Cholesterol Modulates Cellular TGF-β Responsiveness by Altering TGF-β Binding to TGF-β Receptors

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Transforming growth factor-β (TGF-β) responsiveness in cultured cells can be modulated by TGF-β partitioning between lipid raft/caveolae- and clathrin-mediated endocytosis pathways. The TβR-II/TβR-I binding ratio of TGF-β on the cell surface has recently been found to be a signal that controls TGF-β partitioning between these pathways. Since cholesterol is a structural component in lipid rafts/caveolae, we have studied the effects of cholesterol on TGF-β binding to TGF-β receptors and TGF-β responsiveness in cultured cells and animals. Here we demonstrate that treatment with cholesterol, alone or complexed in lipoproteins, decreases the TβR-II/TβR-I binding ratio of TGF-β in all cell types studied. Among cholesterol derivatives and analogs examined, cholesterol is the most potent agent for decreasing the TβR-II/TβR-I binding ratio of TGF-β. Cholesterol treatment increases accumulation of the TGF-β receptors in lipid rafts/caveolae as determined by sucrose density gradient ultracentrifugation analysis of cell lysates. Cholesterol/LDL suppresses TβR-I responsiveness and TβR-II/CD enhances it, as measured by the levels of P-Smad2 and PAI-1 expression in cells stimulated with TGF-β. Furthermore, the cholesterol effects observed in cultured cells are also found in the aortic endothelium of atherosclerotic ApoE-null mice fed a high cholesterol diet. These results indicate that high plasma cholesterol levels may contribute to the pathogenesis of certain diseases (e.g., atherosclerosis) by suppressing TGF-β responsiveness.

to those found in cells grown on 3D gel (Sankar et al., 1996). Cell-surface heparan sulfate negatively modulates TGF-β responsiveness in epithelial cells by decreasing the TβRII/TβRI binding ratio of TGF-β without altering the protein levels of these receptors (Chen et al., 2006).

Cholesterol is an important structural component of lipid rafts/caveolae in plasma membranes (Harder and Simons, 1997; Galbiati et al., 2001). Cholesterol depletion by cholesterol binding agents such as β-methylcyclodextrin (β-CD) and nystatin has commonly been used as an experimental approach to examine the functional role of lipid rafts/caveolae localization of resident membrane proteins (Park et al., 1998). Cholesterol depletion redirects resident membrane proteins to non-lipid raft microdomains by decreasing formation of or destabilizing lipid rafts/caveolae and influences the activities of these membranes proteins. Since lipid rafts/caveolae mediate TGF-β degradation, resulting in suppressed TGF-β responsiveness (Di Guglielmo et al., 2003; Chen et al., 2006), we hypothesize that cholesterol treatment of cells results in increased lipid raft/caveolae localization of TGF-β receptors (via increasing formation of or stabilizing lipid rafts/caveolae) and suppressed TGF-β responsiveness. To test this hypothesis, we have studied the effects of cholesterol on TGF-β binding to TGF-β receptors and TGF-β responsiveness in several cell types. In this study, we demonstrate that treatments of cells with cholesterol, LDL or VLDL decreases the TβRII/TβRI binding ratio of TGF-β and suppresses TGF-β responsiveness (as determined by measurement of TGF-β-stimulated Smad2 phosphorylation and plasminogen activator inhibitor-1 expression) while treatment with cholesterol-lowering agents or other cholesterol-depleting agents increases the TβRII/TβRI binding ratio of TGF-β and potentiates TGF-β responsiveness. We also show that cholesterol increases the accumulation of TβRI and TβRII in lipid rafts/caveolae. Furthermore, we show that the cholesterol effects in cultured cells (exhibiting a low TβRII/TβRI binding ratio of TGF-β and a low level of P-Smad2) are also detected in the aortic endothelium of Apoe-null mice fed a high cholesterol diet.

**Materials and Methods**

**Materials**

Na<sup>125I</sup> (17 Ci/mg) and [methyl-<sup>3</sup>H] thymidine (67 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). High molecular mass protein standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa), chloramidine-T, bovine serum albumin (BSA), LDL, VLDL, HDL, fluvastatin, lovastatin, cholesterol (≥99% pure), cholest-4-ene-3-one, disuccinimidyl suberate (DSS), nystatin, β-cyclodextrin (β-CD), and lipoprotein (a) were obtained from Sigma (St. Louis, MO). 25-Hydroxycholesterol, 7-dehydrocholesterol (7-DHC), 7-ketosterol, 7-ketosterol 5-cholen-7-one and 4-cholen-3-one were obtained from Steraloids (Newport, RI). Oxidized 7-DHC (oxy-7-DHC) was prepared by air drying a thin film of 7-DHC (initially dissolved in chloroform) at ambient room temperature, protected from exposure to light with aluminum foil, for 1 week. Reverse-phase HPLC revealed a mixture of polar products, but no remaining 7-DHC. Phospho-Smad2 (P-Smad2) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). TGF-β, was purchased from Austral Biologicals (San Ramon, CA). Mouse polyclonal antibodies to the transferrin receptor 1 (TR1) were obtained from Zymed Laboratories (San Francisco, CA). Rabbit polyclonal antibodies to caveolin-1, TβRI-1 (ALK5), TβRII-2, and α-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The luciferase assay system was obtained from Promega (Madison, WI).

**Cell culture**

Mink lung epithelial cells (Mv1Lu cells), Chinese hamster ovary cells (CHO-K1 cells), bovine aorta endothelial cells (BAEC) and normal rat kidney fibroblasts (NRK cells) were maintained in DMEM or DMEM-F12 containing 10% fetal calf serum with or without bFGF.

**125I-TGF-β affinity labeling**

Cells grown on 6-well dishes in DMEM or DMEM-F12 containing 10% fetal calf serum were treated with 50 μg/ml cholesterol or cholesterol analogs/derivatives, 5 or 50 μg/ml LDL, HDL, VLDL in serum-free medium at 37°C for 1 h or 1 μM fluvastatin or lovastatin in serum-free medium (volume: 1 ml) at 37°C for 18 h. 125I-TGF-β affinity labeling was then performed at 0°C using the cross-linking agent DSS according to the published procedures (Cheifetz et al., 1988; Huang et al., 2003). 125I-TGF-β, affinity-labeled cell lysates were analyzed by 7.5% SDS–PAGE followed by autoradiography or quantification using a Phosphomager. For all experiments, cholesterol or cholesterol analogs/derivatives were prepared as the stock solution with a concentration of 25 or 50 mg/ml in ethanol. The final concentration of ethanol in the medium was 0.2%. In all of the cell types used in the experiments, the TβRI, which was affinity-labeled by 125I-TGF-β using the bifunctional reagent DSS, was identified as ALK5 as evidenced by immunoprecipitation of 125I-TGF-β1,TβRI complexes using specific antibodies to alk5.

**Northern blot analysis**

Cells grown on 12-well dishes in DMEM containing 10% fetal calf serum were treated with several concentrations of cholesterol or vehicle only in serum-free DMEM (volume: 0.5 ml/well) at 37°C for 1 h. The final concentration of ethanol in the medium was 0.2%. The cholesterol-treated cells were then incubated with 50 or 100 pM TGF-β1 at 37°C for 2 h. The transcripts of plasminogen activator inhibitor-I (PAI-1) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in the cell lysates were analyzed by Northern blot analysis and quantified with a Phosphomager which yields a linearity from 9,000 to 100,000 arbitrary units of the transcript intensity.

**Western blot analysis**

Cells grown to near confluence on 12-well dishes were treated with cholesterol or vehicle only in serum-free DMEM (volume: 0.5 ml/well) at 37°C for 1 h. The treated cells were further incubated with 50 or 100 pM TGF-β1 at 37°C for 30 min (for determining Smad2 phosphorylation) or for 2 h (for determining the protein levels of TβRI and TβRII). Cell lysates were analyzed by 7.5% SDS–PAGE and Western blot analysis using anti-Smad2, anti-Activated Smad2, anti-caveolin-1, anti-TβRI (ALK5) or anti-TβRII antibodies as described (Huang et al., 2003). The antigens on the blots were visualized by using horseradish peroxidase-conjugated anti-rabbit IgG antibody and the enhanced chemiluminescence (ECL) system as described (Huang et al., 2003). The relative amounts of antigen bands on X-ray films were quantified by densitometry.

**125I-TGF-β affinity labeling and Western blot analysis of aortic endothelium from Apoe-null and wild-type mice**

Female (2–3 month old) Apoe-null and wild-type mice (C57BL/6) were fed with high-cholesterol (2%) and normal diets for 4–6 weeks and then sacrificed. The plasma levels of cholesterol in Apoe-null mice fed high-cholesterol and normal diets for 4–6 weeks were estimated to be 1,000–1,400 mg/dl and 500–700 mg/dl, respectively based on the standard assay procedures. The high-cholesterol diet did not significantly affect the plasma levels of cholesterol in wild-type mice (100–120 mg/dl). Apoe-null mice fed a high-cholesterol diet exhibited typical
atherosclerotic lesions (e.g., fatty streaks and plaques) in the aorta as described previously (Palinski et al., 1994). ApoE-null mice fed normal diet had no significant atherosclerotic lesions in the aorta. Aorta (~3 cm length) was removed and cut lengthwise to expose the intima and then incubated with 100 µM 125I-TGF-β1 in binding buffer containing 2 mg/ml BSA (Huang et al., 2003). After 2.5 h at 0°C, 125I-TGF-β1-affinity labeling was performed using DSS (Huang et al., 2003). The aortas were then extensively washed and the intimal endothelium was scraped off from the luminal surface of aorta using a razor blade. The aortic endothelium was extracted with 1% Triton X-100. An obtained by scrapping off the endothelium from the luminal surface (blade. For Western blot analysis of aortic endothelium, aorta endothelium could be removed by scrapping off intimal
endothelium as evidenced by the observation that the majority of
intimal endothelium was scrapped off from the luminal surface of
aorta using a razor blade. The aortic endothelium was extracted
with 1% Triton X-100. The Triton X-100 extracts were then
analyzed by 7.5% SDS–PAGE and autoradiography or quantification
with a PhosphoImager. Under the experimental conditions,
125I-TGF-β1-affinity labeling occurred mainly in the intimal
endothelium as evidenced by the observation that the majority of
125I-TGF-β1 radioactivity (>90%) and factor VIII antigen in aortic
endothelium could be removed by scrapping off intimal
endothelium from the luminal surface of the aorta using a razor
blade. For Western blot analysis of aortic endothelium, aorta
(~3 cm length) was cut lengthwise. Intimal endothelium was
obtained by scrapping off the endothelium from the luminal surface
of aorta using a razor blade and extracted with 1% Triton X-100. An
equal protein amount (~200 µg protein) of Triton X-100 extracts
was subjected to 7.5% SDS–PAGE followed by Western blot
analysis using antibodies to P-Smad2, Smad2, TβR-I (ALK5), TβR-II
and α-actin and quantification by ECL and densitometry.

Sucrose density gradient ultracentrifugation
Mv1Lu and NRK cells were grown to near confluence in 100-mm
dishes (5–10 x 10⁶ cells per dish) and incubated with cholesterol
(50 µg/ml) at 37°C for 1 h and then incubated with TGF-β1
(100 pM) for 1 h. After being washed with ice-cold
phosphate-buffered saline, cells were scraped into 0.85 ml
of 500 mM sodium carbonate, pH 11.0. Homogenization was
carried out with 10 strokes of a tight-fitting Dounce homogenizer
(Soniprep 150; Fisher Scientific, Pittsburgh, PA) to disrupt cellular
membranes as described previously (Ito et al., 2004; Chen et al.,
2006). The homogenates were analyzed by sucrose density
gradient ultracentrifugation as described previously (Ito et al.,
2004; Chen et al., 2006). Ten 0.5-ml fractions were collected from
the top of the tube, and a portion of each fraction was analyzed by
SDS–PAGE followed by Western blot analysis using antibodies to
TβR-I, TβR-II, caveolin-1, and TFR-1. The relative amounts of TβR-I
and TβR-II on the blot were quantified by densitometry.

Statistical analysis
One-way ANOVA was used to compare groups. Comparisons
with the control group and experimental group were performed
using the unpaired t-test and the Mann-Whitney test. P < 0.05
was considered significant.

Results
Treatment of cells with cholesterol decreases the
TβR-II/TβR-I binding ratio of TGF-β on the cell surface
The TβR-II/TβR-I binding ratio of TGF-β has been shown to be
positively correlated with TGF-β responsiveness in several cell
systems (McCaffrey et al., 1995; Sankar et al., 1996; Coppa et al.,
1997; McCaffrey et al., 1997; Letamendia et al., 1998; Eckelberg
et al., 2002; Quan et al., 2004; Chen et al., 2006). We recently
reported that the TβR-II/TβR-I binding ratio of TGF-β in
TGF-β receptor oligomeric complexes on the cell surface is a
signal determining TGF-β partitioning between lipid
raft/caveolae- and clathrin-mediated endocytosis pathways and
resultant TGF-β responsiveness (Huang and Huang, 2005;
Chen et al., 2006). When the TβR-II/TβR-I binding ratio of
TGF-β increases, more receptor-bound TGF-β (as Complex I,
which contains more TβR-II than TβR-I and exists in non-lipid
raft microdomains) undergoes clathrin-mediated
endocytosis and generates signaling in endosomes, leading to
promotion of TGF-β responsiveness; when the
TβR-II/TβR-I binding ratio of TGF-β decreases, more
receptor-bound TGF-β (as Complex II, which contains more
TβR-I than TβR-II and exists in lipid rafts/caveolae) undergoes
lipid raft/caveolae-mediated endocytosis and rapid degradation,

Fig. 1. Time dependence of the cholesterol effect on 125I-TGF-β1 binding to TβR-I and TβR-II in Mv1Lu cells. Cells were treated with
50 µg/ml cholesterol or vehicle only at 37°C for the time periods as indicated and 125I-TGF-β1-affinity labeling was performed. 125I-TGF-β1-
affinity-labeled TGF-β receptors were analyzed by 7.5% SDS–PAGE and autoradiography (a) and quantified with a Phospholmager (b). The bracket
indicates the locations of 125I-TGF-β1 affinity-labeled TβR-I and TβR-II. The TβR-II/TβR-I ratio of 125I-TGF-β1 affinity-labeled TβR-II
and TβR-I is plotted against the time of treatment with cholesterol (c).
resulting in suppression of TGF-β responsiveness (Chen et al., 2006). To determine if cholesterol treatment alters the TβR-II/TβR-I binding ratio of TGF-β, we first determined the time course of the effect of cholesterol treatment on the TβR-II/TβR-I binding ratio of 125I-TGF-β, in Mv1Lu cells. These cells were treated with 50 μg/ml cholesterol at 37°C for several time periods. 125I-TGF-β binding to TβR-I and TβR-II was then determined by affinity labeling at 0°C using the bifunctional cross-linking agent DSS followed by 7.5% SDS–PAGE and autoradiography or quantification using a PhosphoImager. As shown in Figure 1, treatment of Mv1Lu cells with 50 μg/ml cholesterol increased binding of 125I-TGF-β to TβR-I in a time-dependent manner (Fig. 1A). The cholesterol-induced increase of 125I-TGF-β binding to TβR-I was observed within 10 min. Cholesterol increased 125I-TGF-β binding to TβR-I by ~2-fold after a 60- or 120-min incubation, whereas it moderately affected 125I-TGF-β binding to TβR-II after the same time incubation (Fig. 1B). Cholesterol treatment for 60 or 120 min appeared to decrease the TβR-II/TβR-I binding ratio of 125I-TGF-β, from 0.37 (in control cells treated without cholesterol) to 0.18 (Fig. 1C). These results indicate that cholesterol exerts its effect promptly.

We then performed analysis of the effect of cholesterol treatment on 125I-TGF-β binding to TβR-I and TβR-II in several cell types. Mv1Lu, BAEC, and NRK cells were treated with 1 h with increasing concentrations of cholesterol. The binding of 125I-TGF-β to TβR-I and TβR-II in these cells was then determined by 125I-TGF-β affinity labeling at 0°C. As shown in Figure 2A–C, cholesterol treatment effectively increased the binding of 125I-TGF-β to TβR-I but slightly affected the binding of 125I-TGF-β to TβR-II in these three cell types. At 50 μg/ml cholesterol increased 125I-TGF-β binding to TβR-I by ~2.5-fold, ~2.5-fold, and ~5.7-fold in Mv1Lu, BAEC, and NRK cells, respectively (Fig. 2A–C). Thus, cholesterol (50 μg/ml) decreased the TβR-II/TβR-I binding ratio of 125I-TGF-β, from 0.4 to 0.2, 3.5 to 1.4, and 2.0 to 0.4 in Mv1Lu, BAEC, and NRK cells, respectively. These results suggest that pretreatment of cells with cholesterol decreases the TβR-II/TβR-I binding ratio of 125I-TGF-β on the cell surface of all three cell types studied.

**Cholesterol does not affect the affinity of TGF-β binding to TGF-β receptors**

To determine if cholesterol treatment affects the affinity of TGF-β binding to TβR-I and TβR-II, Mv1Lu cells were treated with 50 μg/ml cholesterol at 37°C for 1 h and then incubated with increasing concentrations of 125I-TGF-β, at 0°C for 2.5 h. 125I-TGF-β affinity labeling was performed. 125I-TGF-β affinity-labeled TGF-β receptors were then analyzed by 7.5% SDS–PAGE and autoradiography (Fig. 3Aa,Ba) or quantification using a PhosphoImager (Fig. 3Ab,Bb). Cholesterol (50 μg/ml) appeared to increase 125I-TGF-β (125 μM) binding to TβR-I by ~2-fold (from ~350 to ~700 × 10^3 A.U.) but did not increase 125I-TGF-β binding to TβR-II (from ~180 to ~120 × 10^3 A.U.; Fig. 3Bb vs. Fig. 3Ab). The half-maximum concentrations of 125I-TGF-β binding to TβR-I and TβR-II were ~80–100 pM in Mv1Lu cells treated with and without cholesterol. The half-maximum concentrations, ~80–100 pM, were close to the apparent Kₐ of TGF-β binding to TβR-I and TβR-II (Huang et al., 2003). These results suggest that cholesterol treatment significantly increases 125I-TGF-β binding to TβR-I, thus decreasing the TβR-II/TβR-I binding ratio of 125I-TGF-β but
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Fig. 3. Effect of cholesterol on binding of increasing concentrations of 125I-TGF-β1 to TβR-I and TβR-II in Mv1Lu cells. Cells were treated with 50 μg/ml cholesterol (B) or vehicle only (A) at 37°C for 1 h. 125I-TGF-β1, affinity labeling was performed and analyzed (a). The relative amounts of 125I-TGF-β1 affinity-labeled TβR-I and TβR-II, which were quantified with a Phosphoimager, are plotted against TGF-β1 concentrations (b).

does not alter the affinities of 125I-TGF-β1 binding to either TβR-I or TβR-II.

Lipoproteins and statins alter the TβR-II/TβR-I binding ratio of TGF-β on the cell surface

To further define the physiological significance of the cholesterol effect on TGF-β1 binding to TGF-β receptors, we determined the effect of pretreatment at 37°C for 1 h with cholesterol-containing lipoproteins (LDL, VLDL, and HDL), nystatin (a cholesterol-binding agent; Wang et al., 1998) and β-CD (Subtil et al., 1999) 4-cholesten-3-one (a cholesterol derivative) and cholesterol, alone or in combination, on 125I-TGF-β1 binding to TβR-I and TβR-II (as determined by 125I-TGF-β1 affinity labeling) in Mv1Lu cells. As shown in Figure 4A, LDL (50 μg protein/ml) and VLDL (5 μg protein/ml) increased 125I-TGF-β1 binding to TβR-I but not TβR-II, and thus decreased the TβR-II/TβR-I binding ratio of 125I-TGF-β1 from 0.46 to 0.18 and 0.25, respectively (lanes 2 and 5 vs. lane 1). HDL (50 μg protein/ml) slightly decreased 125I-TGF-β1 binding to TβR-I and thus increased the TβR-II/TβR-I binding ratio of 125I-TGF-β1 from 0.46 to 0.56 (Fig. 4A, lane 3 vs. lane 1). Lipoprotein (a) (50 μg/ml), an atherogenic lipoprotein which exerts its atherogenic effect via a cholesterol-independent mechanism (Kojima et al., 1991), did not significantly affect 125I-TGF-β1 binding to either TβR-I or TβR-II (Fig. 4A, lane 4 vs. lane 1). The LDL- or cholesterol-induced increase of 125I-TGF-β1 binding to TβR-I was abolished in the presence of β-CD, nystatin, or 4-cholesten-3-one (Fig. 4B, lanes 3, 5, and 6 vs. lane 2 and Fig. 4C, lanes 5 and 6 vs. lane 4). For example, the TβR-II/TβR-I binding ratio of 125I-TGF-β1 in cells treated with both LDL or cholesterol and β-CD or nystatin was similar to that of cells treated with β-CD or nystatin alone (Fig. 4C, lanes 5 and 6 vs. lanes 2 and 3). Treatment of cells with β-CD or nystatin alone decreased 125I-TGF-β1 binding to TβR-I and thus increased the TβR-II/TβR-I binding ratio of 125I-TGF-β1, as compared to untreated control cells (Fig. 4C, lanes 2 and 3 vs. lane 1). These results indicate that LDL and VLDL significantly increase 125I-TGF-β1 binding to TβR-I, resulting in a decreased TβR-II/TβR-I binding ratio of 125I-TGF-β1. Since treatment with cholesterol-binding agents β-CD or nystatin abolishes LDL- or cholesterol-induced increase of 125I-TGF-β1 binding to TβR-I, these results also suggest that LDL decreases the TβR-II/TβR-I binding ratio of 125I-TGF-β1 via the cholesterol component rather than other lipid or protein components.

Since statins are cholesterol-lowering agents (Alberts, 1988; Yuan et al., 1991), we reasoned that pretreatment of cells with statins (1 μM, at 37°C for 16 h) should reduce the cholesterol content in the plasma membrane (due to cholesterol metabolism or turnover), thereby decreasing TGF-β1 binding to TβR-I and increasing the TβR-II/TβR-I binding ratio of TGF-β1 (i.e., the converse of the cholesterol-induced increase of TGF-β1, binding to TβR-I). As shown in Figure 4D–F, treatment with fluvastatin (F) or lovastatin (L) decreased 125I-TGF-β1 binding to TβR-I in CHO-K1, Mv1Lu, and BAEC cells, respectively (Fig. 4D–F, lanes 2 and 3 vs. lane 1) and thus increased the TβR-II/TβR-I binding ratio of 125I-TGF-β1 from 0.12 to 0.19 or 0.18, 0.88 to 1.3 or 1.18 and 2.47 to 2.91 or 2.6 in CHO-K1, Mv1Lu, and BAEC cells, respectively. The statin effect was abolished by incubation of cells with cholesterol prior to 125I-TGF-β1 affinity labeling in CHO-K1, Mv1Lu, and BAEC cells (Fig. 4D–F, lanes 5 and 6 vs. lanes 2 and 3), suggesting that the statin effect is due to its cholesterol-lowering properties.

Among cholesterol derivatives and analogs examined, cholesterol is the most potent for decreasing the TβR-II/TβR-I binding ratio of TGF-β on the cell surface

To define the specificity of the cholesterol effect, we determined the effects of cholesterol analogs and derivatives on 125I-TGF-β1 binding to TβR-I and TβR-II in Mv1Lu cells. These cells were treated with 50 μg/ml of cholesterol or related oysterol derivatives (25-hydroxycholesterol, 7-DHC, oxy-7-DHC, cholest-5-en-7-one and 7-ketocholesterol) at
37°C for 1 h, 125I-TGF-β1 binding to TβR-I and TβR-II in Mv1Lu (A–E), CHO-K1 (D) and BAEC (F) cells. Cells were treated with LDL (50 μg protein/ml), VLDL (5 μg protein/ml), HDL (50 μg protein/ml), lipoprotein (a) (10 μg/ml), lovastatin (F; 1 μM), lovastatin (L; 1 μM), nystatin (Nys; 25 μg/ml), β-CD (8 mM), cholesta-4,6-diene (Chol; 50 μg/ml) alone or combination of two. After 1 h at 37°C or 16 h at 37°C (only for fluvastatin and lovastatin), 125I-TGF-β1 affinity labeling was performed. For cells treated with fluvastatin or lovastatin, 125I-TGF-β1 affinity labeling was performed following incubation with cholesterol (Chol; 50 μg/ml) or vehicle (ethanol) only at 37°C for 1 h. The relative amounts of 125I-TGF-β1 affinity-labeled TβR-I and TβR-II were quantified with a PhosphoImager. The TβR-II/TβR-I binding ratio of 125I-TGF-β1 was estimated. The numbers above the lane sequence denote the TβR-II/TβR-I binding ratios of 125I-TGF-β1.

Cholesterol increases accumulation of TGF-β receptors in lipid rafts/caveolae

As demonstrated above, cells treated with cholesterol exhibited a low TβR-II/TβR-I binding ratio of 125I-TGF-β1 on the cell surface, which is the characteristic property of TGF-β receptor complexes localized in lipid rafts/caveolae (Chen et al., 2006). We then examined the localization of the TGF-β receptors in lipid rafts/caveolae of the plasma membrane in cells treated with 50 μg/ml cholesterol or vehicle (ethanol) only using sucrose density gradient ultracentrifugation (Ito et al., 2004; Chen et al., 2006). Sucrose density gradient ultracentrifugation has been a standard procedure used to separate lipid rafts/caveolae from other microdomains in lipid rafts/caveolae.

Fig. 4. Effects of cholesterol analogs and derivatives on 125I-TGF-β1 binding to TβR-I and TβR-II in Mv1Lu cells. Cells were treated with 50 μg/ml of cholesterol and cholesterol analogs/derivatives including 25-hydroxycholesterol, 7-DHC, 7-ketocholesterol, 7β,8β-epoxycholesterol, 7β-hydroxycholesterol, oxy-7-DHC, 50 μg/ml LDL and 5 μg/ml VLDL. After 1 h at 37°C, 125I-TGF-β1 affinity labeling was performed, analyzed by autoradiography and quantified with a Phospholmager. The TβR-II/TβR-I binding ratios of 125I-TGF-β1 were estimated. The numbers above the lane sequence denote the TβR-II/TβR-I binding ratios of 125I-TGF-β1.
plasma membranes. As shown in Figure 6A,B, TβR-II was mainly localized in non-lipid raft fractions (fractions 7 and 8, which contain TIR1) of Mv1Lu and NRK cells. TβR-I was not analyzed in Mv1Lu cells due to the poor reactivity of the anti-TβR-I antibody (used in the experiment) to mink TβR-I. After treatment with cholesterol, TβR-I and TβR-II were enriched in the lipid raft/caveolae fractions (fractions 4 and 5) of the plasma membrane in Mv1Lu and NRK cells. Treatment with cholesterol did not affect the total amounts of TβR-I and TβR-II proteins (Fig. 6 Ab,Bb) and localization of caveolin-1 and TIR1 (data not shown) in these cells. These results suggest that cholesterol increases accumulation of TGF-β receptors in lipid rafts/caveolae. They are also consistent with the observation of a low TβR-II/TβR-I binding ratio of TGF-β in cells treated with cholesterol. A low TβR-II/TβR-II binding ratio of TGF-β is the characteristic property of cell-surface TGF-β receptor oligomeric complexes localized in lipid rafts/caveolae (Huang and Huang, 2005; Chen et al., 2006).

Cholesterol suppresses TGF-β responsiveness

Since lipid rafts/caveolae have been shown to mediate downregulation of TGF-β-stimulated signaling and thus TGF-β responsiveness (Di Guglielmo et al., 2003; Le Roy and Wra, 2005; Huang and Huang, 2005), the finding of cholesterol-induced localization of TβR-I and TβR-II in lipid rafts/caveolae suggests that treatment of cells with cholesterol should suppress TGF-β-stimulated signaling and TGF-β responsiveness. To test this, we determined the effect of cholesterol on TGF-β-stimulated Smad2 phosphorylation, which is a key signaling event leading to TGF-β responsiveness (Heldin et al., 1997; Massague, 1998; Miyazono et al., 2000; Moustakas et al., 2002; Feng and Derynck, 2005). Mv1Lu cells were treated with 50 μg/ml cholesterol at 37 °C for 1 h and then incubated with 50 pM TGF-β1 at 37 °C for 30 min. P-Smad2 and Smad2 in the cell lysates were analyzed by 7.5% SDS–PAGE followed by Western blot analysis using anti-P-Smad2 and anti-Smad2 antibodies and ECL and quantified by densitometry. As shown in Figure 7, cholesterol treatment attenuated TGF-β1-stimulated Smad2 phosphorylation by ~50% in these cells (Fig. 7A, lane 4 vs. lane 3, and Fig. 7B). To determine the effect of cholesterol on TGF-β responsiveness, we analyzed the PAI-1 expression in cells treated with and without cholesterol and then stimulated with and without TGF-β1. TGF-β1-stimulated PAI-1 expression is mediated by the Smad signaling pathway (Lund et al., 1987; Heldin et al., 1997; Massague, 1998). We used Northern blot analysis and quantification using a PhosphoImager which provided a linearly

**Fig. 6.** Sucrose density gradient ultracentrifugation analysis of TβR-I and TβR-II in Mv1Lu (A) and NRK (B) cells treated with and without cholesterol. Cells were treated with 50 μg/ml cholesterol or vehicle only at 37°C for 1 h. The cell lysates from these treated cells were directly analyzed by Western blot analysis using antibodies to TβR-I and TβR-II (b) and subjected to sucrose density gradient ultracentrifugation. The sucrose density gradient fractions were then analyzed by Western blot analysis (a). The arrow indicates the locations of TβR-I, TβR-II, caveolin-1, and TIR1. Fractions 4 and 5 contained lipid rafts/caveolae (which contained caveolin-1), whereas fractions 7 and 8 represented non-lipid raft fractions, which contained TIR1. * indicates the increased amount of TβR-I or TβR-II in the fraction as compared to that of the untreated control.
quantitative measurement. Mv1Lu cells were treated with increasing concentrations of cholesterol at 37°C for 1 h and then further incubated with 50 µM TGF-β, at 37°C for 30 min. P-Smad2 and total Smad2 in the cell lysates were analyzed by immunoblotting (A). The quantitative analysis of the immunoblots expressed as arbitrary units (A.U.) is shown (B). The data bar represents the mean ± SD (n=3). *Significantly lower than that in cells treated with TGF-β1 only: P<0.001.

Fig. 7. Effect of cholesterol on Smad2 phosphorylation in Mv1Lu cells stimulated with TGF-β. Cells were treated with 50 µg/ml cholesterol or vehicle only at 37°C for 1 h and then further incubated with 50 pM TGF-β, at 37°C for 30 min. P-Smad2 and total Smad2 in the cell lysates were analyzed by immunoblotting (A). The quantitative analysis of the immunoblots expressed as arbitrary units (A.U.) is shown (B). The data bar represents the mean ± SD (n=3). *Significantly lower than that in cells treated with TGF-β1 only: P<0.001.

To examine the TGF-β responsiveness, we performed 125I-TGF-β affinity labeling and determined the level of P-Smad2 in aortas and other tissues (Kalinina et al., 2004; Phipps et al., 2004). As shown in Figure 9A, the aortic endothelium from ApoE-null mice exhibited increased 125I-TGF-β binding to TGF-β1 and concomitantly decreased 125I-TGF-β binding to TGF-β II (Fig. 9Aa, lane 2 vs. lane 1). The TβR-II/TβR-I binding ratios of 125I-TGF-β in ApoE-null mice appeared to be lower than those found in wild-type mice (Fig. 9Ab, 0.5 vs. 1.08). A low TβR-II/TβR-I binding ratio of 125I-TGF-β was also found in cultured BAEC cells treated with cholesterol (50 µg/ml) as compared with that found in control cells (Fig. 4F, lane 4 vs. lane 1).

To determine the in vivo relevance of the effect of cholesterol, we performed 125I-TGF-β affinity labeling and determined the level of P-Smad2 in the aortic endothelium of wild-type mice and ApoE-null mice fed a high-cholesterol (2%) or normal diet. ApoE-null mice fed a high-cholesterol diet have commonly been used as an animal system to study the mechanisms by which hypercholesterolemia initiates and/or facilitates atherogenesis. ApoE-null mice fed a high-cholesterol diet show typical atherosclerotic lesions such as fatty streaks and plaques in the aorta as described (Palinski et al., 1994). To determine the TβR-II/TβR-I binding ratio of 125I-TGF-β in the aortic endothelium, the aortas were cut lengthwise to expose the endothelium and incubated with 125I-TGF-β in binding buffer. After 2.5 h at 0°C, 125I-TGF-β affinity labeling with DSS was performed; the 125I-TGF-β affinity-labeled aortic endothelium was scraped off from the luminal surface of the aorta and extracted with 1% Triton X-100. The Triton X-100 extracts, which contained factor VIII (an endothelial cell marker), were then analyzed by 7.5% SDS–PAGE and autoradiography. As shown in Figure 9A, the aortic endothelium from ApoE-null mice exhibited increased 125I-TGF-β binding to TβR-II and TβR-I and concomitantly decreased 125I-TGF-β binding to TβR-II (Fig. 9Aa, lane 2 vs. lane 1). The TβR-II/TβR-I binding ratios of 125I-TGF-β in ApoE-null mice appeared to be lower than those found in wild-type mice (Fig. 9Ab, 0.5 vs. 1.08). A low TβR-II/TβR-I binding ratio of 125I-TGF-β was also found in cultured BAEC cells treated with cholesterol (50 µg/ml) as compared with that found in control cells (Fig. 4F, lane 4 vs. lane 1).
Discussion

In this communication, we demonstrate that cells treated with cholesterol, alone or complexed as lipoproteins, decrease the TβR-II/TβR-I binding ratio of TGF-β1 on the cell surface. Conversely, cholesterol-lowering agents and cholesterol-depleting agents increase the TβR-II/TβR-I binding ratio of TGF-β1. Cholesterol may exert this effect by integrating into the plasma membrane as evidenced by the following observations: (1) cholesterol acts rapidly. It effectively increases TGF-β1 binding to TβR-I within 10 min; (2) the cholesterol effect is reversible. It can be reversed by incubation of cholesterol-treated cells with 1% β-CD prior to 125I-TGF-β1 affinity labeling; and (3) the cholesterol effect on 125I-TGF-β1 binding to TGF-β receptors can be reproduced using plasma membranes purified from mouse liver (unpublished results), suggesting that cholesterol treatment is capable of increasing formation or stabilizing lipid rafts/caveolae in the plasma membrane in a cell-free system.

Among the various sterols and cholesterol analogs examined, cholesterol exhibited the most potent activity in increasing 125I-TGF-β1 binding to TβR-I and promoting the formation of Complex II (Huang and Huang, 2005; Chen et al., 2006), which mainly utilizes the lipid raft/caveolae endocytosis pathway. This is consistent with prior reports indicating that cholesterol is a preferred sterol to form lipid rafts/caveolae with sphingolipids and phospholipids. Wang et al. (2004) identified several cholesterol derivatives and other sterols which behave like cholesterol in their ability to be inserted into lipid rafts, using an in vitro phospholipid binding assay. Subsequent studies by Keller et al. (2004), using both an in vitro model system and in vivo studies with rats, demonstrated that
7-DHC is as competent as cholesterol with regard to promoting lipid raft formation in an in vitro system. However, 7-DHC and most of the other sterols analyzed in the present study were unable to function like cholesterol in forming or stabilizing lipid rafts/caveolae in our \( ^{125}\text{I}-\text{TGF-}\beta_{1} \) affinity labeling assay. This suggests that the \( ^{125}\text{I}-\text{TGF-}\beta_{1} \) binding assay may be a useful system to study the structural and functional role of cholesterol in lipid rafts/caveolae in cells. Interestingly, while pure 7-DHC was a poor replacement for cholesterol in the \( ^{125}\text{I}-\text{TGF-}\beta_{1} \) binding assay, exhaustively oxidized 7-DHC (a complex mixture of as yet undefined oxysterols) was nearly as potent as cholesterol in increasing \( ^{125}\text{I}-\text{TGF-}\beta_{1} \) binding to \( \text{T\beta R-I} \). This finding is currently not understood.

Cholesterol treatment appears to promote localization of \( \text{T\beta R-I} \) and \( \text{T\beta R-II} \) (as Complex I, which is characterized by a high \( \text{T\beta R-II}/\text{T\beta R-I} \) binding ratio of \( \text{TGF-}\beta_{1} \)) in non-lipid raft microdomains of the plasma membrane (Chen et al., 2006). This results in subsequent clathrin-mediated endocytosis, endosomal signaling and promoted cellular responsiveness (Huang and Huang, 2005; Chen et al., 2006). For example, depletion of cholesterol by \( \beta\)-CD from the plasma membrane results in potentiated \( \text{TGF-}\beta_{1} \) responsiveness as demonstrated by enhanced PAI-1 expression.

Cholesterol is a major causative agent for atherosclerotic cardiovascular disease. The mechanisms by which cholesterol initiates and/or facilitates atherogenesis have been studied extensively but remain poorly understood. In this communication, we demonstrate that cultured cells treated with cholesterol and the aortic endothelium in ApoE-null mice fed a high cholesterol diet share similar characteristics of suppressed \( \text{TGF-}\beta_{1} \) responsiveness. Aortic endothelial cells of ApoE-null mice fed a high cholesterol diet exhibit a low \( \text{T\beta R-II}/\text{T\beta R-I} \) binding ratio of \( \text{TGF-}\beta_{1} \) and a low level of P-Smad2 as compared with wild-type mice fed a high cholesterol diet. Notably, treatment of cultured cells with cholesterol does not affect the expression of either \( \text{T\beta R-I} \) or \( \text{T\beta R-II} \) whereas the aortic endothelium of ApoE-null mice fed a high cholesterol diet exhibit attenuated expression of \( \text{T\beta R-II} \) and little change in localization of \( \text{T\beta R-I} \) and \( \text{T\beta R-II} \) (as Complex I, which is characterized by a high \( \text{T\beta R-II}/\text{T\beta R-I} \) binding ratio of \( \text{TGF-}\beta_{1} \)) in non-lipid raft microdomains of the plasma membrane (Chen et al., 2006). This results in subsequent clathrin-mediated endocytosis, endosomal signaling and promoted cellular responsiveness (Huang and Huang, 2005; Chen et al., 2006).
expression of TβR-I. The decreased expression of TβR-II protein in the aortic endothelium of these mice may be due to the chronic effect of increased cholesterol on vascular cells in the aortic endothelium of these animals. This possibility is supported by the observation that treatment of BAEc cells with 5 or 15 μg/ml cholesterol in medium containing 10% fetal calf serum for 3 days specifically down-regulates the TβR-II protein level without significantly affecting the TβR-I protein level (unpublished results). A strong causal link between atherosclerosis and low TGF-β responsiveness in vascular cells and/or low TGF-β levels in plasma has been demonstrated in several relevant in vivo models (McCaFFrey et al., 1997; Grainger et al., 2000; Mallat et al., 2001; Reckless et al., 2001; Robertson et al., 2003; Li et al., 2006). The chronically suppressed TGF-β responsiveness in vascular cells caused by hypercholesterolemia might contribute to the pathogenesis of atherosclerosis.

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