G-Protein-Mediated Signaling in Cholesterol-Enriched Arterial Smooth Muscle Cells. 1. Reduced Membrane-Associated G-Protein Content Due to Diminished Isoprenylation of G-γ Subunits and p21ras[†]

Kenneth B. Pomerantz, Harry M. Lander,[‡] Barbara Summers, Janet D. Robishaw,[§] Eric Balcueva,[§] and David P. Hajjar*,[‡]

Departments of Medicine and Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, and Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822

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ABSTRACT: Mechanisms contributing to altered heterotrimeric G-protein expression and subsequent signaling events during cholesterol accretion have been unexplored. The influence of cholesterol enrichment on G-protein expression was examined in cultured smooth muscle cells that resemble human atherosclerotic cells by exposure to cationized LDL (cLDL). cLDL, which increases cellular free and esterified cholesterol 2-fold and 10-fold, respectively, reduced the cell membrane content of Gαi-1, Gαi-2, Gαi-3, Gq/11, and Gαs. The following evidence supports the premise that the mechanism by which this occurs is due to reduced isoprenylation of the Gγ-subunit. First, the inhibitory effect of cholesterol enrichment on the membrane content of Gαi subunits was found to be post-transcriptional, since the mRNA steady-state levels of Gαi(1−3) were unchanged following cholesterol enrichment. Second, the membrane expression of α and β subunits was mimicked by cholesterol and 17-ketocholesterol, both of which inhibit HMG-CoA reductase. Third, inhibition of Gαi and Gβ expression in cholesterol-enriched cells was overcome by mevalonate, the immediate product of HMG-CoA reductase. Fourth, pulse-chase experiments revealed that cholesterol enrichment did not reduce the degradation rate of membrane-associated Gαi subunits. Fifth, cholesterol enrichment also reduced membrane expression of Gγ-5, Gγ-7_{upper}; these γ subunits are responsible for trafficking of the heterotrimeric G-protein complex to the cell membrane as a result of HMG-CoA reductase-dependent post-translational lipid modification (geranylgeranylation) and subsequent membrane association. Cholesterol enrichment did not alter expression of G-γ-5 mRNA, as assessed by reverse transcriptase polymerase chain reaction, supporting a post-transcriptional defect in Gγ subunit expression. Fifth, cholesterol enrichment also reduced the membrane content of p21ras (a low molecular weight G-protein requiring farnesylation for membrane targeting) but did not alter the membrane content of the two proteins that do not require isoprenylation for membrane association—PDGF-receptor or p60-src. Reduced G-protein content in cholesterol-laden cells was reflected by reduced G-protein-mediated signaling events, including ATP-induced GTPase activity, thrombin-induced inhibition of cyclic AMP accumulation, and MAP kinase activity. Collectively, these results demonstrate that cholesterol enrichment reduces G-protein expression and signaling by inhibiting isoprenylation and subsequent membrane targeting. These results provide a molecular basis for altered G-protein-mediated cell signaling processes in cholesterol-enriched cells.

INTRODUCTION

Heterotrimeric and low molecular weight G-proteins occupy a pivotal site in a variety of signal transduction pathways regulating numerous aspects of arterial smooth muscle cell function, including growth (Owens, 1989; Weber et al., 1994a) and blood vessel tone (Flavahan & Vanhoutte, 1990). Diverse G-protein-coupled receptor ligands (thrombin, endothelin, bradykinin, and acetylcholine) are coupled by their cognate receptors to Gαi subunits (Liebmann et al., 1990; Hayashida et al., 1996), which activate common effectors, including phospholipases A_2 and C (Burch & Axelrod, 1987; Antonaccio et al., 1993; Berwins & Fredholm, 1992; Lambert et al., 1986), MAP-kinase (Inglese et al., 1995; Koch et al., 1994), protein kinase C (Marrero et al., 1994; Weber et al., 1994a), and adenylyl cyclase (Hulme, 1990; Birnbaumer, 1992). Similarly, low molecular weight G-proteins, such as p21ras, occupy a central position in transducing signals from receptor tyrosine kinase ligands (i.e., PDGF and FGF[1]) to similar effector molecules to mediate a variety of cellular processes, including proliferation and chemokinesis (Fantl et al., 1993; Klagsbrun & Dluz, 1993; Owens, 1989). Stern and his colleagues have also shown that other cellular processes, such as permeability and cell shape, may also be influenced indirectly by G-protein-mediated signaling (Brett et al., 1989). These observations demonstrate the critical importance of heterotrimeric and low-molecular-weight G-proteins on cytokine and growth factor

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* To whom correspondence and reprint requests should be addressed.
[‡] Department of Biochemistry, Cornell University Medical College.
[§] Geisinger Clinic.

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[1] Abbreviations used: cAMP, cyclic AMP; CE, cholesteryl ester; cLDL, cationized LDL; GTP, guanosine triphosphate; LDL, low-density lipoprotein; PDGF, platelet-derived growth factor, RT-PCR, reverse transcriptase polymerase chain reaction.

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signaling and imply that alterations in G-protein expression may significantly alter smooth muscle function.

Low-molecular-weight G-proteins and G-protein-γ subunits (and hence, heterotrimeric G-protein complexes) are targeted to the cell membrane as a result of isoprenylation of their carboxyl termini (Kinsella & Omahony, 1994; Zhang & Casey, 1996). Since isoprenoid synthesis is dependent upon HMG-CoA reductase, reductions in the activity of this enzyme, as occurs in a variety of cells in response to exogenous cholesterol (Axelson & Larsson, 1995; Angelin, 1988), may alter G-protein dependent expression and signaling. In fact, the expression of pertussis toxin-sensitive G proteins linked to endothelium-dependent vasodilation (nitric oxide) is impaired in a lapine model of atherosclerosis (Shimokawa et al., 1991). In endothelial cells, LDL inhibits the ability of Gαi proteins to generate nitric oxide in response to bradykinin (Liao, 1994), and oxidized LDL reduces Gαi expression in endothelial cells (Liao et al., 1995). These observations suggest that cholesterol or oxidation products of LDL can alter G-protein-mediated signaling events.

Smooth muscle cells comprise a major component of the arterial neointima and accumulate cholesterol during atherogenesis. Currently, there is no information on the influence of cholesterol enrichment of smooth muscle cells on G-protein expression and signaling. In this study, we show that cholesterol enrichment of arterial smooth muscle cells reduces cell membrane expression of G-protein heterotrimers and p21ras. Mechanistic studies suggest that reduced membrane G-protein expression and activity in cholesterol-enriched cells occurs as a consequence of reduced isoprenylation.

EXPERIMENTAL PROCEDURES

Reagents. Antibodies against Gαi-1 (3646), Gαi-2 (1521), Gαi-3 (1518), and a pan-Gαi antibody (8729) were kindly provided by Dr. David Manning (University of Pennsylvania). Antibodies directed against Gαs, Gq/11, pan-Gβ, p21ras, and p60src antibodies were purchased from Santa Cruz Biotechnology, Inc., CA. DNA probes complementary to Gαi-1, Gαi-2, and Gαi-3 were provided by Drs. Randall R. Reed (Johns Hopkins University, MD) and Burton Horowitz (University of Nevada). 17-Ketocholesterol, 25-hydroxycholesterol, cholesterol 5α,6α-epoxide, and mevalonic acid lactone were purchased from Sigma (St. Louis, MO). Linoleic acid was obtained from Nu-Chek Prep (Elysian, MN.). AlF_4^- was prepared from $AlCl_3$ and NaF, diluted into PBS. AlF_4^- mimics the terminal phosphate of GTP and activates otherwise inactive (GDP-bound) Gα subunits. The interaction of AlF_4^- with heterotrimeric G-proteins has been established by spectrophotometric methods (Higashijima et al., 1991). The crystal structure in AlF_4^--transducin complex has been elucidated, and its use as a probe in the mechanism of GTP hydrolysis has been established (Sondek et al., 1994). In numerous biological systems, AlF_4^- specifically stimulates heterotrimeric G-protein activity (Paris & Pouyssegur, 1987; Pimplikar & Simons, 1993; Nakamura et al., 1995; Morishita et al., 1995).

Preparation of Plasma LDL and cLDL. LDL (1.019−1.063 g/mL) was isolated by preparative ultracentrifugation of pooled donor human plasma and cationized using *N*,*N*-dimethyl-1,3-propanediamine (Kodak Chemicals, Rochester, NY) as adduct and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide−HCl (Aldrich Chemicals, Milwaukee, WI) as catalyst at pH 6.5 (Pomerantz & Hajjar, 1989). Both native and cationized LDL (cLDL) contained less than 1 nmol of tetramethoxypropane equivalents/mg protein (Kraemer et al., 1993).

Isolation and Culture of Aortic Smooth Muscle Cells. Cells were propagated from explants of rabbit or rat thoracic aorta and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Logan, UT) and 1% penicillin/streptomycin/amphotericin (v/v/v) in an atmosphere of 5% CO_2 in air at 37 °C (Pomerantz et al., 1984). Cells were identified as smooth muscle by their hill-and-valley morphology at confluence and by positive staining for α-actin. Cells were cholesterol-enriched by incubation with cLDL (75 μg protein/ml) in DMEM containing 10% FBS for 7 days. Fresh media containing cLDL was added on day 4 (Pomerantz & Hajjar, 1989). Cholesterol-enriched cells have 2-fold more free cholesterol and from 7- to 15-fold more CE relative to control cells (Pomerantz & Hajjar, 1989). Cells were rendered quiescent by incubation in DMEM containing 1% insulin−transferrin−selenium (ITS). The presence of cholesterol oxides in cLDL, and in cells exposed to cLDL, was measured as described by Hodis et al. (Hodis et al., 1994). The level of cholesterol oxides in cLDL and in cholesterol-enriched cells was below the limit of detection (data not shown).

Western Analysis: α and β Subunits. Cells were washed in Ca^{+2}−Mg^{+2}−free PBS [PBS(−)] and then scraped into PBS(−), pelleted, and resuspended into 200 μL of homogenizing buffer (25 mM HEPES, 1.0 mM EDTA containing 20 μM leupeptin, 15 μM pepstatin A, and 1.0 mM PMSF). Cells were then sonicated until clarified and then centrifuged at 1000 rpm to remove debris. The supernatant was then subjected to 55000*g* for 1 h at 4 °C in a Beckman ultracentrifuge to pellet microsomes. The cytosolic supernatant was removed, and the membrane pellet was resuspended in homogenization buffer and recentrifuged. Samples were frozen at −80 °C in 25 μL aliquots until assay. For Western analysis of Gα and β subunits, MAP kinase, PDGF-R, and p60src, 30−100 μg of membrane and cytosolic protein was reduced (2.5% SDS/250 μM dithiothreitol for 5 min at 90 °C) and alkylated using *N*-ethylmaleimide (2.0 mM, 15 min at room temperature), prior to the addition of loading buffer (0.25 M Tris, 20% SDS, 50% glycerol, 2% dithiothreitol). Samples were boiled for 5 min and then subjected to electrophoresis (Laemmli, 1970) through 15% polyacrylamide. Proteins were transferred to Immobilon-P (Millipore) in 38 mM glycine, 48 mM Tris, and 0.035% SDS (Towbin et al., 1979). After overnight blocking in 20 mM Tris, 0.154 M NaCl, pH 7.4, containing 0.05% Tween-20 and 5% nonfat dry milk at 4 °C, blots were then exposed to primary antibodies for 2 h at 25 °C. Blots were then washed in PBS-Tween (3 × 150 mL) and PBS (1 × 150 mL) and were visualized using the ECL detection system (Amersham Life Science, U.K.) and Kodak X-O-Mat film. The developed peaks were scanned and imaged using Photoshop (MacIntosh) and quantified using NIH Image 1.42. Data are expressed as arbitrary absorbance units.

Western Analysis: Gγ Subunits. Gγ subunit mass was determined as previously reported (Hansen et al., 1995). Briefly, rabbit cells washed in PBS containing 10 μM PMSF were snap-frozen into liquid N_2. Cells were resuspended into 20 mM HEPES (pH 7.2) containing 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, and 10 μg each of leupeptin

and pepstatin A (HME-PI), lysed by passing through a 25 gauge needle, and then homogenized using a glass/Teflon homogenizer. Membrane fractions were isolated by centrifugation (75000*g* for 1 h) through 40% sucrose, followed by rehomogenation in HME-PI and reisolation by centrifugation (110000*g* for 1 h). The membrane-associated γ subunits were solubilized in HME-PI containing 1% cholate and then subjected to SDS−PAGE in 15% gels after addition of 4x SDS-PAGE sample buffer. Proteins were then transferred to nitrocellulose using a high-temperature transfer procedure (Robishaw & Balcueva, 1993), and the relative mass of Gγ-5 and Gγ-7 proteins was visualized by chemiluminescence and quantified as described above.

Metabolic Labeling and Immunoprecipitation of Gαi Subunits. Metabolic labeling and subsequent immunoprecipitation was performed as described by Liao et al. (Liao & Clark, 1995) with modifications. Briefly, normal and cholesterol-enriched cells were incubated in cysteine- and methionine-free modified Eagle's medium supplemented with 125 μCi/mL of [^{35}S]cysteine/methionine (ExpresSS, New England Nuclear, Boston, MA) for 4 h at 37 °C. After 0, 12, 24, 48, and 72 h, cells were harvested and membranes were prepared as described above. Membranes were resuspended in immunoprecipitation buffer [NaCl (150 mM), Tris−HCl, (50 mM, pH 7.4), SDS (0.2%), Triton X-100 (1.0%), aprotinin (1 μg/mL), leupeptin (1.0 μg/mL), and PMSF (1 mM)]. To aliquots containing 150 μg of protein were added pan-Gαi or pan-β antibodies (1:75). Samples were allowed to incubate for 16 h at 4 °C, followed by addition of protein A−sepharose beads. After 2 h, the beads were washed twice in immunoprecipitation buffer, followed by washing once with 150 mM NaCl, 50 mM Tris−HCl, pH 7.4, and 5 mM EDTA. Samples were then resuspended in sample buffer, boiled for 5 min, and then run out in 10% PAGE, which were then fixed and dried. Radioactivity was detected by autoradiography and quantified as described above.

Isolation of RNA and Northern Analysis. Total RNA was prepared by the method of Chirgwin (Chirgwin et al., 1979). Cells were harvested in 4.0 M guanidinium isothiocyanate (GIT buffer) containing 25 mM Na acetate and 0.1% β-mercaptoethanol. The RNA extract was triturated through an 18-gauge needle and applied to a cushion of 5.7 M CsCl containing 25 mM sodium acetate (pH 6.0). After overnight centrifugation at 35 000 rpm, the RNA pellet was solubilized in 0.3 M sodium acetate, precipitated in 70% ethanol, and quantified by UV spectrophotometry. Northern analysis was performed as previously described by our laboratory (Pomerantz et al., 1993). cDNA probes were labeled [^{32}P]-dCTP by the method of random hexamer primer extension. The amounts of RNA were quantified by phosphorimage analysis and normalized by to the expression of GAPDH, whose mRNA steady-state level was unaffected by cholesterol enrichment (Pomerantz et al., 1993).

RT-PCR Analysis of Rabbit Gγ-5 mRNA. Preliminary data demonstrated that arterial smooth muscle cells contain insufficient Gγ-5 mRNA to quantify by Northern analysis using total or poly-A$^+$ mRNA. Furthermore, the genomic sequence of rabbit Gγ-5 is unknown. Accordingly, the amount of Gγ-5 mRNA in rabbit SMC was determined by reverse transcriptase polymerase chain reaction using PCR primers developed from the cDNA sequence of the rat γ-5 sequence (Fisher & Aronson, Jr., 1992). The 19-mer sequence TGTCGGGTTCTTCTAGCGT (corresponding to nucleotides 27−35 of rat γ-5) was used as the forward (5′) primer (primer A). The nucleotide sequence 207−225 of the rat γ-5 cDNA sequence was used to generate a 19-mer 3′-reverse primer (AAGGAGCAGACTTTCTGG, primer B). RT-PCR using these primers against rat RNA generates a 200 bp PCR product. Briefly, 1 μg of rat RNA was subjected to reverse transcriptase employing MuLV reverse transcriptase and random hexamers; incubations were for 42 °C for 1 h. The RT-product was then subjected to PCR using 1.0 μg primers as follows: 94 °C (2.0 min), followed by 35 cycles of 94 °C (1.0 min), 54 °C (1.0 min), 72 °C (1.0 min). Samples were then incubated at 72 °C (10 min) and then held at 4 °C. PCR products were run out on 2% agarose containing ethidium bromide and visualized under UV. For PCR of rabbit RNA, all conditions were kept the same, except the annealing temperature was lowered to 50 °C, and the number of cycles was increased to 40. The PCR product of rabbit SMC RNA was identical in size to the rat SMC PCR product. The expression of Gγ-5 mRNA by this method was normalized to the expression of GAPDH. Since the rabbit GAPDH cDNA has not been cloned, primers were developed from the cDNA sequence of the rat GAPDH gene (Tso et al., 1985). Sequences were chosen that demonstrated homologies to GAPDH to at least three other species. An 18-mer sequence corresponding to nucleotides 42−59 (CGGTGTCAACGGATTTGG) was used to generate the 5′-PCR primer. A 20-mer sequence corresponding to nucleotides 371−390 was used to generate the 3′-reverse primer (GGAGATGATGACCCTTTTGG). RT-PCR of rat SMC RNA generates a 349 bp PCR product using rat SMC RNA indistinguishable in size to that of rabbit SMC RNA.

Cyclic AMP Content. Cyclic AMP content in cell homogenates was measured by radioimmunoassay (New England Nuclear) according to the manufacturer's specifications.

GTPase Assay. Cells in 100 cm^2 flasks were harvested into 100 μL of homogenizing buffer and membranes prepared as described above. The resultant membrane pellet was resuspended into 100 μL of homogenization buffer. GTPase activity was assayed in 100 μL of assay buffer containing 100 mM NaCl, 5.0 mM $MgCl_2$, 0.25 mM EGTA, 12.5 mM Tris, pH 7.4, 2.0 mM DTT, 1.0 mM ATP, 0.5 mM β,γ-imidoadenosine- 5′-[β,γ-imido]- triphosphate, 10 mM creatine PO_4, 50 U/mL creatine kinase, and 100 000 dpm of γ-[^{32}P]-GTP (5000 Ci/mmol) using 5−10 μg of membrane protein (Lander et al., 1993). After 10 min at 37 °C, 800 μL of Norit A was added and vortexed, and the samples were centrifuged for 3 min in a microfuge at 14000*g*. Radioactivity was determined in 450 μL of supernatant. GTPase activity is expressed fmol/min/mg protein.

MAP Kinase Activity. Quiescent cells exposed to PDGF or AlF_4^- were incubated in 20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton, 25 mM β-glycerophosphate, 1.0 mM $NaVO_3$, 2 mM Na phosphate, 20% glycerol, 1.0 mM PMSF, and 10 μg/mL of leupeptin for 15 min at 4 °C, followed by clarification by centrifugation. To 5.0 μg aliquots of each sample was added protein A−sepharose prebound to anti-MAPK (anti Erk-1/Erk-2, Santa Cruz Biotechnology), and the samples incubated for 1 h at 4 °C. After washing (twice with RIPA buffer and three times with 25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, and 0.1 mM $NaVO_3$), samples were resuspended in kinase buffer containing 15 μg of myelin basic protein and [γ-^{32}P]-ATP (10 μCi/nmol). After 15 min at 37 °C, samples were reduced in 6x Laemmli sample buffer

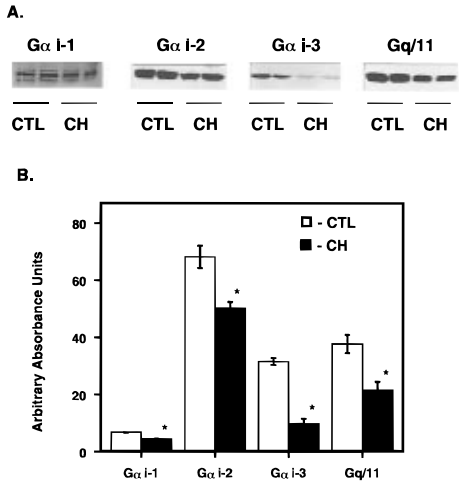

FIGURE 1: Alteration of expression of Gαi subunits by cholesterol enrichment. (A) Normal and cholesterol-enriched rabbit cells grown in 100 mm plates were extracted into homogenization buffer. Membranes were prepared by ultracentrifugation, reduced and alkylated, run out on SDS/PAGE, and transferred to Immobilon-P. Blots were exposed to specific antibodies as described in the Methods. The primary antibodies (Gαi-1, 3646; Gαi-2, 1521; and Gαi-3, 1518) were used at a 1:1000 dilution in blocking buffer containing 0.005% thimerosal) and incubated for 2 h at room temperature. Gq/11 was used at a 1:1000 dilution. After washing in PBS-Tween, blots were exposed to secondary antibodies (donkey anti-rabbit IgG/horseradish peroxidase, 1:10 000), followed by washing and exposure to horseradish peroxidase substrates, followed by autoradiography, and quantification by densitometry as described in the Methods. Each treatment was performed in duplicate (as indicated by lower bars) and is representative of at least three different experiments. Densitometric analysis of blots visualized in A (Gαi-1, Gαi-2, Gαi-3, and Gq/11, by 34%, 26%, 69%, and 43%, respectively). Bars indicate mean ± SEM, * = $p < 0.05$, CTL vs. cholesterol-enriched cells (CH).

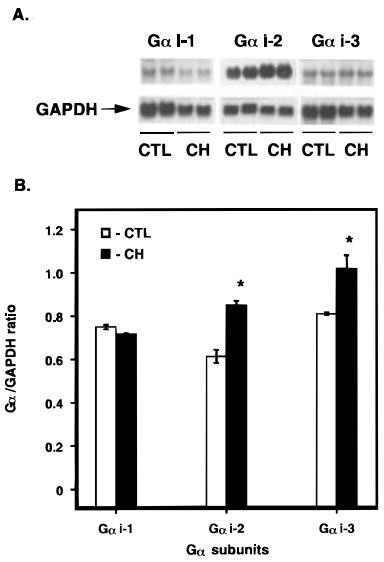

FIGURE 2: Lack of reduction of mRNA steady-state levels by cholesterol enrichment of Gαi subunits. (A) A 20 μg portion of total RNA extracted from normal and cholesterol-enriched rabbit cells grown in 75 cm^2 flasks were subjected to Northern analysis as described in the Methods. Replicate blots were hybridized with Gαi-1, Gαi-2, and Gαi-3-[^{32}P]-labeled probes. Relative amounts of Gαi subunit mRNA were normalized to the expression of GAPDH mRNA. This is representative of two such experiments, each performed in duplicate (as indicated by lower bars). (B) Densitometric analysis of blots visualized in A. Bars indicate mean ± SEM, * = $p < 0.05$, CTL vs. CH.

containing 10 mM DTT, boiled, and subjected to SDS−PAGE. The radioactivity on the dried gels was quantified by phosphorimager analysis or densitometry.

Miscellaneous Assays. Protein was measured by the method of Lowry et al. (Lowry et al., 1951).

Statistical Analysis. All data are expressed as mean ± SEM and analyzed by ANOVA, followed by Newman Keuls test, or Students' T test. Differences with $p < 0.05$ were considered to be significant.

RESULTS

Reduction of Membrane Expression of Gα Subunits by Cholesterol Enrichment. To explore the possibility that the expression of membrane-associated heterotrimeric G-protein subunits is altered following cholesterol enrichment, the mass of membrane-associated Gα subunits was quantified by Western analysis. Arterial cells contained Gαi-1, Gαi-2, Gαi-3, Gq/11 (Figure 1), and Gαs (data not shown). Cholesterol enrichment reduced the mass of membrane-associated Gαi-1, Gαi-2, Gαi-3, and Gq/11 on average approximately 40% (Figure 1). Cholesterol enrichment also reduced membrane-associated Gαs protein (data not shown). Quantitative results demonstrated that cholesterol enrichment reduces the membrane expression of heterotrimeric G-protein α subunits (Figure 1B). Subsequent experiments were then performed to determine the potential mechanisms by which cholesterol enrichment reduced G-protein subunit expression.

Cholesterol Enrichment Does Not Alter Gαi mRNA Steady-State Levels. To determine if cholesterol enrichment reduced Gαi protein expression by inhibiting transcription, the steady-state levels of Gαi mRNAs were evaluated. Each Gαi cDNA probe hybridized to a single band following Northern analysis, which correlated with their protein levels (Gαi-2 > Gαi-3 ≫ Gαi-1) (Figure 2A). Densitometric analysis of Northern blots revealed that when normalized to GAPDH, mRNA levels for Gαi-1, Gαi-2, and Gαi-3 were unaffected by cholesterol enrichment (Figure 2B). Since the protein, but not mRNA, levels of Gαi-1, Gαi-2, and Gαi-3 were reduced following cholesterol enrichment, these results suggest that cholesterol enrichment alters post-transcriptional processes leading to Gαi expression.

Reduction of Gα and β Subunits by Cholesterol and Oxygenated Steroids. To gain insight into the potential mechanisms by which cholesterol enrichment reduces Gα subunit expression, cells were exposed to cLDL, free cholesterol, and 17-ketocholesterol (Figure 3). As determined by Western analysis using a pan-Gαi antibody, cholesterol enrichment reduced Gαi expression 2-fold, while free cholesterol and 7-ketocholesterol reduced Gαi expression approximately 5-fold relative to control levels. As determined by Western analysis using a pan-β antibody, we found that cholesterol enrichment and free cholesterol reduced the expression of the G-β subunit by approximately 25% relative to controls and 7-ketocholesterol reduced expression of the β-subunit nearly 2-fold relative to controls. Other oxygenated sterols, including 25-hydroxycholesterol and cholesterol 5α,6α-epoxide, also reduced membrane expression of Gαi and Gβ subunits (data not shown). These results support the concept that cholesterol accumulation may reduce trafficking of heterotrimeric G-proteins to the cell membrane.

Inhibition of Reduction of Membrane-Associated Gαi and Gβ Subunits by Cotreatment with Mevalonate. Membrane association of G-protein heterotrimers is dependent upon isoprenylation of the Gγ subunit (Iniguez-Lluhi et al., 1992b; Muntz et al., 1992). To provide evidence to support the

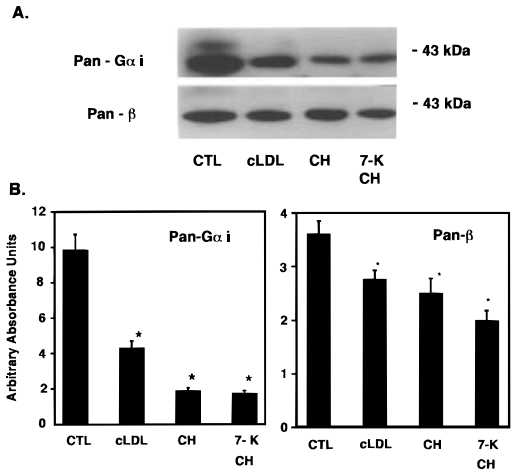

FIGURE 3: Reduction of membrane heterotrimeric G-protein expression by cholesterol and its oxides. (A) Confluent rat smooth muscle cells were exposed to media alone or media containing cationized LDL (75 μg/mL), cholesterol (100 μM), and 7-keto-cholesterol (10 μg/mL) for 1 week with one media change. Membrane preparations (30 μg of protein) were subject to Western analysis, employing a pan-Gαi antibody (8729) at 1:1000, or the β subunit, using a pan-β antibody (1:1000) followed by ECL detection, and quantification by densitometry. This is representative of two experiments. (B) Densitometric analysis of blots visualized in A. Bars indicate mean ± SEM, * = $p < 0.05$, treatment vs. CTL.

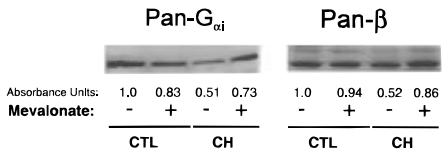

FIGURE 4: Mevalonate attenuation of reduction of G-protein expression by cholesterol enrichment. (A) Confluent smooth muscle cells were exposed to media alone or media containing cationized LDL (75 μg/ml) for 1 week with one media change. Mevalonic acid lactone (100 μM) was added to cells each day. Membrane preparations (30 μg of protein) were subject to Western analysis, employing a pan-Gαi antibody (8729) at 1:1000, or a pan-β antibody (used at similar dilution) followed by ECL detection and quantification by densitometry (indicated as absorbance units). This is representative of three experiments.

hypothesis that cholesterol enrichment reduced membrane expression of heterotrimeric G-protein subunits as a consequence of reduced isoprenylation of Gγ subunits, cells were exposed to media alone or media containing derivatized LDL in the absence and presence of mevalonate (100 μM). We next performed Western analysis of Gαi and Gβ subunits using pan-Gαi and G-β antibodies, respectively (Figure 4). Mevalonate alone did not significantly alter membrane-associated Gαi or Gβ subunits. In contrast, cholesterol enrichment reduced membrane association of Gαi and Gβ subunits; the membrane expression of each of these subunits was partially restored by cotreatment with mevalonate. Since membrane-association of each of these subunits requires prenylation of G-γ, and since mevalonate is the precursor for higher order isoprenoids, these data support the hypothesis that cholesterol enrichment reduces membrane expression of heterotrimeric G-protein subunits by reduction of isoprenylation of Gγ-subunits and subsequent trafficking of heterotrimeric G-protein subunits to the plasma membrane.

Cholesterol Enrichment Does Not Alter Gαi Subunit Half-Life. Pulse-chase experiments were performed to test the hypothesis that cholesterol enrichment reduces membrane content of heterotrimeric G-protein α subunits by decreasing their stability. Smooth muscle cells were exposed to [^{35}S]-cysteine/methionine for 4 h and then chased with isotope-free medium for up to 72 h. Cell lysates were subject to immunoprecipitation using pan-Gαi antibodies, followed by SDS/PAGE and autoradiography. At zero time, the expression of Gαi(1−3) is reduced approximately 55% by cholesterol enrichment. When expressed as a fraction of initial radioactivity at zero time, control cells synthesized 1.8-fold immunoprecipitable isotopic Gαi, which plateaued between 24−36 h and returned to the base line by 72 h. In cholesterol-enriched cells, cells immunoprecipitable isotopic Gαi continued to be synthesized at 24−36 h but only to approximately 50% which was found in control cells (1.5-fold over base line). Importantly, the rates of disappearance of immunoprecipitable isotopic Gαi between control and cholesterol-enriched cells at 36−72 h were similar (data not shown). These results support the hypothesis that cholesterol enrichment reduces translational or post-translational events (isoprenylation) but does not reduce membrane expression of Gαi subunits by increasing their degradation.

Reduction of Membrane-Associated Gγ Protein by Cholesterol Enrichment Without Altering mRNA Steady-State Levels. A major Gγ subunit in smooth muscle cells is Gγ-5. In addition, smooth muscle cells also possess variable amounts of Gγ-7, as well as an additional protein that cross-reacts with Gγ-7 antibodies but is of higher molecular weight, termed Gγ-7$_{upper}$ (Hansen et al., 1995). This latter protein may be either a differentially spliced Gγ-7 or Gγ-12 (based on molecular weight) or a novel Gγ-subunit restricted to smooth muscle cells (J. D. Robishaw, unpublished observations). To directly determine if cholesterol enrichment reduces Gγ-5 and/or Gγ-7 subunit expression, the relative amount of Gγ-5 and Gγ-7 protein in cell membranes of normal and cholesterol-enriched cells was determined by Western analysis (Figure 5). Cholesterol enrichment reduced membrane-associated Gγ-5 by 50%. Gγ-7 antibodies did not detect the lower Gγ-7 band but did detect Gγ-7$_{upper}$. Furthermore, cholesterol enrichment reduced Gγ-7$_{upper}$ subunit expression by approximately 70%. However, cholesterol enrichment did not result in the accumulation of either Gγ subunit into the cytosol (data not shown), suggesting that unprenylated Gγ subunits are degraded. Alternatively, it also possible that cholesterol enrichment reduces transcription of Gγ mRNA. To test this hypothesis, the relative levels of Gγ-5 mRNA were determined using RT-PCR. Cholesterol enrichment did not reduce the expression of γ-5 (Figure 5C) or GAPDH (data not shown). Taken together, these results support the concept that cholesterol enrichment reduces membrane expression of heterotrimeric G-proteins by interfering with post-translational processes (e.g., isoprenylation) of the Gγ subunits.

Reduction of Membrane-Associated p21ras by Cholesterol Enrichment. To determine if cholesterol enrichment specifically reduces the expression of other membrane-associated proteins that require isoprenylation for membrane association, the influence of cholesterol enrichment on the cellular distribution of p21ras (which requires farnesylation for localization to the cell membrane) was determined. Western analysis revealed that cholesterol enrichment reduced membrane expression of p21ras. p21ras was not detectable in the cytosolic fraction of either control or cholesterol-enriched cells. These results demonstrate that, in smooth muscle cells, p21ras is localized to the cell membrane and is reduced in

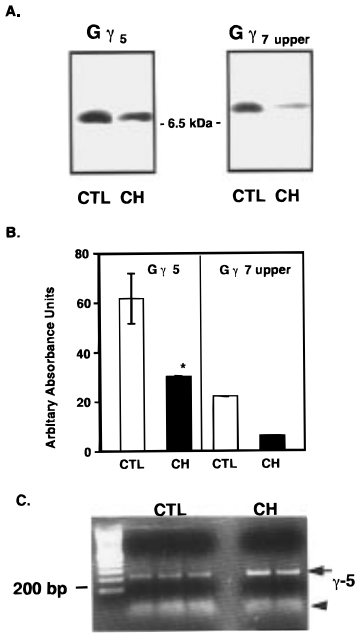

FIGURE 5: Reduction of membrane-associated Gγ-subunit protein but not mRNA levels by cholesterol enrichment. (A) Western analysis: Membranes from normal and cholesterol-enriched rabbit cells grown to confluent density were subjected to Western analysis for the presence of Gγ-5 and Gγ-7 subunits using monospecific antibodies. Gγ-7 antibodies recognize two proteins—lower and upper bands (Hansen et al., 1995). Arterial smooth muscle cells do not express the Gγ-7(lower) band. This is representative of two experiments (Gγ5). (B) Densitometric analysis of blots visualized in A. Bars indicate mean ± SEM, * = $p < 0.05$, CTL vs. CH. (C) RT-PCR of rabbit Gγ-5: Total RNA was subjected to RT-PCR as described in the Methods. Left lane is RNA ladders (100−1000 bp), right arrow indicates Gγ-5 PCR product. Carrot highlights the PCR primers. Each lane represents an individual RT-PCR reaction. Controls were performed in triplicate; cholesterol-enriched cells were performed in duplicate. This is representative of two such experiments.

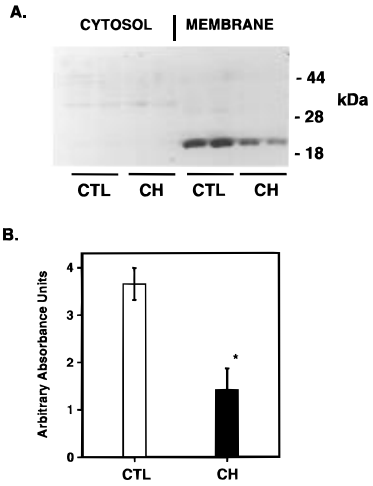

FIGURE 6: Reduction of membrane-associated p21ras by cholesterol enrichment. (A) Western analysis: The mass of p21ras in cell membranes and cytosol from normal and cholesterol-enriched rabbit smooth muscle cells grown in 100 mm plates was determined by Western analysis using a pan-ras antibody (1:500), followed by detection by ECL. This is representative of three experiments, each performed in duplicate (as indicated by lower bars). (B) Densitometric analysis of data shown in A. Bars indicate mean ± SEM, * = $p < 0.05$, CTL vs. CH.

the membranes of cholesterol-enriched cells without accumulating in the cytosol (Figure 6).

Cholesterol Enrichment Does Not Alter Membrane Expression of the PDGF-Receptor or p60src. To determine if

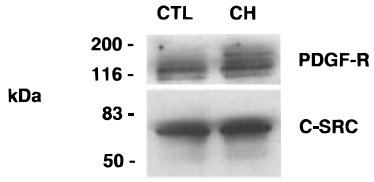

FIGURE 7: Lack of alteration of membrane-associated PDGF-receptor or p60src by cholesterol enrichment. Cell membranes of control and cholesterol-enriched rabbit cells were prepared as described in Figure 6. The relative mass of the PDGF-receptor and p60src was measured in cell membranes from normal and cholesterol-enriched cells employing primary antibodies (PDGF-R 1:1000) and p60src (1:1000), followed by detection by ECL.

cholesterol enrichment reduced other membrane proteins that do not require isoprenylation for membrane localization, the influence of cholesterol enrichment on the membrane expression of the PDGF receptor (a transmembrane protein) and p60src (an inner leaflet protein associated with cell membranes as a result of N-terminal myristoylation) was examined (Figure 7). Cholesterol enrichment did not alter the membrane expression of the PDGF-R or p60src. These results demonstrate that the influence of cholesterol enrichment is specific and interferes with proteins that are dependent upon isoprenoid modification for localization to the cell membrane.

Reduction of Membrane-Associated Gαi Activities by Cholesterol Enrichment. Since cholesterol enrichment reduced the expression of Gαi subunits, we next correlated these findings with the ability of G-protein-linked receptors to alter activities associated with Gαi subunits. First, GTPase activity in membrane preparations from normal and cholesterol-enriched cells was examined following stimulation by ATP. This agonist has been shown to couple to pertussis toxin-sensitive G-proteins (Felder et al., 1991). In normal cells, ATP stimulated GTPase activity 34% over basal levels (Figure 8A). Since this assay measures total membrane GTPase activity encompassing all membrane-bound G-proteins, the small, but statistically significant, increase in GTPase activity following treatment of cells with ATP is indicative of G-protein-coupled receptor activation. Cholesterol enrichment did not reduce basal GTPase activity relative to untreated controls (Figure 8A); in a cell membrane pool of numerous G-proteins, the lack of difference in basal GTPase activity between normal and cholesterol-enriched cells may be due to a selective loss of a specific set of membrane associated G-proteins. Importantly, cholesterol enrichment reduced ATP-receptor-coupled G-protein activation, indicating that ATP-receptor-coupled Gαi activity is reduced. A second pathway mediated by Gαi is thrombin-induced inhibition of adenylate cyclase (Magnaldo et al., 1988). Accordingly, we next tested the hypothesis that cholesterol enrichment would alter thrombin-induced inhibition of adenylate cyclase activity. Thrombin-inhibited adenylate cyclase activity, which was attenuated by cholesterol enrichment (Figure 8B). Cholesterol enrichment also reduced forskolin-stimulated adenylate cyclase activity (72.3 ± 2.1 pmol/mg of protein vs. 59.8 ± 3.0, untreated vs. cholesterol-enriched cells, $n = 4$, mean ± SEM, $p < 0.05$). These data support the concept that cholesterol enrichment reduces Gαi expression and function.

Reduction of MAP Kinase Activity by Cholesterol Enrichment. Phospholipase A_2 is activated by MAP kinase in response to G-protein activation (Gordon et al., 1996; Hawes et al., 1995). Since cholesterol-enriched cells display

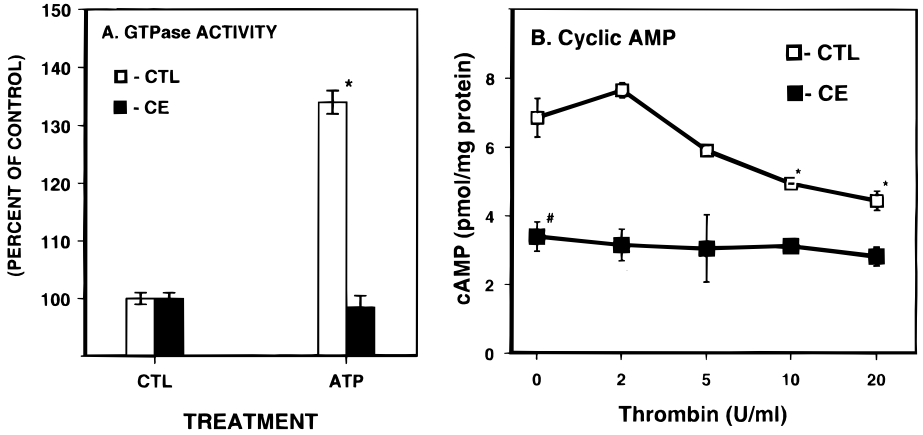

FIGURE 8: Reduction of membrane-associated Gαi activities by CH enrichment. (A) GTPase activity: Cell membranes from control and cholesterol-enriched rabbit cells grown in 100 mM plates were assayed for GTPase activity following exposure to ATP (100 μM) for 10 min at 37 °C. GTPase activity was measured as described in the Methods. Data are expressed as mean ± SEM, $n = 4$, with * = $p < 0.05$. (B) Thrombin-induced inhibition of cAMP accumulation: Normal and cholesterol-enriched cells grown in 35 mm plates were pretreated with isobutylmethylxanthine (IBMX, 100 μM) for 30 min at 37 °C prior to exposure to thrombin (0−20 U/ml) or forskolin (100 μM) for 15 min. cAMP levels were then measured as described in the Methods. Data are expressed as pmol/mg protein (mean ± SEM). This is representative of two experiments, with each treatment performed in duplicate.

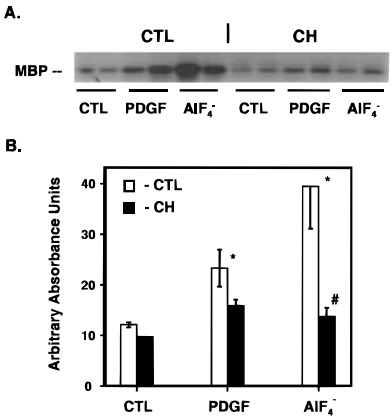

FIGURE 9: Reduction of MAP-kinase by rabbit smooth muscle cells with cholesterol enrichment. Normal and cholesterol-enriched cells grown in six-well cluster plates were exposed to PDGF (5.0 ng/ml) or AlF_4^- (30 mM) for 5 min at 37 °C. Cell lysates were prepared in RIPA buffer and immunoprecipitated with sepharose beads preconjugated with antibodies directed against Erk-1 and Erk-2. After washing, kinase activity was estimated by the incorporation of [γ-^{32}P]-ATP into myelin basic protein. Each sample was performed in duplicate (as shown in lower bars). Quantification of this data is shown in B. Bars indicate mean ± SEM, * = $p < 0.05$, CTL vs. agonist, # = $p < 0.05$, CTL vs. CH.

enhanced phospholipase A_2 activity in response to G-protein activation but show reduced phospholipase A_2 activity in response to PDGF, we tested the hypothesis that cholesterol enrichment may enhance G-protein-mediated MAP kinase activity. Normal and cholesterol-enriched smooth muscle cells were exposed to AlF_4^- and PDGF for 20 min, followed by assessment of Erk-1/ERk-2 activity (Figure 9A). In control cells, PDGF and AlF_4^- stimulated MAP kinase activity 2-fold and 4-fold, respectively, based on densitometric analyses. Cholesterol enrichment significantly inhibited basal MAP-kinase activity approximately 20% and inhibited PDGF- and AlF_4^- induced MAP kinase activity by 35% and 70%, respectively. These data demonstrate that cholesterol enrichment reduces MAP-kinase activity in response to receptor tyrosine kinase (PDGF) and G-protein-mediated activation.

DISCUSSION

Heterotrimeric G-proteins couple extracellular vasoactive molecules, such as bradykinin, angiotensin II, ATP, and endothelin, to effectors that promote the release of vasodilatory second messengers, such as PGI_2 (Huang et al., 1991; Schlondorff et al., 1989). The importance of G-proteins in mediating the release of this second messenger becomes manifest by observations that cellular cholesterol accumulation impairs pertussis-toxin-sensitive pathways leading to endothelium dependent arterial vasodilation (Shimokawa et al., 1991). However, the mechanisms by which cholesterol enrichment of arterial cells alter G-protein expression have been unexplored.

In this study, we demonstrate that cholesterol enrichment reduces the membrane expression of Gαi-1, Gαi-2, Gαi-3, and Gq/11 (Figure 1) and Gαs (data not shown). The results of our experiments strongly support the hypothesis that cholesterol enrichment reduces heterotrimeric G-protein assembly by inhibiting Gγ-subunit isoprenylation. First, since isoprenoid synthesis is dependent upon HMG-CoA reductase activity (the committed step in cholesterol synthesis), it is apparent that inhibition of HMG-CoA reductase could potentially alter heterotrimeric G-protein assembly and signaling. In our studies, the effects of cholesterol enrichment on membrane content of α and β subunits were reduced by cholesterol and a number of cholesterol oxides (Figure 3). Indeed, cholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol are potent HMG-CoA-reductase inhibitors (Axelson & Larsson, 1995; Faust et al., 1982; Gil et al., 1988). Since cholesterol oxides were not detectable in isolated cLDL or in cholesterol-enriched cells (data not shown), cholesterol itself most likely mediates its inhibitory effects on heterotrimeric G-protein expression. Second, the inhibitory effect of cholesterol enrichment on membrane-associated Gαi and β subunit expression could be attenuated by cotreatment with mevalonate, the immediate product of HMG-CoA reductase (Figure 4). Third, cholesterol enrichment reduced the membrane expression of isolated β-subunits (Figure 3), Gγ-5 and $G\gamma\text{-}7_{upper}$ subunits (Figure 5), and p21ras (Figure 6). It is noteworthy that the membrane content of each of these proteins is critically dependent upon

isoprenylation (Zhang & Casey, 1996). Fourth, the effect of cholesterol enrichment on membrane-associated G-protein expression is specific since cholesterol enrichment did not alter the membrane expression of either the PDGF-receptor, a transmembrane protein that is not lipid-modified, or p60src, a membrane protein whose N-terminal is myristoylated (Figure 7). Fifth, results of metabolic labeling/immunoprecipitation experiments revealed that cholesterol enrichment reduces the appearance of isotopically-labeled Gαi in cell membranes but did not alter the degradation rate of Gαi subunits (data not shown). Finally, the steady-state mRNA levels for Gαi-1, Gαi-2, and Gαi-3 subunits were unchanged following cholesterol enrichment (Figure 2). Furthermore, the mRNA level of G-γ-5 was not reduced in cholesterol-enriched cells as determined by RT-PCR (Figure 5C), further supporting the hypothesis that reduced Gαi protein expression occurs as a result of reduced post-translational processing. These data are consistent with the observations that the expression of Gα protein is not regulated at the level of transcription (Li et al., 1994; Li et al., 1996). The expression of the G-protein β and Gγ subunits are critically dependent upon the coexpression of each. Neither will be expressed in the absence of the other (Simonds et al., 1991; Graber et al., 1992). Thus, in the absence of post-translational lipid modification, the expression of both heterotrimeric G-proteins and p21ras is reduced. These data strongly suggest that cholesterol enrichment reduces heterotrimeric G-protein expression by a mechanism that includes reduction in the isoprenylation of Gγ-subunits.

Our results are consistent with previous observations demonstrating the necessity of Gγ-subunit isoprenylation on heterotrimeric G-protein assembly. For example, Chinese hamster ovary cells transfected with cDNA encoding Gγ subunits whose isoprenylation site had been mutated resulted in inefficient translocation of both β and Gγ subunits to the cell membrane. It is through this post-transcriptional mechanism that the membrane content of heterotrimeric G-proteins is regulated (Muntz et al., 1992). It has been found that Gγ subunit isoprenylation was indispensable for high affinity binding of $\beta\gamma$ to Gα subunits in the formation of membrane-associated heterotrimeric complexes (Iniguez-Lluhi et al., 1992a). Our results are also consistent with previous observations demonstrating the necessity of Gγ subunit unit isoprenylation in G-protein-mediated signaling. For example, lovastatin (an HMG-CoA reductase inhibitor) decreased the mass of Gαs and Gαi in plasma membranes of pituitary cells and was correlated with decreased adenylyl cyclase activity but could be restored by treatment with mevalonate (Chiloeches et al., 1995). Similarly, mevalonate restored the inhibition by simvastatin (another HMG-Co-A reductase inhibitor) of vasopressin-induced Ca^{+2} flux in smooth muscle cells (Ng et al., 1994). The necessity of isoprenylation in the regulation of cell function is made clear by observations that mevalonate, farnesol, and geranylgeraniol (Corsini et al., 1993), but not cholesterol (Munro et al., 1994), can mitigate the antiproliferative effects of HMG-CoA reductase inhibitors (Munro et al., 1994; Corsini et al., 1993). However, our data are in contrast to those by Liao et al., who demonstrated that oxidized LDL reduced Gαi-2 (but not Gαi-1 or Gαi-3) expression at the transcriptional and post-transcriptional level (Liao & Clark, 1995). The underlying mechanisms responsible for these differences are unknown, but may be due to the delivery of cholesterol and noncholesterol lipid peroxides. In our model of cholesterol enrichment, neither cLDL nor cholesterol-enriched smooth muscle cells contained detectable cholesterol oxides. Collectively, these observations indicate a common mechanism by which cholesterol enrichment and HMG-CoA reductase inhibitors reduce heterotrimeric G-protein content, i.e., by reduction of the synthesis of isoprenoids necessary for G-protein membrane targeting. These findings imply that conditions that result in reduced HMG-CoA-reductase activity (such as in hypercholesterolemia) may include altered G-protein expression, signaling, and cell function.

We next correlated the alterations in G-protein expression following cholesterol enrichment to alterations in proximal signaling pathways elicited by G-protein activation. Commensurate with the reductions in Gαi expression following cholesterol enrichment, we observed the loss in Gαi activity as evidenced by the inability of ATP to stimulate membrane-associated GTPase activity (Figure 8A) or thrombin to inhibit cAMP accumulation (mediated through Gαi) (Figure 8B). The observation that cholesterol enrichment also reduced forskolin-induced cyclic AMP accumulation may be due to reduced adenylate cyclase protein or decreased Gαs, since Gαs and forskolin stimulate several adenylyl cyclase isoforms in a synergistic manner (Sutkowski et al., 1994). Cholesterol enrichment can also reduce MAP-kinase activity in response to PDGF, which activates a transmembrane receptor kinase (Figure 9), although cholesterol enrichment did not alter expression of the PDGF receptor (Figure 7). PDGF has been shown to activate MAP kinase in a p21ras-dependent manner (Bornfeldt et al., 1995), which is inhibitable by lovastatin (Xu et al., 1996). We reason that cholesterol enrichment reduces PDGF-induced MAP-kinase by diminishing the membrane content of p21ras. Cholesterol enrichment also reduced the induction of MAP-kinase activity in response to AlF_4^- (Figure 9). It has been previously reported that G-protein $\beta\gamma$ subunits activate MAP kinase by activating p21ras and raf (Inglese et al., 1995; Ito et al., 1995). Since we observed reductions in the expression of both $\beta\gamma$ and p21ras in the membranes of cholesterol-enriched cells, we suggest that the reduction in MAP-kinase activity following AlF_4^- occurs as a consequence of reduced membrane expression of both $\beta\gamma$ and p21ras.

In summary, these new findings show that cholesterol enrichment diminishes the membrane expression of heterotrimeric G-proteins and p21ras as a result of inefficient isoprenylation. The molecular sequelae of reduced G-protein expression correlated with the inhibitory effect of cholesterol enrichment on a number of proximal cell signaling pathways, including agonist-induced GTPase activity, adenylyl cyclase activity, and MAP-kinase activity. In the setting of recent studies of others (Liao, 1994; Pritchard et al., 1995) where LDL can alter G-protein-mediated nitric oxide generation, our results may also provide a novel mechanism to explain the observed reductions in G-protein-mediated nitric oxide production under conditions of cholesterol accretion (Shimokawa et al., 1991).

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