Cholesterol Depletion Results in Site-Specific Increases in EGF Receptor Phosphorylation Due to Membrane Level Effects: Studies with Cholesterol Enantiomers.

Emily J. Westover*, Douglas F. Covey*, Howard L. Brockman#, Rhoderick E. Brown#, and Linda J. Pike+§

Washington University School of Medicine
*Department of Molecular Biology and Pharmacology and
+Department of Biochemistry and Molecular Biophysics
660 South Euclid
St. Louis, MO 63110

#University of Minnesota
The Hormel Institute
801 16th Ave NE
Austin, MN 55912

Running Title: Cholesterol Effects on EGF Receptor Phosphorylation
Author to whom correspondence should be addressed.

Washington University School of Medicine, Dept. of Biochemistry and Molecular Biophysics, 660 So. Euclid, Box 8231, St. Louis, MO 63110. Tel: 314-362-9502; Fax: 314-362-7183; e-mail: pike@biochem.wustl.edu
Summary

In A431 cells, depletion of cholesterol with methyl-β-cyclodextrin induced an increase in both basal and EGF-stimulated EGF receptor phosphorylation. This increase in phosphorylation was site-specific, with significant increases occurring at Tyr845, Tyr992 and Tyr1173 but only minor changes at Tyr1045 and Tyr1068. The elevated level of receptor phosphorylation was associated with an increase in the intrinsic kinase activity of the EGF receptor kinase, possibly as a result of the cyclodextrin-induced enhancement of the phosphorylation of Tyr845, a site in the kinase activation loop known to be phosphorylated by pp60^Src. Cholesterol and its enantiomer, ent-cholesterol, were used to investigate the molecular basis for the modulation of EGF receptor function by cholesterol. Natural (nat)-cholesterol was oxidized substantially more rapidly than was ent-cholesterol by cholesterol oxidase, a protein that contains a specific binding site for the sterol. By contrast, the ability of natural (nat)-cholesterol and ent-cholesterol to interact with sphingomyelins and phosphatidylcholine and induce lipid condensation in a monolayer system was the same. These data suggest that whereas protein-cholesterol interactions may be sensitive to the absolute configuration of the sterol, lipid-sterol interactions are not. Nat-cholesterol and ent-cholesterol were tested for their ability to physically reconstitute lipid rafts following depletion of cholesterol. Nat- and ent-cholesterol reversed to the same extent the enhanced phosphorylation of the EGF receptor that occurred following removal of cholesterol. Furthermore, the enantiomers showed similar abilities to reconstitute lipid rafts in cyclodextrin-treated cells. These data suggest that cholesterol most likely affects EGF receptor function because of its physical effects on membrane properties, not through direct, enantioselective interactions with the receptor.
Introduction

Cholesterol is an essential component of mammalian membranes. It alters membrane fluidity, thickness, curvature, and permeability (1-6). In addition, cholesterol is an important constituent of lipid rafts, specialized membrane microdomains that are rich in cholesterol, sphingolipids, and saturated phospholipids (1,6,7). Through interactions with cholesterol, the acyl chains of phospholipids in lipid rafts pack tightly together and extend fully to create a liquid-ordered phase (7-9).

A subset of plasma membrane proteins selectively partition into the ordered environment of lipid rafts (1,7,10). Because of the large number of signaling proteins that are localized to lipid rafts, it has been postulated that these domains serve as regulatory platforms for some signal transduction pathways (11,12). The EGF receptor is one of the proteins involved in signaling that is known to be enriched in lipid rafts (13-15).

A variety of studies have shown that EGF receptor function is affected by the levels of cholesterol which is present at higher concentrations in lipid rafts than in the surrounding plasma membrane (16). Depletion of cholesterol from cells leads to an increase in both basal (17,18) and EGF-stimulated (18,19) receptor phosphorylation. The enhanced receptor tyrosine phosphorylation appears to be due to a rise in the intrinsic kinase activity of the receptor (19). Cholesterol depletion has also been shown to result in an increase in the number of cell surface EGF binding sites (19,20). The increased EGF binding seems to result from an unmasking of receptors that are present on the surface of cells but unable to bind EGF in the presence of elevated levels of cholesterol (19,20).

In addition to altering the intrinsic binding and kinase activity of the EGF receptor, cholesterol also modulates signaling events directly downstream of the EGF receptor. For example, depletion of cholesterol impairs the ability of EGF to stimulate phosphatidylinositol turnover (21). By contrast, cholesterol depletion leads to the enhancement of EGF-stimulated
MAP kinase activity (22).

These observations suggest that cholesterol plays a significant role in modulating EGF receptor-mediated signaling. However, the molecular basis for these effects of cholesterol on EGF receptor function is not known. Cholesterol could affect EGF receptor function indirectly by influencing the physical properties of the membrane, such as thickness, fluidity or lateral domain formation (3,23). Indeed, depletion of cholesterol leads to the loss of the EGF receptor from low-density, lipid raft domains (19,21) suggesting that some of the effects of cholesterol on EGF receptor function could be mediated through the sterol’s ability to promote lateral membrane domain formation. Alternatively, cholesterol could bind specifically to the receptor, regulating its activity through allosteric mechanisms. In this report, we further characterize the effects of cholesterol depletion on EGF receptor function and address the question of whether the effects of cholesterol are due to membrane level effects of the compound or result from specific molecular recognition of the sterol.

We report here that the enhanced basal and hormone-stimulated phosphorylation of the EGF receptor that occurs upon cellular cholesterol depletion is due to a selective increase in the phosphorylation of a subset of the phosphorylatable tyrosine residues in the C-terminal tail of the receptor. Using natural cholesterol (nat-cholesterol) and its enantiomer, ent-cholesterol, we provide evidence that the effects of cholesterol on EGF receptor function are most likely due to non-enantioselective effects of the sterol on membrane properties, such as fluidity or the ability to form rafts.
Experimental Procedures

Materials

EGF was prepared by the method of Savage and Cohen (24). The monoclonal anti-phosphotyrosine antibody PY20 was from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against (phosphorylated) tyrosines PY845, PY992, PY1045 and PY1068 in the EGF receptor were purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody against (phosphorylated) PY1173 of the EGF receptor was from Upstate Biotechnology (Lake Placid, NY). The monoclonal flotillin antibody was from BD Transduction Laboratories (San Diego, CA). Polyvinylidene fluoride membranes were from Osmonics (Westborough, MA). The Enhanced Chemiluminescence Kit was from Amersham Pharmacia Biotech (Piscataway, NJ). The Cholesterol CII and Free Cholesterol E assay kits were from Wako (Richmond, VA). Methyl-β-cyclodextrin was from Aldrich (Milwaukee, WI). Egg sphingomyelin and (2S, 3R, 4E)-2-stearoylaminooctadec-4-ene-3-hydroxyl-1-phosphocholine (N-stearoylsphingomyelin) were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma (St. Louis, MO).

Methods

Cell Culture. A431 cells were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 7% newborn calf serum and 3% fetal calf serum. Cells were incubated overnight in DMEM containing 0.1% newborn calf serum prior to use. CHO cells were maintained at 37°C and 5% CO2 in Hams F12 medium containing 10% fetal calf serum. Cells were incubated overnight in Hams F12 containing 0.1% fetal calf serum prior to use.

Synthesis of Ent-Cholesterol. Ent-cholesterol was prepared from ent-desmosterol through a minor modification of methods described previously (25). Silyl-protected ent-desmosterol was subjected to catalytic hydrogenation (300 psi H2, 15 min, Pt/C) and then treated to remove the
silyl protecting group to give ent-cholesterol (mp 147.5-148° C, [α] =+40.0 (c=1.0, CHCl₃)). Figure 1 shows the structures of natural cholesterol and ent-cholesterol.

Preparation of Cholesterol/Methyl-β-Cyclodextrin Complexes. Cholesterol/methyl-β-cyclodextrin complexes were synthesized as described by Klein et al. (26). Briefly, 30 mg of nat- or ent-cholesterol was dissolved in 400 µL of isopropanol/chloroform (2:1, v/v). Methyl-β-cyclodextrin (1 g) was dissolved in 11 mL phosphate-buffered saline (PBS) and heated to 80° C with stirring. The solubilized sterol was added in small aliquots to the heated solution over 30 min.

Cholesterol Depletion and Repletion. For cholesterol depletion, cells were incubated for 30 min at 37° C in DMEM containing 50 mM HEPES, pH 7.2, 0.1 % bovine serum albumin (DMEM-BSA), and the indicated concentration of methyl-β-cyclodextrin. Control cells were incubated in the same medium lacking cyclodextrin. To replete cells with cholesterol following cyclodextrin treatment, cells were incubated at 37° C for 30 min in DMEM-BSA containing the indicated concentration of sterol/cyclodextrin complex. For both cholesterol depletion and repletion, the tissue culture plates were swirled every 10 min to ensure continuous mixing of the components in the media.

Cholesterol Assay. Cells were washed twice with 1 mL cold PBS, and lipids were extracted with 2 mL hexane/isopropanol (3:2, v/v) for 1 h at room temperature. The organic extract was removed from the cell monolayer and the solvent was removed in a Speed Vac. The lipid residue was solubilized in 1 mL of the Cholesterol CII assay kit buffer solution. As reported previously (27), color generation from nat-cholesterol standards reached a plateau after 5 min at 37° C and remained stable through 60 min at this temperature, whereas ent-cholesterol standards
required 60 min at 37° C for complete color development. Samples were incubated for 1 h at 37° C prior to measuring absorbance at 505 nm.

After lipid extraction, the residual cell monolayers were solubilized with 10 mM sodium borate and 1% SDS. Aliquots of the solubilized material were then used for determination of total protein content using the bicinchoninic acid protein assay.

Cholesterol Oxidase Assay. Assay of cholesterol oxidase utilized the Wako Free Cholesterol E assay kit. The Free Cholesterol E assay reagent contains cholesterol oxidase plus several additional components (peroxidase, 4-aminoantipyrine, and DEHSA) that permit the colorimetric detection of the hydrogen peroxide produced upon oxidation of cholesterol. For assay, 5 µg of either nat- or ent-cholesterol in complex with cyclodextrin was placed in a glass tube. One mL of the Free Cholesterol E assay kit buffer solution was then added. Samples were incubated at 37° C for the indicated times. Absorbance (A$_{600}$) of the samples was measured immediately. Equal concentrations of free cholesterol and cyclodextrin-complexed cholesterol gave equivalent absorbance measurements in this assay.

Cell Surface EGF Binding. A431 cells were grown to confluence in 24-well dishes and incubated overnight in DMEM containing 0.1% fetal calf serum. After treatment to alter cholesterol content, cultures were washed with ice-cold PBS and incubated for 2 hr at 4° C in 1 mL DMEM-BSA. 50 pM $^{125}$I-EGF and increasing concentrations of unlabeled EGF. At the end of the incubation, cells were washed three times with ice-cold PBS. Cell monolayers were dissolved in 1 mL 1M NaOH and counted for $^{125}$I. Data were analyzed using the LIGAND computer program (28).

Stimulation of Cells with EGF and Preparation of Cell Lysates. Cells in 35 mm dishes were
treated to alter cholesterol content as outlined above. At the end of the incubation, EGF was added to the medium for 5 min. Cells were then washed in cold PBS and lysed by scraping the monolayers into 300 µL of RIPA buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing 1 µg/mL leupeptin, 100 µM sodium o-vanadate, 10 mM p-nitrophenylphosphate, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 10 min with periodic vortexing and then clarified by centrifugation at 12000 x g for 10 min. Aliquots were then taken for determination of total protein concentration. Receptor tyrosine phosphorylation was determined by Western blotting.

**EGF Receptor Dephosphorylation.** To assess the rate of EGF receptor dephosphorylation, cholesterol-depleted or repleted cells were stimulated with 1.25 nM EGF for 5 min at 37°C. The medium was removed and cells were washed with cold PBS. Residual cell-surface bound EGF was removed by incubating the cells twice for 2 min in 50 mM glycine, 100 mM NaCl, pH 4.0 on ice. After an additional wash with cold PBS, warmed DMEM-BSA was added and the cells were incubated at 37°C for the indicated time. Cells were washed with cold PBS and lysed with RIPA buffer as usual. Receptor tyrosine phosphorylation was determined by Western blotting.

**Membrane Preparation and In Vitro Phosphorylation Assays.** Cells were treated to alter cholesterol levels and then lysed by homogenization in 25 mM HEPES, pH 7.2. Membranes were pelleted by centrifugation for 10 min at 12000 x g and then resuspended in 70 mM β-glycerophosphate, 250 mM NaCl, 25% glycerol, pH 7.2. Assays were carried out in a final volume of 50 µL containing: 20 mM β-glycerophosphate, 100 µM ATP, 12 mM MgCl₂, 2 mM MnCl₂, 20 mM p-nitrophenylphosphate, 100 µM sodium o-vanadate, and 1 µg membrane protein. When included, EGF was added at a final concentration of 25 nM. Membranes were incubated with growth factor for 5 min at room temperature. Assays were begun by the addition
of ATP and metal ions. After incubation at 30°C for 15 sec, reactions were stopped by the addition of 50 µL SDS sample buffer. Samples were boiled, run on a 10% SDS-polyacrylamide gel and analyzed by Western blotting.

Preparation of Lipid Rafts. All manipulations were performed at 4°C. After appropriate treatments, one 150-mm plate of A431 cells was washed five times in PBS and scraped into 0.4 mL of 0.25 M sucrose, 1 mM EDTA, 20 mM Tris, pH 7.8. Cells were lysed by passage through a 23-gauge needle 10 times. The lysates were sonicated 5 times for 15 s using a Branson 250 sonicator set at maximum power output for a microtip. After centrifugation at 1000 x g for 10 min, the post nuclear supernatant was mixed with an equal volume of 85% sucrose in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) and placed in the bottom of a centrifuge tube. A 15-35% discontinuous sucrose gradient was formed above the lysate, by adding sucrose-containing buffers as follows: 2 mL 35% sucrose, 2 mL 28% sucrose, 2 mL 22% sucrose, and 4 mL 15% sucrose, all in MES-buffered saline. The gradient was centrifuged for 18 h at 210,000 x g in an SW41 rotor (Beckman Instruments, Palo Alto, CA). After discarding the uppermost 4 mL, the gradient was fractionated into 8 1-mL fractions. Then, 100 µL aliquots of each fraction were subjected to SDS-PAGE and analyzed by Western blotting.

Western Blotting. Samples containing 25 µg - 100 µg of protein mixed with SDS sample buffer, were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, and incubated with primary antibody for 1 h at room temperature (PY20, PY1173, DB-1, flotillin) or overnight at 4°C (PY845, PY992, PY1045, PY1068). Antibody detection was carried out using Enhanced Chemiluminescence.

Langmuir Film Balance. Water for the subphase buffer was purified by reverse osmosis,
activated charcoal adsorption, and mixed-bed deionization; passed through a Milli-Q UV Plus System (Millipore Corp., Bedford, MA), and filtered through a 0.22-µm Millipak 40 Membrane. Subphase buffer (pH 6.6) consisting of 10 mM potassium phosphate, 100 mM NaCl, and 0.2% sodium azide was stored under argon until use. Glassware was acid cleaned and rinsed thoroughly with deionized water and then with hexane/ethanol (95:5). Solvent purity was verified by dipole potential measurements prior to use (29). Final stock concentrations of sterols were determined gravimetrically using a Cahn microbalance (model 4700) and of sphingomyelins, by lipid phosphate analysis (30).

Surface pressure-molecular area isotherms were measured using a computer-controlled, Langmuir-type balance, described in detail previously (31,32) and calibrated according to the equilibrium spreading pressures of known lipid standards (33). The subphase was maintained at fixed temperature using a thermostatted, circulating water bath. The film balance was housed in an isolated laboratory supplied with clean air by a Bioclean Air Filtration system equipped with charcoal and HEPA filters. The trough was separately enclosed under humidified argon, cleaned by passage through a seven-stage series filtration set-up consisting of an Alltech activated charcoal gas purifier, a LabClean filter, and a series of Balston disposable filters consisting of two adsorption (carbon) and three filter units (93% and 99.99% efficiency at 1 µm). Film balance features that contribute to isotherm reproducibility include automated lipid spreading via a modified HPLC autoinjector, automated surface cleaning by multiple barrier sweeps between runs, and highly accurate, reproducible setting of the subphase level by an automated aspirator. Lipids were mixed and spread (51.67 µl aliquots) from stock solutions dissolved in hexane/ethanol (95:5). Films were compressed at a rate of 4 Å²/molecule/min after an initial delay period of 4 min. Standard errors of the resulting force-area isotherms were routinely less than 2%.
Results

Site-Specific Effect of Cholesterol on EGF Receptor Phosphorylation

A431 cells were treated with increasing concentrations of methyl-β-cyclodextrin for 30 minutes to remove cholesterol. Lysates were prepared and analyzed for receptor tyrosine phosphorylation by SDS-polyacrylamide gel electrophoresis and Western blotting with an anti-phosphotyrosine antibody. As shown in Figure 2A, cholesterol depletion increased basal tyrosine phosphorylation of the EGF receptor up to 11-fold. The dose of methyl-β-cyclodextrin yielding maximal stimulation was 7.5 mM when confluent cultures were used. However, when significantly subconfluent cells were used, the optimal concentration of methyl-β-cyclodextrin was 2- to 3-fold less than this. This indicates that it is the ratio of cells to reagent that is important rather than the absolute concentration of reagent added.

In addition to enhancing basal EGF receptor phosphorylation, cholesterol depletion also increased EGF-stimulated receptor phosphorylation at all doses of EGF tested (Figure 2B). At the maximal dose of EGF, receptor phosphorylation was enhanced from 1.2- to 2.0-fold in different experiments. The EC₅₀ for EGF was approximately 2 nM and was essentially unchanged following cholesterol depletion. Thus, the increase in EGF-stimulated tyrosine phosphorylation of the receptor following cholesterol depletion appears to be due to a change in the maximum level of phosphorylation rather than to a change in the EC₅₀ for EGF.

The increase in EGF receptor phosphorylation was associated with only a modest increase in the number of cell surface EGF binding sites (Figure 3). Scatchard analysis indicated the presence of a single class of EGF binding sites on control cells that exhibited a K_d of ~10 nM. Cyclodextrin treatment resulted in a ~10% increase in the number of EGF binding sites present on A431 cells with no change in the binding affinity of EGF.

The EGF receptor contains several tyrosine residues that become phosphorylated in response to EGF. To determine whether all sites were similarly affected by cholesterol
depletion, EGF receptor phosphorylation was analyzed using a panel of antibodies that recognize specific phosphorylated tyrosine residues on the receptor. The data in Figure 4 show that cholesterol depletion differentially affected the phosphorylation of individual tyrosine residues. Cholesterol depletion routinely enhanced the EGF-stimulated phosphorylation of tyrosines 845, 992 and 1173. By contrast, the level of hormone-stimulated phosphorylation of tyrosines 1045 and 1068 was relatively unaffected by removal of cholesterol. In all cases, cholesterol depletion did not significantly alter the EC50 for receptor phosphorylation. These data indicate that cholesterol depletion induces site-specific changes in phosphorylation of the EGF receptor.

Comparison of the Enantiomers of Cholesterol

The effect of cholesterol depletion on EGF receptor phosphorylation could be due either to an effect of cholesterol on the physical properties of the membrane or to direct interaction of the sterol with the EGF receptor or another protein that regulates receptor phosphorylation. Theoretically, the use of cholesterol enantiomers should allow discrimination between these two possibilities. Because the physical properties of natural cholesterol (nat-cholesterol) and its enantiomer, ent-cholesterol, are identical, their effects on general membrane properties should not be significantly different. However, because the two enantiomers have mirror image shapes, they should interact differently with molecules, such as proteins, that contain a stereospecific binding site for the sterol. To determine whether cholesterol affects EGF receptor function due to general membrane level effects or to direct binding to a protein, the enantiomer of cholesterol was synthesized (25) and used as a tool to probe the effects of cholesterol on EGF receptor function.

The ability of nat- and ent-cholesterol to interact with lipids and proteins was first compared in model systems to characterize the behavior of these two enantiomers in sterol-protein and sterol-lipid interactions. To assess the characteristics of nat- and ent-cholesterol when interacting specifically with a protein, the ability of cholesterol oxidase to use these
enantiomers as substrates was compared. As shown in Figure 5, nat-cholesterol was rapidly oxidized by cholesterol oxidase in a standard cholesterol assay system. The oxidation of nat-cholesterol was essentially complete within 30 seconds under these conditions and showed a t$_{1/2}$ of less than 10 seconds. By contrast, ent-cholesterol was much more slowly oxidized by cholesterol oxidase under the same conditions. Approximately 30 minutes were required for complete oxidation of this enantiomer. The reaction had a t$_{1/2}$ of 10 minutes. Thus, nat- and ent-cholesterol show clearly distinct behaviors when interacting with a protein that has a specific sterol binding site.

The capacity of nat- and ent-cholesterol to interact with other lipids was first examined by comparing the ability of these two enantiomers to condense sphingomyelins in a monolayer system. Because lipid rafts are thought to be held together via cholesterol/sphingolipid interactions, this represents a good measure of the raft-forming capabilities of these enantiomers. Figure 6 shows the surface pressure-average molecular area isotherms for mixtures of 70 mol% egg sphingomyelin and 30 mol% nat- or ent-cholesterol (open and closed circles, respectively). The isotherms were indistinguishable for the two enantiomers. This similarity in behavior was not the result of heterogeneity in the acyl chains present in the egg sphingomyelin mixture. When enantiomerically pure N-stearoylsphingomyelin was used in place of the egg sphingomyelin, the isotherms observed for mixtures with nat- and ent-cholesterol were again indistinguishable (Figure 6, open and filled squares). The isotherms of pure nat- and pure ent-cholesterol (open and filled triangles, Figure 6) were also identical. In addition, the force-area behavior of 1:1 mixtures of either nat- or ent-cholesterol with palmitoyl oleoyl phosphatidylcholine were the same (open and filled diamonds). The condensing effect of cholesterol is a well-established indicator of its lateral interaction and non-ideal mixing with membrane phospholipids and sphingolipids (29,34,35). Therefore, these data indicate that the absolute configuration of the sterol does not affect its interaction with either phosphatidylcholine or sphingomyelin.
The Effects of Nat- and Ent-Cholesterol on the EGF Receptor are Similar

The cholesterol enantiomers were next used in the A431 cell system to determine whether the effect of cholesterol on EGF receptor phosphorylation is the result of general sterol-lipid or stereospecific sterol-protein interactions. A431 cells were depleted of cholesterol and subsequently repleted with either nat- or ent-cholesterol using complexes of each sterol with methyl-β-cyclodextrin. The data in Table I confirm that treatment of cells with the same concentration of either sterol/cyclodextrin complex added a similar amount of each sterol to cholesterol-depleted cells.

As shown in Figure 7, nat- and ent-cholesterol exhibited similar abilities to reverse the effects of cholesterol depletion on both basal (Figure 7A) and EGF-stimulated (Figure 7B) receptor phosphorylation. Basal EGF receptor phosphorylation was enhanced 6-fold by the depletion of cholesterol. Nat-cholesterol reversed this effect, exhibiting an EC\textsubscript{50} between 0.07 and 0.2 mM cholesterol. Ent-cholesterol reversed the effects of cholesterol depletion on basal EGF phosphorylation to the same extent as nat-cholesterol and exhibited an EC\textsubscript{50} in the same range as that for the natural enantiomer. Nat- and ent-cholesterol also exhibited similar potencies and efficacies for reversing the enhancement of EGF-stimulated receptor phosphorylation induced by cholesterol depletion (Figure 7B).

To determine whether the similar effects of nat- and ent-cholesterol were restricted to A431 cells which express ~3 x 10\textsuperscript{6} EGF receptors/cell, we performed a similar depletion/repletion experiment in Chinese hamster ovary cells which have been engineered to express a more normal 28,000 EGF receptors/cell. As shown in Figure 8, cholesterol depletion led to an enhancement of EGF-stimulated receptor phosphorylation. Basal EGF receptor phosphorylation was only slightly affected by cholesterol depletion in this cell line. The effect on EGF-stimulated receptor phosphorylation was reversed to the same extent and over the same dose range by repletion of the cells with nat- and ent-cholesterol. Thus, the effects of these enantiomers on EGF receptor autophosphorylation appear to be independent of cell type and the
level of receptor expression.

**Cholesterol Affects EGF Receptor Kinase but Not Phosphatase Activity.**

The observed increase in receptor phosphorylation upon cholesterol depletion could be due either to a decrease in the activity of a protein tyrosine phosphatase or an increase in the intrinsic kinase activity of the receptor. To determine whether cholesterol affects the dephosphorylation of the EGF receptor, A431 cells were depleted of cellular cholesterol and then repleted with either nat- or ent-cholesterol. Following a 5 minute stimulation with EGF, cells were washed with a low pH glycine buffer to remove cell surface EGF. Subsequently, the cells were incubated for increasing lengths of time in EGF-free media at 37º C. Cells were lysed and analyzed for EGF receptor phosphorylation.

The data in Figure 9 show that, as expected, the absolute amount of receptor phosphorylation was higher at all times in cholesterol-depleted cells than in untreated control cells. However, the rate of receptor dephosphorylation was similar in these two treatment groups. Control and cholesterol-depleted cells both exhibited t1/2’s for receptor dephosphorylation in the range of 20 to 25 min. Cells repleted with either nat- or ent-cholesterol showed similar rates of dephosphorylation with t1/2’s of approximately 20 min. These data suggest that receptor dephosphorylation was not significantly affected by either the absolute level of cholesterol in the membrane or its absolute configuration.

To determine if cholesterol levels or the absolute configuration of the sterol affected the intrinsic kinase activity of the EGF receptor, A431 cells were depleted of cholesterol and subsequently repleted with equal amounts of either nat- or ent-cholesterol. A total membrane fraction was then prepared and assayed for EGF receptor autophosphorylation in an in vitro system containing phosphatase inhibitors. The results are shown in Figure 10. Both basal and EGF-stimulated receptor phosphorylation were increased in membranes prepared from cells depleted of cholesterol. Repletion with nat- or ent-cholesterol reversed the effect of cholesterol
depletion on basal and EGF-stimulated EGF receptor phosphorylation to a similar extent. These data suggest that intrinsic EGF receptor kinase activity is affected by cholesterol levels but not by the absolute configuration of the sterol.

**Nat- and Ent-Cholesterol Support Lipid Raft Formation**

Cholesterol depletion disrupts lipid rafts and leads to the loss of the EGF receptor from these low-density membrane domains (19,21). Repletion of cholesterol leads to the reconstitution of lipid rafts and the re-localization of the EGF receptor to these domains (19,21). An experiment was therefore performed to determine whether nat- and ent-cholesterol exhibited similar abilities to reconstitute lipid rafts in cholesterol-depleted cells.

A post-nuclear supernatant was prepared from A431 cells that had been depleted of cholesterol and then repleted with ent-cholesterol. Assay of this material for cholesterol indicated that 40% of the cholesterol present in these starting membranes was ent-cholesterol. Non-detergent lipid rafts were then prepared from the post-nuclear supernatant. In the lipid raft fraction derived from these starting membranes, 41% of the cholesterol was determined to be ent-cholesterol. In a separate experiment, a 1% Triton X-100 extract was made from cells that had been depleted of cholesterol and repleted with ent-cholesterol. In this experiment, 54% of the cholesterol in the Triton extract was ent-cholesterol. After flotation in a sucrose gradient, the resulting Triton-resistant lipid rafts were assayed and found to contain 60% ent-cholesterol. These data indicate that ent-cholesterol partitions into both non-detergent and Triton-resistant lipid rafts in direct proportion to its concentration in the starting membranes.

A431 cells were depleted of cholesterol using methyl-β-cyclodextrin and repleted with equal amounts of either nat- or ent-cholesterol. Non-detergent lipid rafts were then prepared by sucrose density gradient centrifugation and the distribution across the gradient of the EGF receptor and flotillin, a lipid raft marker protein, was assessed. The results are shown in Figure 11. As expected, both the EGF receptor and flotillin selectively partitioned into the low-density
portion of the gradient in untreated cells. Depletion of cholesterol using methyl-β-cyclodextrin resulted in the loss of both proteins from the low density region of the gradient and their recovery in higher density fractions. Repletion of cells with nat-cholesterol reconstituted the lipid rafts, restoring the partitioning of flotillin and the EGF receptor into the low density fractions of the gradient. Similarly, repletion with ent-cholesterol led to the movement of both flotillin and the EGF receptor back into the low density region of the gradient. These data suggest that nat- and ent-cholesterol have similar capacities to reconstitute lipid rafts and promote partitioning of the EGF receptor into these low density membrane domains.

Discussion

Cholesterol Depletion

The ability of cholesterol depletion to enhance EGF receptor phosphorylation has been observed by several groups (17-19). In this study, we provide additional details regarding the characteristics of cholesterol-modulated EGF receptor phosphorylation and provide insight into the molecular basis of this phenomenon.

In A431 cells, cholesterol depletion by methyl-β-cyclodextrin was found to enhance both basal and EGF-stimulated receptor tyrosine phosphorylation. The increase in ligand-dependent phosphorylation of the EGF receptor observed in these studies was found to be due to an increase in the maximal level of receptor phosphorylation rather than a change in the EC50 for EGF. Only a ~10% increase in EGF receptor number was observed in A431 cells following cholesterol depletion. Because this is substantially smaller than the percent increase in EGF-stimulated autophosphorylation observed in our experiments, the enhancement in EGF receptor phosphorylation cannot be attributed solely to a rise in surface-exposed EGF receptors.

We (19) and others (18,20) have observed that cholesterol depletion leads to an increase in the number of cell surface EGF receptors in a variety of other cell types. Evidence suggests that
this increase is due to the unmasking of cryptic receptors present on the cell surface (18,19). Recent structural studies of the extracellular domain of the EGF receptor offer insight into a possible molecular mechanism for this effect. The unliganded EGF receptor exists in a bent, auto-inhibited configuration that is stabilized by interactions between loops in the second and fourth extracellular subdomains (36). It apparently binds EGF only weakly (36). This bent, inactive conformation is likely in equilibrium with an open configuration that binds EGF with high affinity and is capable of forming functionally active receptor dimers (37,38). EGF shifts this equilibrium from the inactive to the active configuration of the receptor by binding to and stabilizing the open form (36,38). The observation that cholesterol depletion results in an increase in EGF binding with no change in the amount of receptor protein detectable at the cell surface (19) suggests that high cholesterol levels may stabilize the intramolecular interactions, increasing the fraction of the EGF receptor that is in the inactive conformation. Cholesterol depletion would reduce this intramolecular stabilization, allowing the receptor to more readily adopt the open conformation necessary for tight EGF binding.

The increase in EGF receptor phosphorylation in response to cholesterol removal could be due to an increase in the intrinsic kinase activity of the receptor or a decrease in the rate of receptor dephosphorylation. Cell culture experiments demonstrated that the $t_{1/2}$ for receptor dephosphorylation was similar in control and cyclodextrin-treated cells. By contrast, following cholesterol depletion, EGF receptor autophosphorylation was enhanced in *in vitro* experiments, in which kinase activity was assayed in the presence of phosphatase inhibitors. Together, these data suggest that it is primarily an increase in intrinsic receptor kinase activity that gives rise to enhanced receptor phosphorylation in intact cells depleted of cholesterol. This is consistent with recent observations made in NIH 3T3 cells (19).

A novel finding with respect to the increase in EGF receptor phosphorylation following cholesterol depletion is that there was a selective increase in the phosphorylation of some sites with little or no effect on the phosphorylation of other sites. Tyrosines 992, 1045, 1068, and
1173 are all known sites of EGF receptor autophosphorylation (39,40). However, the phosphorylation of tyrosines 992 and 1173 was routinely enhanced 1.5- to 2-fold by cholesterol depletion whereas the phosphorylation of tyrosines 1045 and 1068 was minimally affected by cholesterol withdrawal. The observation that there was not a uniform increase in the phosphorylation of all sites available to the EGF receptor kinase implies that either the specificity of the kinase was altered or that the accessibility of the kinase to the individual sites differs in control and cholesterol-depleted cells. Cholesterol depletion is accompanied by the disruption of lipid rafts and the loss of the EGF receptor from this compartment (19,21). It is therefore tempting to speculate that the intracellular domain of the EGF receptor adopts a different conformation when it is outside of lipid rafts than when it is localized to this compartment. This would give rise to differences in the accessibility of the various sites of autophosphorylation and would be reflected in the overall pattern of receptor phosphorylation.

Tyrosine 845 is a site on the EGF receptor known to be phosphorylated by pp60src (41,42). The observation that the phosphorylation of this site was selectively enhanced by cholesterol depletion implies that either this site becomes more accessible to pp60src in cholesterol-depleted cells or that cholesterol depletion enhances the activation of pp60src in response to EGF. Phosphorylation of Tyr845, which is in the activation loop of the kinase domain, is associated with an increase in the kinase activity of the EGF receptor (41). It is therefore possible that the increase in the intrinsic kinase activity of the EGF receptor that occurs following cholesterol depletion is a result of the enhanced phosphorylation of the receptor on Tyr845. Additional studies will be required to clarify the molecular basis of this effect.

Specific phosphorylated tyrosine residues on the EGF receptor serve as binding sites for adaptor proteins and enzymes (43-46). The finding that a decrease in membrane cholesterol levels leads to selective increases in the phosphorylation of specific tyrosine residues suggests that cholesterol depletion could preferentially enhance signaling via specific pathways. For example, Shc binds selectively to Tyr1173, a residue whose phosphorylation was selectively
enhanced in cholesterol-depleted cells (44). Thus, the MAP kinase pathway would be expected to be activated to a greater extent in cyclodextrin-treated cells than other pathways that depend on tyrosine residues whose phosphorylation were not selectively increased. Interestingly, the phosphorylation of Tyr1045, the binding site for the ubiquitin ligase, c-Cbl (46), that down regulates the EGF receptor, was not enhanced in cyclodextrin-treated cells. This suggests that the increased kinase activity of the receptor is not counterbalanced by a Cbl-mediated increase in receptor internalization and down-regulation. The up-regulation of kinase activity without a concomitant increase of the negative regulatory mechanisms could serve to further enhance the relative signaling capacity of the EGF receptor in cholesterol-depleted cells.

Enantiomers of Cholesterol

The ability of cholesterol to modulate EGF receptor function could arise from several sources including alterations in specific protein-sterol interactions or from more general effects of the sterol on membrane structure. To distinguish between these two possibilities the enantiomer, or mirror image, of cholesterol was synthesized and used in experiments in which nat-cholesterol was replaced by its enantiomer, ent-cholesterol. Preliminary experiments using cholesterol oxidase demonstrated that an enzyme possessing a specific binding site for cholesterol is capable of distinguishing between the enantiomers of cholesterol. Such enantioselective interactions appear to be absolutely required for viability of organisms, as our previous work has shown that substitution of ent-cholesterol for nat-cholesterol in the growth medium of C. elegans results in 100% lethality in second generation animals (47). Thus, proteins and intact organisms can distinguish nat- from ent-cholesterol.

By contrast, in the monolayer experiments in which sterol/lipid interactions were examined, the enantiomers of cholesterol were indistinguishable in terms of their abilities to condense sphingomyelin and phosphatidylcholine. This result is consistent with recent studies that have found that artificial bilayers containing sphingomyelin and either nat- or ent-
cholesterol are indistinguishable as assessed by differential scanning calorimetry, X-ray diffraction, and neutral buoyant density measurements (48). In addition, we and others (49-52) have found no enantioselectivity in the interaction of cholesterol with phospholipids. Thus, with respect to their interactions with membrane lipids, nat- and ent-cholesterol appear to function similarly.

We took advantage of the difference in the behavior of the enantiomers of cholesterol in situations involving specific molecular recognition or more general sterol-lipid interactions to gain insight into the molecular mechanism underlying the effects of cholesterol on EGF receptor function. By replacing nat-cholesterol with ent-cholesterol in cholesterol-depleted cells, it was possible to compare the effect of the two enantiomers on EGF receptor phosphorylation.

Ent-cholesterol was found to partition into lipid rafts in direct proportion to its concentration in total cell membranes, indicating that it behaves similarly to nat-cholesterol in its ability to distribute into cellular membranes. Furthermore, our studies indicate that nat- and ent-cholesterol have equal abilities to reverse the increase in EGF receptor phosphorylation observed following cholesterol depletion. Both the extent of the reversal and the dose of the sterol required to observe reversal were the same for nat- and ent-cholesterol. In addition, in cholesterol-depleted cells, the EGF receptor as well as the raft marker protein, flotillin, shifted out of the low density portion of sucrose density gradients, reflecting the disruption of cholesterol-enriched lipid rafts (19,21). Nat- and ent-cholesterol were equally capable of reconstituting the lipid rafts, as evidenced by the movement of the EGF receptor and flotillin back into the low density portion of the gradient following repletion with either sterol. Thus, no distinction was made at any level between nat- and ent-cholesterol. These observations indicate that the effect of cholesterol on EGF receptor function and raft integrity is not enantioselective.

Since proteins that possess specific binding sites for cholesterol appear to readily distinguish between nat- and ent-cholesterol, while sterol/lipid interactions appear to be non-enantioselective, the present observations suggest that the effects of cholesterol on EGF receptor
function are due to effects of the sterol on the physical properties of the membrane. We have shown previously that sterol analogs such as 25-hydroxycholesterol and 7-ketocholester differentially affect EGF receptor function when exchanged into cell membranes (21). Because of their additional chemical groups, these analogs impart different properties on the membrane than cholesterol. However, these analogs have an overall molecular shape that is more similar to that of nat-cholesterol than does ent-cholesterol. Nonetheless, the receptor discriminated among these sterol analogs that have similar shapes but impart different properties on the membrane. The present findings show that the receptor does not discriminate between nat-cholesterol and ent-cholesterol that have different shapes but impart similar properties on the membrane. Thus, there appears to be a correlation between the ability of a sterol to alter membrane properties (as compared to cholesterol) and its ability to alter EGF receptor function (as compared to cholesterol). Therefore, while we cannot rule out the possibility that cholesterol binds in a non-enantioselective manner to the EGF receptor, we favor the interpretation that the effects of cholesterol on EGF receptor function are due to cholesterol-induced changes in the physical properties of the cell membrane. It seems likely that any non-enantioselective binding of cholesterol to the EGF receptor would occur at hydrophobic sites that are not specifically regulatory in nature. Such non-specific interactions would probably be dependent on the lipid composition and physical properties of the membrane and hence would be encompassed in the general membrane-level effects of sterols on EGF receptor function.

Since cholesterol is critical for inducing the formation of lipid rafts and because EGF receptors are normally localized to lipid rafts, it is possible that the effects of cholesterol on receptor function are ultimately due to the ability of cholesterol to induce lateral domain formation in membranes. The high concentration of cholesterol within lipid raft domains may affect EGF receptor conformation and function through lipid-based mechanisms involving changes in membrane fluidity, thickness or stabilization of a particular conformation of the protein.
In summary, our data indicate that cholesterol depletion induces an increase in the intrinsic kinase activity of the EGF receptor and leads to enhanced, site-specific phosphorylation of the receptor. The observation that changes in membrane cholesterol levels alters the pattern of EGF receptor phosphorylation suggests that the extent of activation of individual downstream signaling pathways by EGF could differ depending on the level of cholesterol in the cell membrane. As a variety of pathological conditions, including atherosclerosis and Niemann-Pick type C1 (53), lead to alterations in cellular cholesterol levels, it is possible that changes in hormonal signaling occur in diseased cells as a result of underlying problems in cholesterol trafficking. Our data also suggest that the effects of cholesterol on receptor signaling are most likely due to the ability of cholesterol to interact with membrane lipids and possibly promote the formation of lateral membrane domains. Through this mechanism, increased membrane cholesterol content could have widespread effects on membrane protein function.
References

Footnotes

1 This work was supported by National Institutes of Health grant GM64491 (to L.J.P), National Institutes of Health grant GM47969 (to D.F.C.), the Lucille P. Markey Predoctoral Fellowship and Cardiovascular Research Training Grant NIH 55935 (E.J.W.) and the Hormel Foundation and USPHS grants GM45928 (to R.E.B). We thank Maureen Momsen for expert technical assistance with the monolayer measurements.

2Abbreviations used: BSA, bovine serum albumin; CDX, methyl-ß-cyclodextrin; CHO cells, Chinese Hamster Ovary cells; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate
**Figure Legends**

**Figure 1.** *The structure of natural cholesterol (nat-cholesterol) and its unnatural enantiomer (ent-cholesterol).*

**Figure 2.** *Effect of cholesterol depletion on EGF receptor phosphorylation.* (A) A431 cells were treated without or with the indicated concentration of methyl-β-cyclodextrin for 30 min to remove cholesterol. Cells were lysed and analyzed for basal receptor phosphorylation by Western blotting with the anti-phosphotyrosine antibody, PY20. Results were quantitated by densitometry. (B) Cells were treated without (CON) or with (CDX) 7.5 mM methyl-β-cyclodextrin for 30 min and were then stimulated with the indicated dose of EGF for 5 min. Cells were lysed and analyzed as in (A). Results from a representative experiment were quantified by densitometry and subjected to non-linear curve fitting using GraphPad Prism.

**Figure 3.** *Effect of cholesterol depletion on 125I-EGF binding.* A431 cells were treated without (closed circles) or with (open circles) 5 mM methyl-β-cyclodextrin and assayed for 125I-EGF binding as described in Experimental Procedures. Points represent the mean of triplicate determinations.

**Figure 4.** *Effect of cholesterol on site-specific tyrosine phosphorylation of the EGF receptor.* A431 cells were treated without (CON) or with (CDX) 7.5 mM methyl-β-cyclodextrin to remove cholesterol and then stimulated with the indicated dose of EGF for 5 min. Cells were lysed and analyzed for receptor phosphorylation by Western blotting with antibodies that recognize phosphorylated tyrosine residues 845, 992, 1045, 1068, or 1173 on the EGF receptor. Results were quantified by densitometry and subjected to non-linear curve fitting using GraphPad Prism. Results from a representative experiment are shown.
**Figure 5.** *Oxidation of nat- and ent-cholesterol by cholesterol oxidase.* Equal amounts of nat- and ent-cholesterol were subjected to oxidation by cholesterol oxidase as described in Experimental Procedures. Samples were incubated for the indicated times at 37° C and analyzed for absorbance at 600 nm.

**Figure 6.** *Condensation of lipids by nat- and ent-cholesterol in monolayers.* Surface pressure versus average molecular area behavior was measured as described in Experimental Procedures for pure nat-cholesterol or ent-cholesterol or mixtures of these enantiomers with sphingomyelin.

Open and filled triangles represent the isotherms for pure nat-cholesterol and pure ent-cholesterol, respectively. Circles represent the isotherms for 30 mol% cholesterol mixed with 70 mol% egg sphingomyelin. (Open circles, nat-cholesterol; closed circles, ent-cholesterol). The squares show isotherms for 30 mol% cholesterol mixed with 70 mol % N-stearoylsphingomyelin ((2S, 3R, 4E)-2-stearoylaminoaooctadec-4-ene-3-hydroxy-1-phosphocholine, the naturally occurring form of this lipid). (Open squares, nat-cholesterol; closed squares, ent-cholesterol). Diamonds represent the isotherms for 1:1 mixtures of cholesterol with POPC (open diamonds, nat-cholesterol; closed diamonds, ent-cholesterol). Each trace represents the average of three or more experimental isotherms. The standard errors are shown.

**Figure 7.** *Effect of cholesterol repletion on phosphorylation of the EGF receptor.* (A) A431 cells were treated without (CON) or with (CDX) 7.5 mM methyl-β-cyclodextrin to remove cholesterol. Some cultures were subsequently repleted with cholesterol by incubation with either nat-cholesterol (NAT) or ent-cholesterol (ENT) in complex with methyl-β-cyclodextrin. Concentrations given refer to the amount of cholesterol added. Cells were lysed and analyzed for receptor phosphorylation by Western blotting with the anti-phosphotyrosine antibody, PY20. Results were quantified by densitometry. (B) Cells were treated as in (A), but prior to lysis, cells were stimulated with 5 nM EGF for 5 min.
Figure 8. **Effect of cholesterol repletion on phosphorylation of the EGF receptor.** CHO cells expressing EGF receptors were treated without (CON) or with (CDX) 7.5 mM methyl-β-cyclodextrin to remove cholesterol. Some cultures were subsequently repleted with cholesterol by incubation with either nat-cholesterol or ent-cholesterol in complex with methyl-β-cyclodextrin. Concentrations given refer to the amount of cholesterol added. Cells were then stimulated with 25 nM EGF for 5 min, lysed, and analyzed for receptor phosphorylation by Western blotting with the anti-phosphotyrosine antibody, PY20. Results were quantified by densitometry.

Figure 9. **Effect of cholesterol depletion and repletion on dephosphorylation of the EGF receptor.** A431 cells were treated without (CON) or with (CDX) 7.5 mM methyl-β-cyclodextrin to remove cholesterol. Some cultures were subsequently repleted by incubation for 30 min with 0.2 mM of either nat-cholesterol (NAT) or ent-cholesterol (ENT) in complex with methyl-β-cyclodextrin. Cells were stimulated with 1.25 nM EGF for 5 min and washed to remove residual EGF as described in Experimental Procedures. Cells were incubated in DMEM-BSA for the indicated time and then analyzed for EGF receptor phosphorylation by Western blotting with an anti-phosphotyrosine antibody (PY20). Results from a representative experiment were quantified by densitometry.

Figure 10. **Effect of repletion with nat- or ent-cholesterol on in vitro autophosphorylation of the EGF receptor.** A431 cells were treated without (CON) or with (CDX) 10 mM methyl-β-cyclodextrin. Cholesterol-depleted cells were subsequently repleted by incubation for 30 min with 0.2 mM of either nat-cholesterol (NAT) or ent-cholesterol (ENT) in complex with methyl-β-cyclodextrin. Membranes were prepared and aliquots were subjected to *in vitro* autophosphorylation assays as described in the Experimental Procedures. Results from a representative experiment were quantified by densitometry.
Figure 11. Reconstitution of lipid rafts by nat- and ent-cholesterol. A431 cells were treated without (CON) or with (CDX) methyl-β-cyclodextrin for 30 min. Some CDX-treated cultures were subsequently repleted with cholesterol by incubation for 30 min with 0.2 mM nat-cholesterol (NAT) or ent-cholesterol (ENT) in complex with methyl-β-cyclodextrin. Lipid rafts were prepared as described in Experimental Procedures. Equal aliquots of fractions from the sucrose density gradient were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting for the EGF receptor or flotillin.
### Table I.

*Cholesterol Content of A431 Cells Treated with Methyl-β-Cyclodextrin Complexes with Either Enantiomer of Cholesterol*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cholesterol µg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>+ Cyclodextrin</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>+ Nat-Cholesterol</td>
<td></td>
</tr>
<tr>
<td>0.02 mM</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>0.07 mM</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>0.20 mM</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>+ Ent-Cholesterol</td>
<td></td>
</tr>
<tr>
<td>0.02 mM</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>0.07 mM</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>0.20 mM</td>
<td>39 ± 4</td>
</tr>
</tbody>
</table>

A431 cells were treated without (Control) or with 7.5 mM methyl-β-cyclodextrin (+Cyclodextrin) for 30 min at 37°C. Cyclodextrin-treated cells were then incubated for 30 min at 37°C with the indicated concentration of either nat-cholesterol (+Nat-Cholesterol) or ent-cholesterol (+Ent-Cholesterol) in complex with methyl-β-cyclodextrin. The medium was removed and the cell monolayers were washed twice with cold PBS. Lipids were then extracted and cholesterol levels were determined using a colorimetric cholesterol assay as described in the Methods. Total cellular protein content was determined by the bicinchoninic acid method. The values shown represent the mean ± SD of triplicate determinations.