin inhibits IGF-I-induced cell cycle progression at the downstream step of Ca^{2+} entry and that it does not inhibit priming effect of TSH or Bt2cAMP.

HMG-CoA reductase, which is the rate limiting enzyme on sterol biosynthetic pathway, also plays a important role in providing farnesyl acids to some membrane proteins when anchoring to cellular membranes. Many low moleculer weight GTP binding proteins and some of G protein subunits of Yeast are thought to be among these proteins (9). HMG-CoA reductase inhibitors inactivate newly synthesized these proteins by leaving them free from the plasma membrane. Previously, we reported that insulin and IGF-I can induce Ca^{2+} channel activation via some pertussis toxin sensitive G protein (1). Our present data shows that this G protein might not have pravastatin sensitivity, because no inhibition of Ca^{2+} influx was observed. Although further investigations are required to determine the pravastatin-sensitive target protein situated on the downstream pathway of the Ca^{2+} channel activation, there are many reports that show the involvement of $p21^{ras}$ in cell proliferation (10). In addition, some papers suggests that $p21^{ras}$ might be on the signal transduction pathway of Insulin and IGF-1 (4).

The priming effect of TSH or Bt2cAMP on the growth-arrested FRTL-5 cells to be able to respond progression factors needs at least several hours' presence of these factors (1). Moreover, the primed cells become to fail to respond progression factors 24 hours after the washing-removal of the effective concentrations of TSH. So, in inducing priming effect TSH or Bt2cAMP might need some synthetic process of the limited-lived substances which our data shows to be not pravastatin sensitive. There are some reports that PDGF which is a competence factor in fibroblasts, would activate $p21^{ras}$ (11,12). If pravastatin that we used are sufficient to inhibit ras protein and/or other small G proteins, our result might mean priming effect of TSH doesn't need activation of ras protein in FRTL-5 cells.

Acknowledgments

This work was supported by a research grant from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, and a Grant-in Aid for Scientific research (to N.A.; no. 61480178) from the Ministry of Education, Science, and Culture of Japan. The authors are grateful to Miss Yuki Hirano for her secretarial assistance.

References

1. Takada,K., Amino,N.,Tada,H., and Miyai,K. (1990) J.Clin.Invest. **86**, 1548-1555.
2. Kojima,I., Matsunaga,H., Kurokawa,K., Ogata,E., and Nishimoto,I. (1988) J.Biol.Chem. **263**, 16561-16567.
3. Hancock,J.S., Magee,A.I., Childs,J.E., and Marshall,C.J. (1989) Cell **57**, 1167-1177
4. Burgering, B.M.T.,Snijders, A.J.,Maassen, J.A., van der EB,A.J., and Bos,J.L. (1989) Mol. Cell.Biol. **9**, 4312-4322
5. Ambesi-Impiombato,F.S., Picone,R., and Coon,H.G. (1980) Proc.Natl.Acad.Sci.USA **77**, 3455-3459
6. Damante,G., Cox,F., and Rapoport,B. (1988) Biochem. Biophys. Res. Commun. **151**, 1194-1199
7. Mauger,J.P., Poggioli,J., Guesdon,F., and Claret,M. (1984) Biochem.J. **221**, 121-127
8. Muldoon,L.L., Rodland,K.D., and Magun,B.E. (1988) J.Biol.Chem. **263**, 18834-18841
9. Schafer,W.R., Kim,R., Sterne,R., Thorner,J., Kim,S.H., and Rine,J. (1989) Science **245**, 379-385
10. Mulcahy,L.S., Smith,M.R., and Stacy,D.W. (1985) Nature **313**, 241-243
11. Gibbs,J.B., Marshall,M.S., Scolnick,E.M., Dixon,R.A.F., and Vogel,U.S. (1990) J.Biol. Chem. **265**, 20437-20442
12. Satoh,T., Endo,M., Nakafuku,M., Nakamura,S., and Kaziro,Y. (1990) Proc.Natl.Acad. Sci.USA **87**, 5993-5997