Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 399-406

LOVASTATIN DISRUPTS EARLY EVENTS IN INSULIN SIGNALING: A POTENTIAL MECHANISM OF LOVASTATIN'S ANTI-MITOGENIC ACTIVITY

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Received August 11, 1994

The mechanism by which lovastatin lowers cholesterol levels is well characterized but little is known about its anti-mitogenic and anti-tumorigenic mechanism. Here we demonstrate that lovastatin disrupts early events in the mitogenic signaling pathways of insulin. Insulin treatment (200 nM) of quiescent HIR rat-1 fibroblasts results in an 8-fold stimulation of phosphatidylinositol-3-kinase (PI-3-K) activity. Overnight pretreatment of cells with lovastatin (20μM) inhibits insulin stimulation of PI-3-K activity by 75%. Immunoprecipitation and immunoblotting experiments using antibodies against the regulatory subunit of PI-3-K (p85), phosphotyrosine, and insulin receptor α and β subunits demonstrate that lovastatin inhibits the association of p85 with tyrosine phosphorylated insulin receptor substrate-1 and the β subunit of the insulin receptor. Furthermore, lovastatin dramatically reduces (70-100%) the level of tyrosine phosphorylated insulin receptor β subunit following insulin stimulation. These results clearly demonstrate that lovastatin disrupts early events of insulin mitogenic signaling by reducing the levels of tyrosine phosphorylated β subunit and suggest that this disruption is a potential mechanism for the anti-mitogenic effect of lovastatin.

The discovery of potent inhibitors of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate limiting step in cholesterol biosynthesis, has been of great therapeutic benefit to hypercholesterolemia patients (1-4). The prototype of these inhibitors, lovastatin, has been thoroughly studied and much is known about its cholesterol-

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Abbreviations: IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI-3-K, phosphatidylinositol-3-kinase; P-Y, phosphotyrosine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor.

0006-291X/94 $5.00

lowering mechanism (1,4). Little attention has been paid, however, to the mechanism(s) by which lovastatin inhibits growth factor-induced DNA synthesis and cell proliferation (8-10) as well as inhibits tumor growth in animals (5-7). Recently, several proteins that are involved in growth factor signal transduction have been shown to be lipid-modified by cholesterol biosynthesis intermediates such as farnesylpyrophosphate and geranylgeranylpyrophosphate (11). Lovastatin inhibits the biosynthesis of these two isoprenoids and one possible mechanism by which lovastatin inhibits cell growth may be by interfering with growth factor signaling pathways that require farnesylated or geranylgeranylated proteins.

Insulin is a mitogen which stimulates DNA synthesis by acting through a high affinity plasma membrane receptor which is composed of two α and two β subunits. The α subunit (p130) is responsible for insulin binding whereas the β subunit (p95), an integral membrane protein with a large cytoplasmic domain, has an intrinsic tyrosine kinase activity (12,13). Binding of insulin to its receptor results in activation of the receptor tyrosine kinase, autophosphorylation of the β-subunit and tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (14). The phosphotyrosine residues on IR and IRS-1 serve as sites for the recruitment of proteins that contain Src homology 2 (SH-2) domains (15-18). Among these SH-2-containing proteins are important signaling molecules such as phosphatidylinositol-3-kinase (PI-3-K) (16,17).

Activation of the PI-3-K limb of growth factor signal transduction pathways is believed to play a critical role in growth factor-induced DNA synthesis (19). PI-3-K is a heterodimeric enzyme composed of 110,000 and 85,000 dalton subunits (p110 and p85) (20,21). p110 is the catalytic subunit of PI-3-K that phosphorylates PI, PI-4-P and PI-4,5-P_2 on the 3' position of the inositol ring (20,21). p85 is the regulatory subunit that contains two SH-2 domains allowing the enzyme to directly associate with specific tyrosine phosphorylated motifs (YXXMs). Thus, stimulation of cells with either insulin or platelet-derived growth factor (PDGF), another mitogen that mediates its effects through a tyrosine kinase receptor, results in receptor association and activation of PI-3-K as well as the appearance of its p85 subunit in anti-phosphotyrosine immunoprecipitates (22,23). Interestingly, in the PDGF signaling pathway, association of p85 with PDGF receptor (PDGFR) has been shown to be required for PDGF-induced mitogenesis (19). Recently, we showed, using NIH3T3 cells, that lovastatin inhibits both PDGF and insulin activation of PI-3-K activity (24). For PDGF, our data also indicated that inhibition of PI-3-K activity by lovastatin correlated with a decreased level of association between the p85 subunit of PI-3-K and the tyrosine phosphorylated PDGFR (24). It is not known at this point, however, if this decreased p85/PDGFR complex formation is a reflection of reduced PDGFR tyrosine phosphorylation. Although this is an important question that carries with it implications on not only PI-3-K activation but potentially all receptor tyrosine kinase-dependent events of PDGF,

our ability to address this question was not possible using NIH3T3 cells and PDGF (see Results and Discussion). Therefore, we have used rat-1 fibroblasts that stably overexpress human IR to examine the effects of lovastatin on early events of IR signaling. Utilizing specific antibodies directed against PI-3-K p85 subunit, phosphotyrosine, and IR α and β subunits, we demonstrate that lovastatin not only disrupts insulin-induced association between p85 and IR/IRS-1, but does so by reducing the levels of tyrosine phosphorylated IRβ subunit.

MATERIALS AND METHODS

All experiments were performed with HIR cells (rat-1 fibroblasts that stably overexpress the human insulin receptor (25)) that were plated in 100 mm dishes in Dulbecco's modified Eagles's medium supplemented with F12 nutrient solution (1:1) (DMEM/F12) containing 10 % fetal bovine serum (FBS) and 1 % Pen-Strep (GIBCO laboratories). After 3 or 4 days in culture, cells were confluent and were switched to DMEM/F12 containing 1 % BSA but no FBS. Cells starved 24 h were next treated overnight with either 20 μM lovastatin (a gift from Dr. Alfred W. Alberts, Merck Sharp and Dohme, Rahway, NJ, USA) or with vehicle (PBS, pH 7.5) and then stimulated with either 200 nM insulin or vehicle (PBS, pH 7.5) at 37°C for 10 min. Cells were lysed in buffer containing 1 % Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 2 mM vanadate, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1 mg/ml trypsin inhibitor and 20 mM p-nitrophenylphosphate, vortexed, and subsequently cleared by centrifugation at 14,000 rpm for 30 min at 4°C.

Measurement of PI-3-kinase activity in cleared lysates (1600 μg protein) was performed in a similar manner as described previously (24). The reaction product [^{32}P]PI-3-P was extracted from the reaction mixture, resolved by thin layer chromatography (TLC), and visualized by autoradiography. PI-3-K activity was quantitated by densitometric scanning of [^{32}P]PI-3-P spots on X-ray film (24).

Immunoprecipitations were achieved by incubating cleared lysate with either 0.5 μl rabbit anti-PI-3-kinase (anti-p85 subunit) antiserum (a gift from Brian Schaffhausen, Department of Biochemistry, Tufts University and Lewis Cantley, Department of Cellular and Molecular Physiology, Harvard University), 40 μl anti-phosphotyrosine antibody (prepared from culture supernatants of FB2 clone, ATCC), 50 μl anti-insulin receptor α subunit (anti-IRα; monoclonal antibody 83.7) (26), or 2 μg of anti-IRβ monoclonal antibody CT-1 (26). Both anti-IRα and anti-IRβ antibodies were prepared in the laboratory of Kenneth Siddle, (University of Cambridge, England) and were the gift of Peter Isakson, (Monsanto, Saint Louis, MO). Immunoprecipitations for each antibody were performed with a constant amount of lysate protein in each sample (200 μg for anti-p85, 250 μg for anti-IRα, and 400 μg for anti-phosphotyrosine FB2). Antibody/antigen complexes were precipitated using 15 μl of either protein A-agarose (for anti-p85) or anti-mouse IgG-agarose (for all other antibodies) and incubated overnight at 4°C with constant mixing. Immunoprecipitates were then washed four times with lysis buffer and subsequently incubated with 40 μl SDS-PAGE sample buffer for 2 min at 100°C. Proteins in the supernatants were electrophoresed on 7.5 % polyacrylamide gels and transferred to nitrocellulose filters. Filters were blocked with 5 % nonfat dry milk in PBS, 0.1% Tween-20 (PBS-T) then probed with either anti-phosphotyrosine antibody 4G10 (diluted 1000-fold with 3 % nonfat dry milk in PBS-T), anti-p85 antiserum or anti-IRβ monoclonal antibody CT-1 (diluted 2000-fold with 3 % nonfat dry milk in PBS-T). Positive antibody reactions were visualized using an appropriate peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection system (ECL, Amersham Corp.).

RESULTS AND DISCUSSION

Lovastatin inhibits insulin-induced activation of PI-3-K activity in HIR rat-1 fibroblasts. Recently, in an effort to investigate potential anti-mitogenic mechanisms of lovastatin, we examined the effect of this compound on early events of growth factor signal transduction in NIH3T3 mouse fibroblasts. It was demonstrated that lovastatin inhibited both insulin- and PDGF-induced activation of PI-3-K (24). However, the mechanism by which lovastatin decreases growth factor-induced PI-3-K activation is not known. Our studies with PDGF suggested that lovastatin reduces association of PI-3-K with tyrosine phosphorylated PDGFR (24). However, direct investigation as to whether this observation is a reflection of lower PDGFR phosphotyrosine levels was not possible due to unavailability of anti-PDGFR antibodies capable of efficient immunoprecipitation and blotting. Therefore, in order to address this question, we have investigated the mechanism by which lovastatin decreases insulin activation of PI-3-K since effective antibodies against IR are readily available. In choosing this system, it was also necessary to switch to HIR rat-1 fibroblasts that stably overexpress human IR since NIH3T3 cells express low levels of IR. Quiescent HIR rat-1 cells were treated with or without lovastatin (20μM) overnight and then stimulated for 10 minutes with insulin (200nM) or vehicle. Cells were lysed and the lysates immunoprecipitated with antiphosphotyrosine antibody. Immunoprecipitates were assayed for PI-3-K activity as described in Materials and Methods and (24). Figure 1 shows that, in the absence of lovastatin, insulin stimulated PI-3-K activity by 8-fold. However, in cells pretreated with lovastatin, insulin stimulation of PI-3-K activity was inhibited by 75 % (Figure 1, A and B).

Lovastatin disrupts the association of PI-3-K with the IR/IRS-1 complex. Activation of PI-3-K by receptor tyrosine kinases is believed to be dependent on the association of PI-3-K with these receptors. In order to determine the effects of lovastatin on complex formation between PI-3-K p85 subunit and IR and/or IRS-1, quiescent HIR rat-1 cells were treated with or without lovastatin (20 μM) for 16 hours, stimulated with insulin (200nM) for 10 minutes, and cell lysates were immunoprecipitated with an anti-p85 antibody as described in Materials and Methods. Tyrosine phosphorylated proteins that co-immunoprecipitated with the p85 subunit were separated by SDS-PAGE and detected by an anti-phosphotyrosine antibody. Insulin stimulation of HIR cells not treated with lovastatin, resulted in an insulin-dependent association of PI-3-K with two tyrosine-phosphorylated proteins: the β subunit of the insulin receptor (p95) and IRS-1 (p185) (Figure 2A). Both IRS-1, which contains nine p85 YXXM binding motifs, as well as IRβ subunit, which possesses a single C-terminal YTHM motif, have been shown to bind the p85 subunit of PI-3-K (15). Overnight pretreatment with lovastatin reduced the association of the p85 subunit with tyrosine-phosphorylated p95 and p185 by 57 % and 70 %,

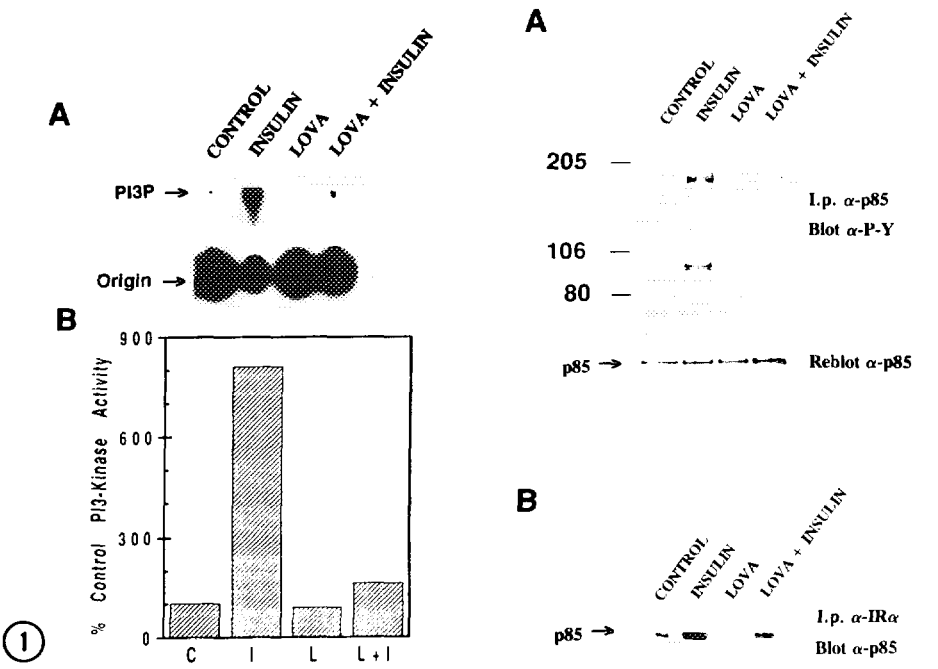

Fig. 1. Lovastatin inhibition of insulin-induced PI-3-kinase activity. Starved HIR cells were treated with or without lovastatin, insulin stimulated, lysed and PI-3-kinase activity measured as described under "Materials and Methods." *A*, ^{32}P-phospholipids separated by TLC. *Arrows* indicate the origin and the spot corresponding to PI-3-P. *B*, a graphical presentation of the PI-3-kinase activity. C, I, L designate control, insulin and lovastatin, respectively.

Fig. 2. Lovastatin inhibition of PI-3-Kinase (p85)/IRS-1/IRβ subunit complex formation. HIR cells were treated with or without lovastatin, stimulated with insulin and lysed. Immunoprecipitation of proteins with anti-p85 (*A*) or anti-IRα (*B*) antibodies, electrophoresis, and blotting with either anti-phosphotyrosine (*A, upper panel*) or anti-p85 antibodies (*A, lower panel* and *B*) were performed as described under "Materials and Methods." The same nitrocellulose was used for probing for phosphotyrosine and p85 in the *upper* and *lower* panels of *A*, respectively.

respectively (Figure 2A). Although it is not known whether the association of p85 with IRβ is one that is direct or indirect with IRS-1 serving as an adaptor, it is clear from these results that the extent of complex formation between p85 and tyrosine phosphorylated IR/IRS-1 is reduced by lovastatin. This decrease in the association of p85 with IR and IRS-1 is not due to inhibition of the expression of p85 since lovastatin had no effect on the actual amount of p85 subunit in these cells (Figure 2A, bottom). Furthermore, expression of the IR β subunit was unchanged after overnight pretreatment of HIR rat-1 cells with lovastatin (data not shown). Thus, the results suggest that inhibition by lovastatin of insulin-induced activation of PI-3-K (Figure 1) is due to a decrease of the levels of the insulin receptor/IRS-1/PI-3-K complex formed upon insulin stimulation.

The effect of lovastatin on the association of PI-3-K with insulin receptor was confirmed by immunoprecipitating HIR rat-1 cell lysates with an antibody to the α subunit of the insulin receptor and western blotting the electrophoretically-separated proteins with the anti-p85 antibody. Stimulation with insulin resulted in an insulin-dependent increase in the amount of p85 associated with the insulin receptor (Figure 2B). Quantitation of the p85 bands by densitometric scanning showed that insulin induced a 2.7- fold increase in the amount of p85 associated with insulin receptor. Overnight pretreatment of cells with lovastatin prior to insulin stimulation inhibited this association by 41% (Figure 2B). These results clearly demonstrate that insulin-dependent recruitment of PI-3-K to IR as well as subsequent activation of PI-3-K is impaired by lovastatin.

Lovastatin reduces insulin-stimulated levels of tyrosine phosphorylated β subunit of the insulin receptor. One plausible mechanism by which lovastatin could inhibit the formation of the insulin receptor/IRS-1/PI-3-K complex is by decreasing the levels of tyrosine-phosphorylated IR β subunit. To address this question we treated quiescent HIR rat-1 cells with or without lovastatin (20μM) for 16 hours, stimulated the cells with insulin (200nM) for 10 minutes, then immunoprecipitated the insulin receptor with an antibody to the α subunit. The immunoprecipitated proteins were separated by SDS-PAGE and western blotted with an anti-phosphotyrosine antibody as described in Materials and Methods. In insulin-stimulated cells (in the absence of lovastatin), the β subunit of the insulin receptor was the major tyrosine phosphorylated protein that co-immunoprecipitated with IRα (Figure 3A). Lysates from cells pretreated with lovastatin prior to insulin stimulation had an 80% lower level of tyrosine phosphorylated β subunit (Figure 3A). The effect of lovastatin on β subunit phosphotyrosine levels was directly assessed by immunoprecipitating the tyrosine phosphorylated subunit with an anti-phosphotyrosine antibody, separating the proteins by SDS-PAGE and blotting with an antibody specific for the β subunit. Figure 3B shows that lysates from cells stimulated with insulin in the absence of lovastatin contained tyrosine phosphorylated β subunit whereas unstimulated cells did not. Overnight pretreatment of cells with lovastatin prior to insulin stimulation resulted in cells with no detectable levels of tyrosine phosphorylated β subunit (Figure 3B). As mentioned above, lovastatin did not affect IRβ expression as assessed by anti-IRβ western blotting (data not shown). This combination of immunoprecipitation/blotting studies with antibodies against IRα, IRβ, and phosphotyrosine clearly demonstrates that lovastatin inhibits the levels of insulin-dependent tyrosine phosphorylation of the β subunit of the insulin receptor. Whether this reduction of the level of tyrosine phosphorylation of the β subunit is due to inhibition of the intrinsic tyrosine kinase activity of IRβ or activation of a phosphotyrosine phosphatase is not known.

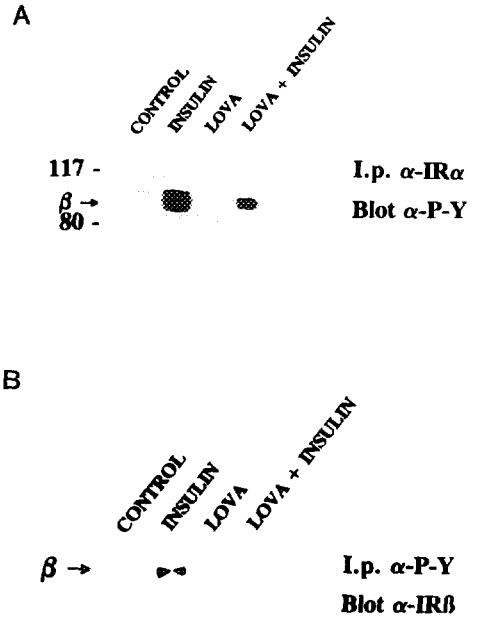

Fig. 3. Lovastatin inhibition of insulin receptor β subunit tyrosine phosphorylation. HIR lysates, prepared as described in the legend of Fig. 2, were either immunoprecipitated with anti-IRα antibody and subsequently western blotted with anti-phosphotyrosine antibody (*A*) or immunoprecipitated with anti-phosphotyrosine antibody and subsequently western blotted with anti-IRβ antibody (*B*). β designates IRβ subunit.

Lovastatin inhibits farnesylpyrophosphate and geranylgeranylpyrophosphate biosynthesis and therefore can interfere with the functions of proteins that require these lipid modifications. The present work suggests that some of these proteins might be involved in the early events that occur shortly after insulin stimulation. Of the many proteins that have been implicated in growth factor signaling, p21ras is one potential candidate that is known to require farnesylation for its biological activity and lovastatin is known to inhibit p21ras farnesylation. Availability of farnesylation-specific inhibitors (27,28) will allow us to further examine this question.

The results presented in this manuscript demonstrate that one effect of lovastatin on growth factor receptor tyrosine kinase signal transduction is to decrease the level of receptor phosphotyrosine and, thus, to reduce association/activation of the second messenger-generating enzyme PI-3-K. The finding that lovastatin reduces levels of IRβ phosphotyrosine carries with it possible implications for reduced activities of all receptor tyrosine kinase-dependent effects. Thus, these results provide evidence for possible biochemical mechanisms of lovastatin's anti-mitogenic activity.

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