Advances in Experimental Medicine and Biology 1115

Avia Rosenhouse-Dantsker
Anna N. Bukiya *Editors*

Cholesterol Modulation of Protein Function

Sterol Specificity and Indirect Mechanisms

Advances in Experimental Medicine and Biology

Volume 1115

Editorial Board:

IRUN R. COHEN, *The Weizmann Institute of Science, Rehovot, Israel*
ABEL LAJTHA, *N.S. Kline Institute for Psychiatric Research, Orangeburg, NY, USA*
JOHN D. LAMBRIS, *University of Pennsylvania, Philadelphia, PA, USA*
RODOLFO PAOLETTI, *University of Milan, Milan, Italy*
NIMA REZAEI, *Tehran University of Medical Sciences, Children's Medical Center Hospital, Tehran, Iran*

More information about this series at http://www.springer.com/series/5584

Avia Rosenhouse-Dantsker • Anna N. Bukiya
Editors

Cholesterol Modulation of Protein Function

Sterol Specificity and Indirect Mechanisms

Editors
Avia Rosenhouse-Dantsker
Department of Chemistry
University of Illinois
Chicago, IL, USA

Anna N. Bukiya
The University of Tennessee Health
Science Center
Memphis, TN, USA

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-04277-6 ISBN 978-3-030-04278-3 (eBook)
https://doi.org/10.1007/978-3-030-04278-3

Library of Congress Control Number: 2018966812

© Springer Nature Switzerland AG 2019, Corrected Publication 2021
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Cholesterol is one of the most talked-about molecules of modern times. This sterol was first obtained from gallstones in the mid-eighteenth century by French chemist François Poulletier de la Salle. His work was not published, but was referred to by his colleagues and collaborators. Another French chemist, Michel Eugène Chevreul, named the compound "cholesterine" in 1815, and this recognition opened an era of studies on cholesterol biosynthesis and physiological function that has persisted into the twenty-first century.

Cholesterol is a vital component of all animal cells. Cholesterol insertion into biological membranes conditions their physical properties by maintaining a proper rigidity, adjusting membrane thickness and curvature. In addition, cholesterol serves as a precursor for steroid hormones and as an essential component of lipoproteins. The human body produces around 1 g of cholesterol each day, resulting in total cholesterol levels in the body exceeding 30 g. Twenty-five percent of this amount is located in the brain, highlighting the vital role of cholesterol in maintaining neuronal cell excitability and homeostasis.

Despite over 200 years of scientific search into cholesterol properties, the role of cholesterol in physiology and pathology still remains a subject of investigation. On the one hand, excessive cholesterol consumption with Western diets and excessive production of cholesterol in the human body constitute a major risk factor for common pathological conditions, cardiovascular disease in particular. At the other extreme, very low cholesterol levels serve as indicators of a poor prognosis in critical illness. Our understanding of the multiple health consequences of cholesterol levels that depart from a normal "middle ground" is often hampered by the difficulty in interpreting the molecular mechanisms that underlie the cellular effects of cholesterol and, most importantly, the modulation of the effector molecular targets of cholesterol, cell proteins. This volume is the first of two volumes that captures the current state of our understanding of the molecular mechanisms that underlie cholesterol modulation of protein function.

Consistent with the two major physiological roles of cholesterol as a structural and signaling molecule, the general view of the molecular mechanisms that govern cholesterol modulation of protein function is conceptualized in two modes of

action: (a) indirect effects via cholesterol modulation of membrane physical properties and (b) protein targeting via direct interaction of the cholesterol molecule with sterol-sensing protein sites. This first volume focuses on sterol specificity as a means to distinguish between direct and indirect effects of cholesterol and on indirect mechanisms, whereas the second volume covers direct cholesterol-protein interactions.

Experimental discrimination between indirect and direct mechanisms of cholesterol effects on protein function is not straightforward. Cholesterol stereoisomers and cholesterol derivatives that exert differential effects on the physical properties of cell membranes are often used as tools to help distinguish between the potential mechanisms that underlie cholesterol-protein interactions. Thus, the first part of this volume introduces the reader to cholesterol chemistry and the use of both naturally occurring and synthetic derivatives that help to distinguish between indirect and direct modulations of protein function by cholesterol. Examples in this part include the well-studied G-protein-coupled receptors and two classes of potassium channels.

The second part of this volume focuses on studies that successfully use modern technologies to elucidate the effects of cholesterol on the physical properties of membranes and highlight these major driving forces behind this sterol's effect on proteins. These include the following studies on the various aspects of cholesterol's effects: modulation of the physical properties of membranes by means of nuclear magnetic resonance, modifications of dipole potential of lipid membranes, and mapping using mass spectrometry imaging. The volume concludes with a chapter on the cholesterol-dependent gating of a voltage-gated potassium channel demonstrating the lipid property-driven effect of cholesterol on protein function.

As the reader will discover, the depiction of cholesterol effects on protein function as either indirect or direct is somewhat oversimplified. In nature, these mechanisms are not mutually exclusive and likely coexist in the finely tuned cellular environment. Moreover, our knowledge of cholesterol modulation of protein function is far from being complete. There is little doubt that the field of cholesterol-protein interactions will remain an active and intriguing area of research for years to come.

The editors are deeply thankful to all the authors who contributed to this project aimed at portraying the complexity of the biomechanisms involving this lipid discovered 200 years ago. The editors are also grateful to senior mentors, collaborators, and emerging junior colleagues for the inspiration, for the fruitful exchange of ideas, and for providing a nurturing environment for the completion of this collection of important contributions to the field.

Chicago, IL, USA Avia Rosenhouse-Dantsker
Memphis, TN, USA Anna N. Bukiya

Contents

Part I Sterol Specificity in Modulating Protein Function

Chirality Effect on Cholesterol Modulation of Protein Function 3
Jitendra D. Belani

A Critical Analysis of Molecular Mechanisms Underlying Membrane Cholesterol Sensitivity of GPCRs 21
Md. Jafurulla, G. Aditya Kumar, Bhagyashree D. Rao, and Amitabha Chattopadhyay

Regulation of BK Channel Activity by Cholesterol and Its Derivatives 53
Anna N. Bukiya and Alex M. Dopico

Chiral Specificity of Cholesterol Orientation Within Cholesterol Binding Sites in Inwardly Rectifying K^+ Channels 77
Nicolas Barbera and Irena Levitan

Part II Indirect Modulation of Protein Function by Cholesterol

Cholesterol Effects on the Physical Properties of Lipid Membranes Viewed by Solid-state NMR Spectroscopy 99
Trivikram R. Molugu and Michael F. Brown

Effect of Cholesterol on the Dipole Potential of Lipid Membranes 135
Ronald J. Clarke

Mass Spectrometry Imaging of Cholesterol 155
Stephanie M. Cologna

Cholesterol-Dependent Gating Effects on Ion Channels 167
Qiu-Xing Jiang

Correction to: Cholesterol Effects on the Physical Properties of Lipid Membranes Viewed by Solid-state NMR Spectroscopy C1

Index 191

Contributors

G. Aditya Kumar CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

Nicolas Barbera Division of Pulmonary and Critical Care, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Jitendra D. Belani Thomas Jefferson University, College of Pharmacy, Philadelphia, PA, USA

Michael F. Brown Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA

Department of Physics, University of Arizona, Tucson, AZ, USA

Anna N. Bukiya The University of Tennessee Health Science Center, Memphis, TN, USA

Amitabha Chattopadhyay CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

Academy of Scientific and Innovative Research, Ghaziabad, India

Ronald J. Clarke University of Sydney, School of Chemistry, Sydney, NSW, Australia

Stephanie M. Cologna Department of Chemistry and Laboratory of Integrated Neuroscience, University of Illinois at Chicago, Chicago, IL, USA

Alex M. Dopico The University of Tennessee Health Science Center, Memphis, TN, USA

Md. Jafurulla CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

Qiu-Xing Jiang Department of Microbiology and Cell Science, IFAS, University of Florida, Gainesville, FL, USA

Irena Levitan Division of Pulmonary and Critical Care, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Trivikram R. Molugu Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA

Bhagyashree D. Rao CSIR-Indian Institute of Chemical Technology, Hyderabad, India

Academy of Scientific and Innovative Research, Ghaziabad, India

Part I
Sterol Specificity in Modulating Protein Function

Chirality Effect on Cholesterol Modulation of Protein Function

Jitendra D. Belani

Abstract Cholesterol is a key steroidal, lipid biomolecule found abundantly in plasma membranes of eukaryotic cells. It is an important structural component of cellular membranes and regulates membrane fluidity and permeability. Cholesterol is also essential for normal functioning of key proteins including ion-channels, G protein-coupled receptors (GPCRs), membrane bound steroid receptors, and receptor kinases. It is thought that cholesterol exerts its actions via specific binding to chiral proteins and lipids as well as through non-specific physiochemical interactions. Distinguishing between the specific and the non-specific interactions can be difficult. Although much remains unclear, progress has been made in recent years by utilizing *ent*-cholesterol, the enantiomer of natural cholesterol (*nat*-cholesterol) as a probe. *Ent*-Cholesterol is the non-superimposable mirror image of *nat*-cholesterol and exhibits identical physiochemical properties as *nat*-cholesterol. Hence, if the biological effects of cholesterol result solely due to membrane effects, it is expected that there will be no difference between *ent*-cholesterol and *nat*-cholesterol. However, when direct binding with chiral proteins and lipids is involved, the enantiomer is expected to potentially elicit significantly different, measurable effects due to formation of diastereomeric complexes. In this chapter, we have reviewed the literature related to *ent*-cholesterol and its use as a probe for various biophysical and biological interactions of cholesterol.

Keywords Cholesterol · Enantiomer · *ent*-Cholesterol · Synthesis · Stereospecificity · Sterol–membrane interactions

J. D. Belani (✉)
Thomas Jefferson University, College of Pharmacy, Philadelphia, PA, USA
e-mail: Jitendra.belani@jefferson.edu

© Springer Nature Switzerland AG 2019

A. Rosenhouse-Dantsker, A. N. Bukiya (eds.), *Cholesterol Modulation of Protein Function*, Advances in Experimental Medicine and Biology 1115,
https://doi.org/10.1007/978-3-030-04278-3_1

1 Introduction

A chiral racemic compound consists of an equimolar mixture of two enantiomeric forms. In the last few decades, scientists have tried to understand biogenesis and role of naturally occurring enantiomerically pure compounds. Chiral triterpenes found in nature undergo stereospecific cyclizations to produce enantiomerically pure steroids. Cholesterol and other steroids are thus produced in optically pure form as one enantiomer. Many of these steroidal biomolecules play a vital role in cellular functions. Molecular mechanisms underlying these cellular functions are still largely unclear. The unnatural enantiomers of these steroids can serve as valuable probes as their chemical composition, bonding pattern, and relative configuration are the same as the naturally occurring steroids. Since these unnatural enantiomers are not found in nature, these must be chemically synthesized in the laboratory. The non-superimposable mirror image of naturally occurring cholesterol is referred to as *ent*-cholesterol. A diastereomer of cholesterol with the configuration different only at C3 with α-OH group is referred to as 3-epicholesterol. The structures of cholesterol, 3-epicholesterol, and *ent*-cholesterol are given in Fig. 1. Both *ent*-cholesterol and 3-epicholesterol have been extensively used to elucidate cholesterol mechanisms and when compared to *nat*-cholesterol, these stereoisomers are expected to elicit different specific and non-specific interactions with lipids and proteins present in the membranes.

2 Chemical Structure and Synthesis

Chemical structure of natural cholesterol contains four rings (labeled A, B, C, and D) and a "side-chain" attached at the C17 of ring D. The structure contains eight stereocenters (C3, C8, C9, C10, C13, C14, C17, C20), seven of which are contiguous. In addition, there is an isolated double bond between C5 and C6 that contributes significantly to the three-dimensional shape of the steroid. Cellular biosynthesis of cholesterol is a tightly regulated process and involves enantioselective epoxidation of squalene followed by polyene cyclization to provide lanosterol in a concerted fashion. Lanosterol is further converted into cholesterol in a multistep process by the removal of three methyl groups, the reduction of one double bond by NADPH, and the migration of the other double bond [1]. Racemic syntheses of cholesterol in the laboratory by Woodward in 1952 [2] and by Johnson in 1964 [3, 4] are considered as scientific milestones. These two early and unique syntheses paved the way for synthesis of the enantiomer of this complex biomolecule.

Enantiomer of cholesterol, *ent*-cholesterol is a non-superimposable mirror image of the natural cholesterol. To prepare the enantiomer, the stereochemistry at all eight chiral carbons in cholesterol must be inverted. There is no simple way to achieve this and the enantiomer of cholesterol remains inaccessible without enormous synthetic effect [5]. Steroid nuclei with "near symmetry" such as in *ent*-19-

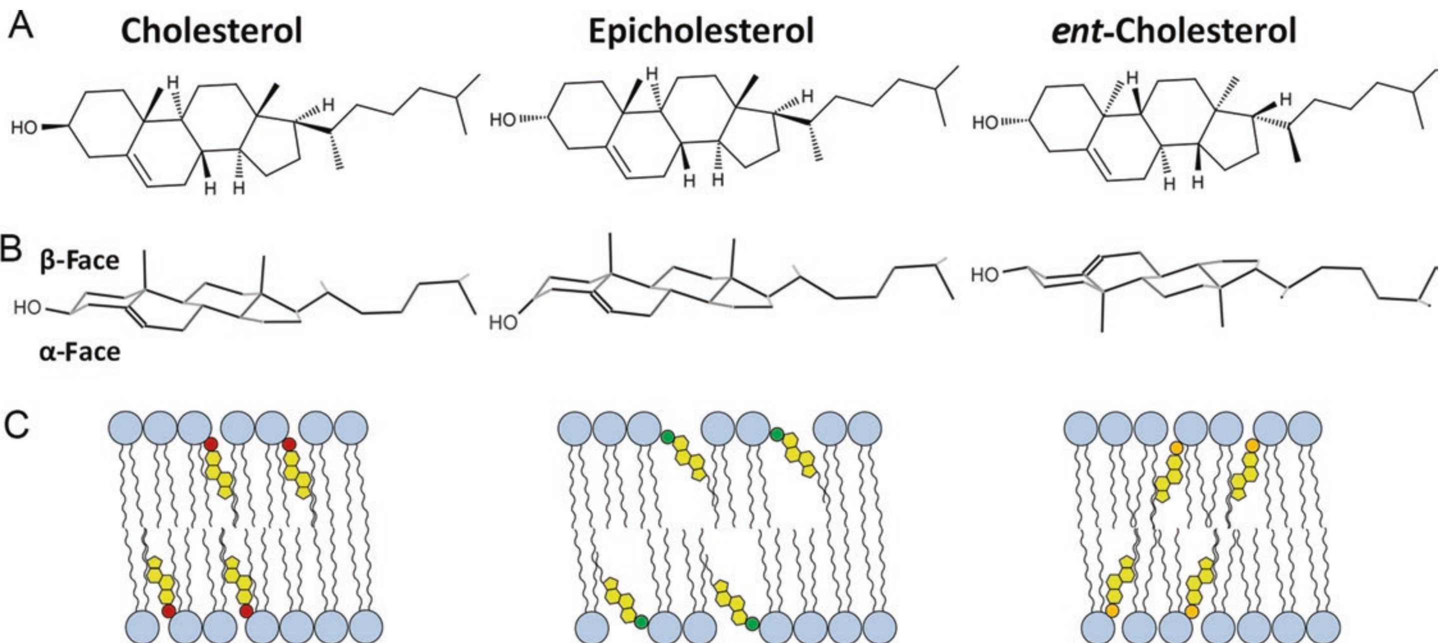

Fig. 1 (**a**) Chemical structures of cholesterol, *ent*-cholesterol, and epicholesterol; (**b**) Three-dimensional representation of the three steroids; (**c**) Schematic of the localization of the three cholesterol isomers within phospholipid bilayers and their effects on membrane packing (Adapted from Barbera et al. Current Topics in Membranes, 80, 2017, 25–50)

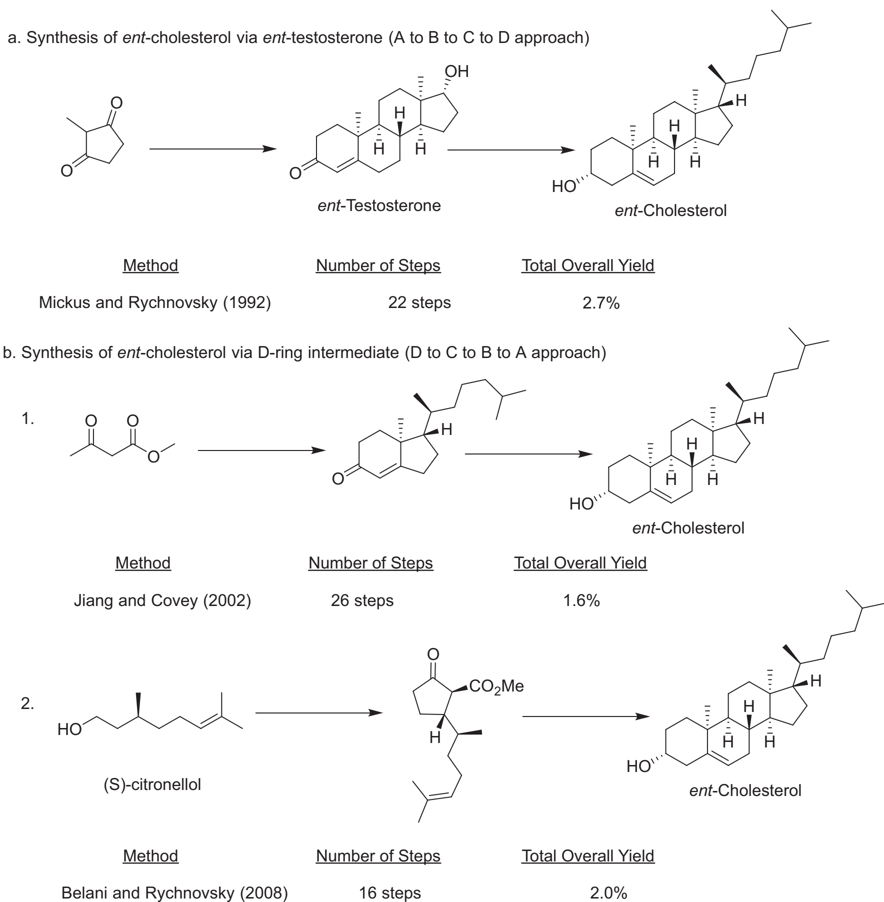

Fig. 2 The Two Different Synthetic approaches to *ent*-Cholesterol

nortestosterone have been synthesized from naturally occurring 19-nortestosterone. However, installation of the side-chain present in cholesterol poses significant additional challenges and the “inversion approach” for cholesterol offers no advantages over traditional total synthesis. Hence, the enantiomer of cholesterol must be prepared via total synthesis. There are essentially two different approaches to enantioselective synthesis of *ent*-cholesterol reported in the literature. The first involves synthesis of ABCD rings followed by installation of side chain on ring D. The other more efficient way is to first prepare a D-ring synthon with an intact side-chain and subsequent elaboration of CBA rings. The first stereoselective total synthesis was reported by Rychnovsky and Mickus in 1992 [6] and involved synthesis of the tetracyclic core of *ent*-testosterone using a modified Hoffman La Roche synthesis of 19-nor steroids [7]. Subsequently, the side chain on steroidal C17 was installed by a multistep protocol. Approximately 18 mg of *ent*-cholesterol was thus synthesized in 22 linear steps from commercially available starting materials in 2.7% overall yield (Fig. 2a). Jiang and Covey subsequently reported the first synthesis of *ent*-cholesterol

from a precursor containing the D-ring and the entire side of the steroid [8]. In this synthesis, approximately 80 mg of the enantiomer was synthesized in 26 linear steps and 1.6% overall yield from commercially available methylacetoacetate (Fig. 2b1). The third synthesis was reported in 2008 by Rychnovsky and Belani and was based on a ring D to C to B to A approach [9]. The synthesis provided approximately 120 mg of *ent*-cholesterol in 16 steps from commercially available (*S*)-citronellol with an overall yield of 2% (Fig. 2b2). Although all the syntheses described above are long and have some low yielding and limiting steps, they make *ent*-cholesterol more readily available to be used as a probe of function and metabolism of cholesterol. The last two routes also allow incorporation of ^{13}C-or ^{2}H-labels toward the end of the syntheses by using CD_3I or $^{13}CH_3I$ for the installation of the axial C-18 methyl group in the A-ring of the steroid. These isotope labels allow for easy analysis of the steroid and its metabolites using various spectral techniques. Two other methods for standalone installation of the isooctyl side-chain on the steroid nucleus have been reported with good yield but preparation of steroid core is still tedious and low yielding [10, 11]. There is still an urgent need for more efficient routes with less number of steps that can allow gram scale preparation of *ent*-cholesterol. A convergent synthesis that can provide about 10% overall yield would certainly be a game-changer for this field.

3 Differential Effects of Cholesterol and Its Stereoisomers

It is now well understood that the biological interactions and functions of the molecule are related to its chirality [12]. Classical examples include (*S*)-thalidomide that is selectively teratogenic; ibuprofen, an anti-inflammatory drug which is sold as a racemate but only the (*S*)-enantiomer is active; and carvone, a monoterpene that interacts differently with olfactory receptors and smells like spearmint in the *R* form and caraway in the *S* form. For steroids, it was first shown that even simple diastereomers that are easily accessible have distinct interactions with lipids [13, 14]. It is anticipated that *ent*-steroids may also have biophysical and biological interactions that are different than those of the natural steroids [15]. In the last two decades, scientists have tried to study to understand these differences; however, many fascinating puzzles and anomalies still remain.

3.1 Effects on Membrane Proteins

Cholesterol plays a very important role in cellular function. It is vital for proper membrane protein function and plays a critical role in signal transduction and overall human health. For cholesterol, the first total synthesis of its enantiomer in >97% ee permitted investigation of the role of sterols in ion-channel formation [16]. Amphotericin B formed different channels in the presence of cholesterol and

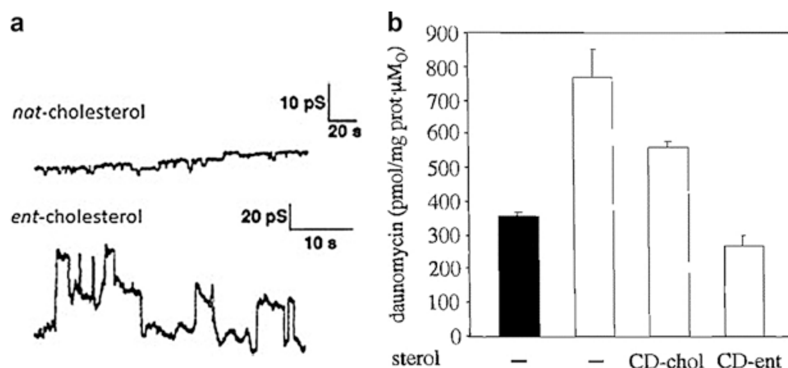

Fig. 3 (**a**) Amphotericin B ion channels in racemic glycerol monooleate (a) with cholesterol and (b) with *ent*-cholesterol with 2 × 10^{-8} M amphotericin B, (Adapted from [16]); (**b**) Accumulation of [^{3}H]-daunomycin in CHO cells in response to cholesterol depletion and repletion (Adapted from [18])

ent-cholesterol. Unlike natural cholesterol, membranes containing *ent*-cholesterol did not support any ion channels at amphotericin B concentrations of 2 × 10^{-8}, but ion channels were observed at a tenfold higher amphotericin B concentration (Fig. 3a). This constituted the first direct proof that amphotericin B binds to cholesterol in the ion channel structure. Further, to confirm if the antifungal activity of amphotericin B solely depends on changes in the physical properties of the phospholipid membrane and if they are stereospecific, Rychnovsky and coworkers used *ent*-cholesterol to probe the extent of suppression of antifungal activity of amphotericin B when compared with exogenous *nat*-cholesterol and ergosterol. It is known that when an exogenous sterol is added to fungal cell cultures, it results in suppression of the antifungal activity of amphotericin B. Ergosterol, the major phytosterol found in fungal cell membrane, was the most effective (62%) in suppressing the antifungal activity of amphotericin B. The antibiotic retained only 73% of its activity at 60 ppm of *nat*-cholesterol and 87% of activity at 30 ppm of *ent*-cholesterol. The results provide strong evidence in favor of enantiospecific interactions between amphotericin B and sterol being responsible for the activity of the antifungal agent [17].

Absolute configuration of cholesterol was however found to have minimal effects on localization of multidrug resistant P-glycoprotein (Pgp) in whole cell systems. These proteins generally localize in low density cholesterol enriched membranes. The structure of low density membrane domains of HepG2 cells was first disrupted by removing cholesterol using cyclodextrin (CD) and then repleted by with cholesterol or *ent*-cholesterol. Repletion with either sterol was equally effective in restoring total cellular content of cholesterol to baseline levels. In a separate experiment, effect of cholesterol on Pgp transport of daunomycin was studied using CHO cells in presence or absence of specific class I Pgp inhibitors. It was observed that daunomycin concentrations in CHO cells were increased three to four folds after cholesterol depletion by CD and treatment with Pgp inhibitors. Restoring the sterol

concentration with *nat*-cholesterol partially reversed the effect of CD on accumulation of daunomycin. However, treatment with the enantiomer of cholesterol before incubation with daunomycin led to significantly less accumulation of daunomycin (Fig. 3b) This observation predicted a specific interaction between cholesterol and the binding site of daunomycin on Pgp [18].

Cholesterol is required for activity and specificity of bacterial pore-forming toxins [19]. Palmer and coworkers studied sterol requirements of two different cholesterol-binding cytolysins—streptolysin O (SLO) and *Vibrio cholerae* cytolysin (VCC). Both SLO and VCC were able to permeabilize liposomal membranes containing *nat*-cholesterol and were inactive with vesicles lacking cholesterol. When cholesterol was replaced by *ent*-cholesterol, VCC had very low activity. However, SLO was only slightly less active with *ent*-cholesterol. The study suggested that although the cholesterol interaction with VCC is stereospecific, it is not with SLO [20]. Covey and coworkers also investigated the role of elevated levels of cholesterol in advanced atherosclerosis. Macrophages normally have cholesterol poor endoplasmic reticulum (ER) membrane. However, in advanced atherosclerotic lesions, free cholesterol accumulates in these ER membranes which subsequently inhibits ER calcium ATPase-2b pump (SERCA2b). To investigate the mechanism of cholesterol-induced SERCA2b inhibition, the authors incubated SERCA2b containing ER membranes with methyl-β-cyclodextrin-complexes with either *nat*-cholesterol or *ent*-cholesterol. It was observed that similar amounts of *nat*-cholesterol and *ent*-cholesterol were incorporated into the membranes and SERCA2b was inhibited to similar extent. This observation confirmed that the interaction between cholesterol and SERCA2b is not stereospecific and the excess free cholesterol simply reduces the conformational freedom of SERCA2b and depletion of ER calcium stores occurs [21].

Antibodies are proteins that are extremely specific and have been used to distinguish and separate enantiomers [22, 23]. Addadi and coworkers investigated interactions between a monoclonal antibody 36A1 and monolayers of sterols: *nat*-cholesterol, *ent*-cholesterol, and *epi*-cholesterol. The hydroxyl group at C3 was in α-position resulting in a diastereomer. When compared, layers of *nat*-cholesterol and *ent*-cholesterol interacted with the antibody to the same extent. The packing of the epimer in monolayer was distinct as the configuration at C3 position imposed a more acute angle between the hydroxyl group and the rigid steroid backbone. Hence the interaction of the antibody was not observed with the epimer. It was proposed that the interaction was dependent on the structure even more than on the chemical composition of the molecules comprising the monolayer. The antibody was able to overcome minor topographical differences between hydrophobic enantiomeric surfaces and bind equally well to the two cholesterol enantiomers. The study highlighted the fact that antibody recognition depends both on molecular structure and on the molecular packing [24, 25].

Structural features of sterols required for binding to and activating ACAT (acyl-CoA:Cholesterol acyltransferase), a membrane bound ER enzyme, were investigated by Covey and coworkers. The ACAT enzyme converts cholesterol and other sterols to long chain fatty acid acyl-CoA esters and consists of two isoforms ACAT1

and ACAT2. The enzymes contain a substrate binding site and an allosteric activator site. It is known that binding of cholesterol on the activator site makes the ACAT enzyme less discriminatory toward esterification and various modifications on sterol structure are tolerated [26]. Activation by cholesterol with equatorial 3β-OH group was compared with *ent*-cholesterol (equatorial, 3α-OH) and epicholesterol (axial, 3α-OH). Not surprisingly, epicholesterol was found to be a poor substrate for ACAT1 indicating axial orientation of the hydroxyl group at C3 is detrimental to the binding of the steroid. What was even more remarkable was the finding that *ent*-cholesterol, with equatorial C3–OH group and physical properties that were identical to *nat*-cholesterol, was also not a strong substrate for ACAT1. Rogers et al. separately studied esterification of pregnenolone, a poor ACAT substrate without the isooctyl side chain, and its activation by cholesterol and its enantiomer. Cholesterol was found to increase the V_{max} for pregnenolone esterification by 100 folds but *ent*-cholesterol only slightly activated the ACAT enzyme. *Ent*-Cholesterol is thus not only a poor substrate but also a poor activator. It has been proposed that the substrate binding site on ACAT enzyme prefers to bind with cholesterol and the interaction is stereospecific. It does not allow *ent*-cholesterol to bind. *Ent*-Cholesterol however does bind to the allosteric activating site albeit poorly [27]. These studies together clearly suggest that the overall shape of the molecule is very critical for ACAT1 substrate binding and allosteric activating site and not just the orientation of the C3-hydroxyl group [28, 29].

Bukiya et al. studied the effect of sterol structure on the activity of large-conductance voltage/Ca^{2+}-gated K^+ (BK) channels. Cholesterol is known to inhibit BK channels and this may affect regulation of neurotransmitter release and neuronal excitability. Although small changes in the steroid ring system or the side-chain had differential efficacy on reducing the activity of the BK channels, some more than the others, the enantiomer repeatedly failed to reduce the BK channel activity. This result again signifies the presence of enantiospecific spatial recognition site on the protein, in this case the BK α subunit [30]. Similar results were observed for bacterial and eukaryotic inward rectifier K^+ (Kir) channels. Cholesterol was found to inhibit purified prokaryotic and eukaryotic Kir channel as confirmed by $^{86}Rb^+$ uptake studies. *Ent*-Cholesterol did not inhibit the $^{86}Rb^+$ uptake even though it is expected to have the same effects on membrane properties as cholesterol. The study confirmed that cholesterol-Kir channel interactions are likely enantiospecific and occur at an evolutionary conserved site [31]. In a separate study conducted to understand mechanisms underlying cholesterol protection against alcohol-induced BK channel inhibition and resulting vasoconstriction, Bisen et al. showed that both cholesterol and *ent*-cholesterol reduced BK channel inhibition by alcohol in inside-out patches excised from freshly isolated cerebral artery myocytes. This observation that the two enantiomers have similar effects on the pharmacological properties of the channel suggests that either a protein site is not involved or that the protein site involved has very lax requirements for sterol recognition. It is more likely that this effect is mediated by non-specific lipid bilayer-mediated mechanism that both the enantiomers have the ability to affect equally [32].

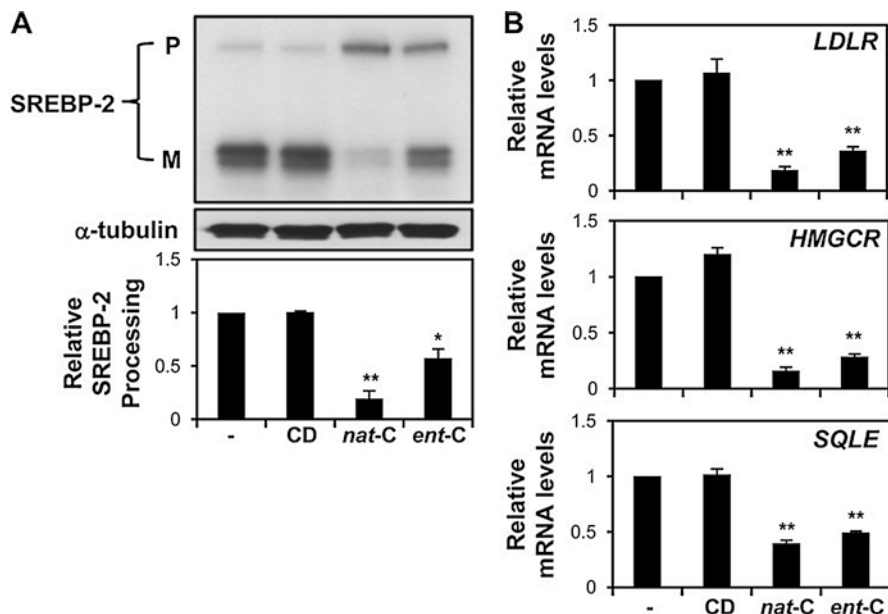

Fig. 4 (**a**, **b**) *Ent*-cholesterol suppresses activation of the SREBP-2 pathway (Adapted from [33])

To understand how cholesterol maintains its homeostasis, Kristiana et al. used the enantiomer to dissect the direct binding ability and its ability to alter the membrane properties [33]. It is known that low cellular cholesterol levels cause activation of sterol regulatory elem*ent*-binding protein-2 (SREBP) transcription factors. Alternatively at high concentrations, cholesterol binds to SREBP cleavage activating protein (Scap), causes a conformational change and eventually traps SREBP-2 in the ER and reduces the cholesterol concentration. In addition, at high concentration, cholesterol accelerates proteolytic cleavage of squalene monooxygenase (SM), a key enzyme required for biosynthesis of cholesterol. Understanding how these feedback mechanisms change when cholesterol is replaced by *ent*-cholesterol will allow elucidation of the role of non-specific membrane effects on regulation of cholesterol homeostasis. In-vitro assays with CHO-7 cells showed that *ent*-cholesterol not only suppressed SREBP-2 processing but also reduced the expression of three SREBP-2 target genes-LDLR, HMGCR, and SQLE (Fig. 4a, b). *ent*-Cholesterol was also equally able to induce a conformational change in Scap as determined by a trypsin-cleavage assay. And finally, *ent*-cholesterol also accelerated degradation of endogenous squalene monooxygenase in SRD-1 cells that lack sterol regulation as well as in CHO-7 cells that overexpress SM. All these findings suggest that the sterol-protein interactions involved in regulating cholesterol levels are not enantiospecific and that *ent*-cholesterol can also elicit these membrane effects that contribute significantly to cholesterol homeostasis [33].

3.2 Effects on Lipid-Modified Proteins

Both lipids and proteins affect formation of cholesterol-rich domains or rafts in membranes [34]. Since both proteins and most lipids are chiral, their interactions with cholesterol could require chiral recognition and affect formation of these rafts. Covey and coworkers compared capacity of the enantiomers to condense sphingomyelins in a monolayer system. Lipid-raft-forming capabilities of the enantiomers were compared by obtaining the surface pressure-average molecular area isotherms for mixtures of 70 mol% egg sphingomyelin and 30 mol% *nat-* or *ent-*cholesterol. These isotherms were identical for the two enantiomers indicating that the absolute configuration of the sterol does not affect its interaction with lipids such as sphingomyelin or phosphatidylcholine. In addition, both the enantiomers reversed to the same extent the enhanced phosphorylation of the EGF receptor that occurred following depletion of cholesterol with methyl-β-cyclodextrin [35]. This was direct evidence that the effects of cholesterol on EGF receptor function are most likely not enantioselective. Separately, Epand et al. studied how the enantiomers of cholesterol modulate lipid organization by small peptides. Interactions of two proteins, a 19-amino-acid, *N*-terminally myristoylated fragment (myristoyl-GGKLSKKKKGYNVNDEKAK-amide) of the protein NAP-22 [neuronal axonal membrane protein], and the segment LWYIK (Leu-Trp-Tyr-Ile-Lys) from the gp41 protein of HIV, were studied with *nat-*cholesterol and *ent-*cholesterol using differential scanning calorimetry (DSC) and nuclear Overhauser enhancement spectroscopy (NOESY-MAS-NMR). Phase transition properties of two lipid mixtures containing the lipid SOPC (1-stearoyl-2-oleoylphosphatidylcholine) and the phospholipid PtdIns(4,5)*P*2] were measured using DSC in presence of either cholesterol or *ent-*cholesterol. No significant difference was observed when *ent-*cholesterol replaced cholesterol. However, when 10 mol% myristoylated fragment of NAP-22 was added, a large increase in enthalpy of the chain melting transition was observed. The lipid mixture containing cholesterol underwent separation into cholesterol-rich and cholesterol-depleted domains. When *ent-*cholesterol was used instead of cholesterol, the chain melting transition had completely disappeared indicating stereospecific requirement for peptide-induced formation of cholesterol domains. Additionally, *N*-acetyl-LWYIK-amide increased the transition enthalpy of the phospholipid-sterol mixture containing cholesterol indicating separation of a cholesterol-depleted domain of SOPC. The effect on chain melting transition with *ent-*cholesterol was much less pronounced. Very similar effects were observed with the all D-isomer of N-acetyl-LWYIK providing evidence that peptide chirality is not essential for interaction with cholesterol containing membranes [36].

3.3 Effects on Sterol-Lipid Interactions

Covey and coworkers subsequently studied enantiospecific interactions between cholesterol and egg yolk sphingomyelin (SPM) measured as a difference in the plots of surface pressure (P) *vs.* mean molecular area (mmA) during the compression of