Université de Montréal

REGULATION OF INTESTINAL CHOLESTEROL TRANSPORT AND METABOLISM BY HIGH GLUCOSE LEVELS

RÉGULATION INTESTINALE DU TRANSPORT ET DU MÉTABOLISME DU CHOLESTÉROL PAR LE GLUCOSE

par

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REGULATION OF INTESTINAL CHOLESTEROL TRANSPORT AND METABOLISM BY HIGH GLUCOSE LEVELS

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Abstract

Growing evidence suggests that the small intestine may contribute to excessive postprandial lipemia, which is highly prevalent in insulin-resistant/type 2 diabetic individuals increasing the risk of cardiovascular disease. The aim of the present study was to determine the role of high glucose levels on intestinal cholesterol absorption, cholesterol transporter expression, enzymes controlling cholesterol homeostasis and the status of transcription factors. To this end, we employed highly differentiated and polarized intestinal cells, Caco-2 cells, plated on permeable polycarbonate filters. Four major technical approaches were used, immunocytochemistry in electron microscopy, western blot, RT-PCR and the assessment of enzymatic activities. The levels of cellular cholesterol uptake were measured by radio-labeling.

In the presence of radiolabeled cholesterol, glucose at 25 mM stimulated its uptake as compared to 5 mM glucose. The high concentration of glucose enhanced the protein expression of the critical cholesterol transporters NPC1L1 and CD36 and concomitantly decreased SR-BI protein expression. No significant changes were observed for ABCA1 and ABCG8, which act as efflux pumps favoring cholesterol export out of absorptive cells. At the same time, HMG-CoA reductase activity was significantly decreased, whereas ACAT activity remained unchanged. Finally, increments were noted in the transcription factors LXRα, LXRβ, PPARβ and PPARγ along with a drop in the protein expression of SREBP-2.

Collectively, our data indicate that glucose at high concentrations may regulate intestinal cholesterol transport and metabolism, thus suggesting a potential influence on the cholesterol absorption process in type 2 diabetes.

Key words: ABCA1, ABCG5/G8, SR-BI, CD36, NPC1L1, PPAR, LXR, SREBP, ACAT and HMG-CoA reductase

Résumé

Plusieurs évidences suggèrent que l'intestin contribue à l'hyperlipidémie postprandiale fortement répandue chez les individus souffrant de résistance à insuline et de diabète de type–2, augmentant ainsi les risques de développer des maladies cardio-vasculaires. Le but de notre étude fut de déterminer, au niveau intestinal, le rôle du glucose dans l'absorption du cholestérol, l'expression des transporteurs du cholestérol et celle des enzymes qui régulent l'homéostasie du cholestérol et le statut des facteurs de transcription. À cet effet, nous avons ensemencé des cellules intestinales différenciées et polarisées, des cellules Caco-2, sur des filtres en polycarbonate perméables. Quatre grandes approches techniques ont été utilisées, l'immuno-cytochimie en microscopie électronique, l'immunobuvardage, le RT-PCR, et la mesure des activités enzymatiques. Les niveaux d'absorption du cholestérol cellulaire ont été mesurés par radio marquage.

En présence de cholestérol radio marqué, le glucose à 25 mM stimule l'absorption du cholestérol comparativement aux cellules supplémentées avec 5 mM de glucose. La concentration élevée de glucose accroît l'expression protéique des transporteurs NPC1L1 et CD36 et diminue l'expression de la protéine SR-BI. On n'a observé aucun changement significatif dans l'expression des protéines ABCA1 et ABCG8, pompes d'efflux favorisant l'exportation du cholestérol hors des cellules intestinales. L'activité de l'enzyme HMG-CoA réductase diminue tandis que celle de l'ACAT demeure inchangée. Finalement, une augmentation des facteurs de transcription LXRα, LXRβ, PPARβ et PPARγ et une baisse de l'expression protéique de SREBP-2 ont été observées à fortes concentrations de glucose.

En conclusion, nos données indiquent que le glucose à des concentrations élevées, peut moduler le transport et le métabolisme intestinal du cholestérol suggérant une influence potentielle sur le processus d'absorption du cholestérol dans le diabète de type 2.

Mots clés: ABCA1, ABCG5/G8, SR-BI, CD36, NPC1L1, PPAR, LXR, SREBP, ACAT and HMG-CoA réductase

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List of Symbols and Abbreviations

ABC	ATP Binding Cassette transporter
CE	cholesteryl ester
CHD	coronary heart disease
DMEM	Dulbecco's Modified Eagle Medium
ER	endoplasmic reticulum
FA	fatty acid
FAT/CD36	fatty acid translocase/cluster determinant 36
FBS	fetal bovine serum
FC	free cholesterol
HDL	high-density lipoprotein
LDL-C	low-density lipoprotein-cholesterol
LXR	liver X receptors
NPC1L1	Niemann Pick C1-Like1
PBS	phosphate buffered saline
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferators response element
RXR	retinoid X receptor
SR-BI	scavenger receptor class B type I
SREBP-2	sterol regulatory element binding protein-2
T2DM	type 2 diabetes mellitus
TG	triacylglycol

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Foreword

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Presentations

- XXIIIe Congress of graduate and postdoctorate students of the Sainte-Justine Research Centre (June 2008). Poster "Potentiel du glucose a moduler le transport et l'homéostasie du cholestérol dans l'intestin". Montreal, Canada

- VI Symposium of digestive tube physiopathology (June 2008). Poster "Régulation du transport et de l'homéostasie du cholestérol par le glucose dans les cellules Caco-2/15". Orford, Canada

- IX Société québécoise de lipidologie de nutrition et de métabolisme congress (May, 2008) Poster "Régulation du transport du cholestérol par le glucose dans les entérocytes". Laval, Canada

- Diabète Québec congress (September 2007). Poster "Regulation by glucose of cholesterol metabolism in intestinal epithelial cells". Quebec, Canada

- Scientific Day, Medicine Faculty, Université de Montréal (June, 2007). Poster "Glucose effect on cholesterol transporters in enterocytes". Montreal, Canada

Introduction

Cholesterol is an essential component of cellular membranes, a precursor of steroid hormones, vitamin D and bile acids, and plays a crucial role in transcriptional gene regulation. Excessive cholesterol, however, is cytotoxic and may cause atherosclerotic lesions. Therefore, a balance must be maintained between cholesterol intake, absorption/excretion and synthesis. For its transport within the enterocytes, cholesterol requires a protein-dependent machinery, including SR-BI, NPC1L1 and CD36, involved in mediating intestinal cholesterol uptake. Other proteins such as ABCA1 and ABCG5/ABCG8 favor the exit of cholesterol from the enterocytes into the intestinal lumen or through the basolateral membrane. Several of these cholesterol carriers influence intracellular cholesterol homeostasis and are controlled by transcription factors, including RXR, LXR, SREBP-2 and PPAR.

Previous studies showed that lipid components exert a regulatory effect on intestinal fat uptake. However, the role of carbohydrates has barely been investigated. Therefore, we aimed to evaluate the effect of glucose on cholesterol transport and metabolism. Establishing this relation may contribute to find new alternative therapeutic treatments, which improve the conditions of metabolic glucose- and lipid-related diseases such as obesity, coronary heart disease and diabetes mellitus among others.

In the present study, we show in an *in vitro* model closely resembling *in situ* intestinal cells that high glucose concentrations enhance cholesterol transport by upregulating the protein expression of NPC1L1 and CD36. In addition, we found a reduced SR-BI protein expression and HMG-CoA reductase activity, a key enzyme in the cholesterol biosynthesis pathway, without altering the proteins ABCA1 and ABCG8. Moreover, our studies document that the expression of particular transcription factors are regulated by glucose levels. Our data indicate that glucose

at high concentrations may regulate intestinal cholesterol transport and metabolism, thus suggesting a potential influence on the cholesterol absorption process in type 2 diabetes.

1. Cholesterol

Cholesterol belongs to the sterol family and is present in the membranes of most eukaryotic cells. The characteristic structure of those lipids is the steroid nucleus, consisting of fused rings, three with six carbons and one with five. Sterols fulfill several indispensable roles in all eukaryotic cells. In mammals, cholesterol is the most important one. It is amphipathic with a polar head group and a nonpolar hydrocarbon body (Figure 1). Its hydrophobicity is responsible for the valuable property necessary to control cell membrane fluidity (Ohvo-Rekila et al., 2002). However, it makes it very difficult to handle in the aqueous environment of the body, both within and between cells. Therefore, sophisticated mechanisms exist to transport cholesterol to its numerous cellular destinations.

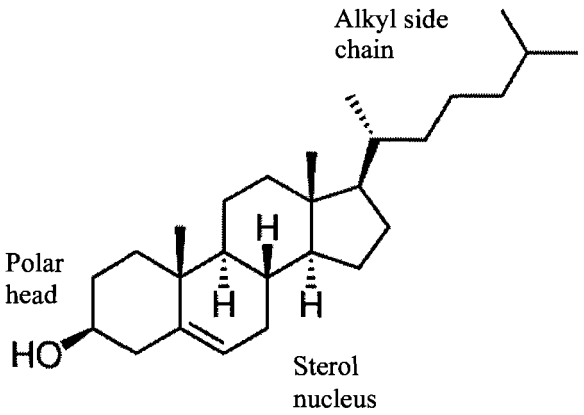

Figure 1. Cholesterol structure

Cholesterol is an integral part of cells as well as organelle membranes and is a precursor of important physiological molecules, like bile salts and steroid hormones and is thus essential for normal cell functioning (Maxfield and Tabas, 2005).

Defects in cholesterol synthesis or transport can have harmful consequences. Furthermore, cholesterol and sterols that are ubiquitously present in the diet also

pose a potential danger. They are critically involved in the development of atherosclerosis. Hence, cellular cholesterol homeostasis and plasma cholesterol levels have to be strictly regulated.

Cholesterol enters the lumen of the small intestine from 3 sources: diet, bile, and desquamated intestinal epithelial cells which are derived from the rapid turnover of intestinal cells. Although the entire length of the small intestine has the capability to absorb cholesterol from the lumen, the main sites of absorption are the duodenum and the proximal jejunum (Charlton-Menys and Durrington, 2008). In humans, 30-50% of the luminal cholesterol is absorbed and returned to the liver, while the rest is eliminated with feces (Turley and Dietschy, 2003). The principal sites of cholesterol biosynthesis are the liver and the intestine. To maintain body cholesterol homeostasis, metabolic adaptations of endogenous *de novo* synthesis and/or catabolism are required in response to fluctuations in dietary intake of cholesterol.(Levy et al., 2007).

The hydrophobicity of cholesterol is related to the main problems associated with increased plasma cholesterol concentrations, i.e., atherosclerosis. Atherosclerosis is at the origin for the majority of cases of coronary heart disease and represents the most prevalent cause of death in industrial countries (Ross, 1995).

The development of atherosclerosis is a process starting already early in life, possible even *in utero* (Napoli et al., 1997). Lipids, such as cholesterol, transported from lipoproteins, accumulate in macrophages on vessel walls (Kruth, 2001). Macrophage cholesterol accumulation converts the macrofages into so-called foam cells and stimulates the macrophages to secrete proteases and tissue factor that contribute to plaque rupture and thrombosis (Zhao et al., 2006).

2. Intestinal Cholesterol Metabolism

2.1. Intestine Generalities

The intestine controls the uptake of water, electrolytes and nutrients as well as secretes ions, enzymes and mucus and excretes endogenous and exogenous compounds from the blood towards the lumen. The barrier function of the intestine is ensured by intestinal mucosa, with includes the epithelial cells with the specialized tight-junction complexes that line the luminal surface. Intestinal motility and micelles cause mixing of the components and ensure absorption and transport along the tract (Scoville et al., 2008).

The morphology of the small intestine influences the absorptive process given its anatomic and physiologic features. Among these are the considerable length of the small intestine (7 m in humans and 90 cm in the rat) (Kaminsky and Zhang, 2003), the distribution of the metabolically competent epithelium as a monolayer of enterocytes and the amplification of the luminal surface by numerous finger-like projections of enterocyte-lined villi and, at their bases, buried crypts. Enterocytes have a very limited life span: after the division of stem cells at the base of the crypt, epithelial cells migrate up to the crypt surface, a process that takes 4 days in humans (3 in rodents). The cells then migrate to the villous tip, where they shed, a passage of 3 days in humans and 2 days in rodents (Kaminsky and Zhang, 2003).

Each region of the intestinal tract consists of the same layers: serosa, muscularis, submucosa and mucosa. The mucosa is lined by a continuous layer of epithelial cells, consisting of enterocytes and goblet cells. Anatomically, the intestine is divided into duodenum, jejunum, ileum and colon. In each of these regions, the enterocytes display particular sets of enzymes and transporters that, as part of the homeostatic function of the intestine, are able to metabolize and transport endogenous and exogenous compounds (Doherty and Charman, 2002).

2.2. Cholesterol Biosynthesis and Esterification: HMG-CoA-reductase and ACAT

Although the structure of cholesterol suggests a complex biosynthetic pathway, the entire formation starts with the acetate precursor.

The first step in this pathway is the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA, catalyzed by the enzyme HMG-CoA synthase. This is followed by the enzymatic action of HMG-CoA reductase to reduce HMG-CoA to mevalonate, wich represents the major rate-controlling step in cholesterol synthesis. Currently, HMG-CoA reductase is considered the rate-limiting key enzyme of the entire process. Mevalonate is converted into isopentenyl-5-pyrophosphate, followed by condensation to squalene and conversion into lanosterol, which is an immediate precursor of cholesterol. To convert lanosterol into cholesterol, the entire process involves 19 reactions (Goldstein and Brown, 1990).

In mammalians, HMG-CoA reductase is a glycoprotein of ~100 kDa. It shows an amino-terminal domain containing seven hydrophobic regions able to anchor the protein in the endoplasmic reticulum membrane, and a carboxy-terminal catalytic domain, which projects into the cytosol (Liscum et al., 1983). Virtually, every tissue and organ can synthesize cholesterol from acetyl-CoA (Dietschy and Siperstein, 1967; Spady and Dietschy, 1983), but the liver and intestine are considered as the major sites (Taylor et al., 1960; Dietschy and Gamel, 1971; Turley et al., 1981).

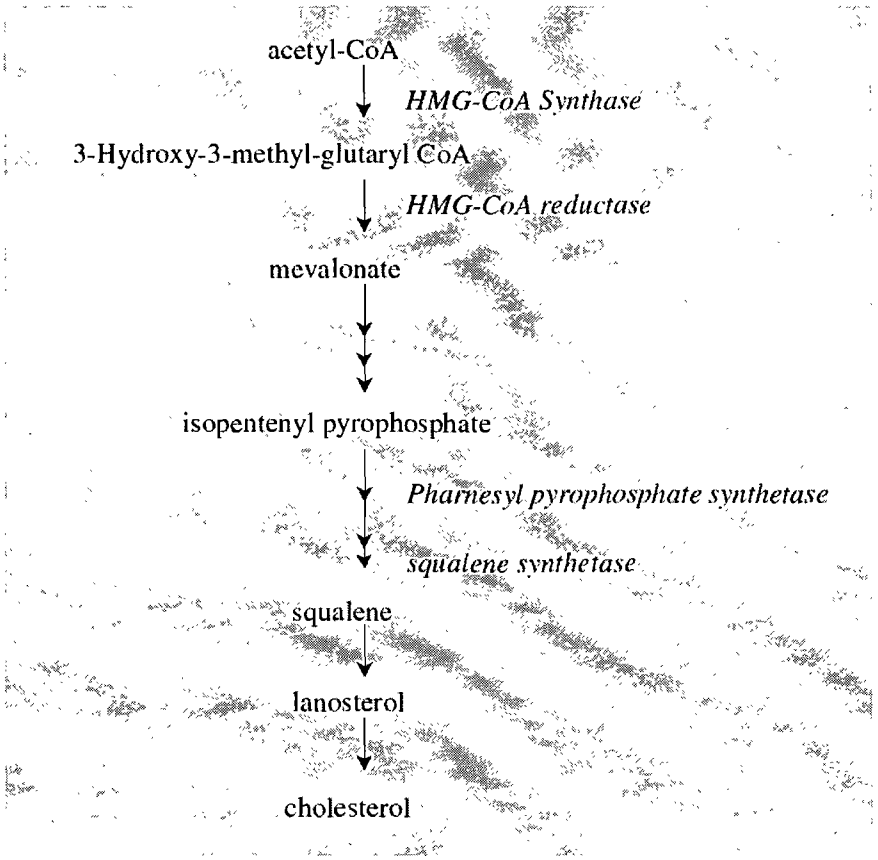

Figure 2. Summary of cholesterol biosynthesis (Adapted from (Charlton-Menys and Durrington, 2008). Cholesterol is an essential molecule in many animals, including humans, but is not required in the mammalian diet. All cells can synthesize it from simple precursors. (Adapted from Lehninger et al., 2002)

HMG-CoA reductase is subject to both short and long-term controls (Goldstein and Brown, 1990). Long-term effects are mediated through alterations in its rate of synthesis and degradation. Short-term actions involve allosteric effects and alterations in its state of phosphorylation. Expression of HMG-CoA reductase and other enzymes in the cholesterogenic pathway is transcriptionally controlled by the sterol regulatory binding protein SREBP-2. Both expression and activity of the enzyme are rapidly reduced under conditions of high intracellular sterol concentrations (Nakanishi et al., 1988).

After biosynthesis and/or uptake by the enterocyte, cholesterol is mainly esterified at C3 with fatty acids to form cholesteryl ester, a reaction catalyzed by acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT-2) (Joyce et al., 1999). Two ACAT genes (*acat-1* and *acat-2*) have been identified in mammals; the two enzymes may function in distinct and complementary manners (Chang et al., 1997; Farese, 1998; Chang et al., 2001; Rudel et al., 2001). In skin cells, macrophages, adrenal cells and CHO cells, ACAT-1 is the major isoenzyme and constitutes 90% or more of the total cellular ACAT activity. In intestinal mucosal cells, ACAT-2 is the major isoenzyme. ACAT-2 may be allosterically regulated by cholesterol (Chang et al., 2000; Liu et al., 2005).

The objective of cholesterol esterification is the storage of cholesterol as cholesteryl esters in cytoplasmic lipid droplets. Cholesteryl esters can be hydrolyzed when necessary and the esterification/hydrolysis cycle provides cells with short-term buffering capacity for cholesterol. In contrast to cholesterol, plant sterols being poor substrates for ACAT-2, are not efficiently esterified and remain in majority unesterified. (Field and Mathur, 1983; Joyce et al., 1999; Temel et al., 2003). This reflects the main difference between plant sterols and cholesterol in all reactions taking place in the enterocyte. Cholesteryl esters can subsequently be secreted into the lymph after their packaging into chylomicrons, to ultimately reach the liver (Chang et al., 2006).

2.3. Cholesterol Biosynthesis Regulation: SREBP

Cholesterol biosynthesis is controlled by a family of transcription factors of the helix-loop-helix family designated Sterol Regulatory Element Binding Proteins (SREBPs). SREBPs activate the expression of more than 30 genes required for the synthesis of cholesterol, fatty acids, triglycerides and phospholipids, and are thus considered key regulators of cholesterogenesis and lipogenesis (Brown and Goldstein, 1997; Horton and Shimomura, 1999; Edwards et al., 2000; Sakakura et al., 2001).

SREBPs are encoded by two genes, *srebp-1* and *srebp-2*. Alternative promoter usage and alternative splicing of SREBP-1 drive the production of two isoforms, SREBP-1a and SREBP-1c. The 29 additional amino acids present in the SREBP-1a NH2 terminus are enriched in acidic residues and might be responsible for the higher transcriptional activity of SREBP-1a, compared with that of SREBP-1c. SREBP-1c was initially cloned in rats and called adipocyte determination and differentiation factor-1 (ADD1) (Rosen and Spiegelman, 2000).

SREBP-1a has been mainly studied in cell lines, showing a strong expression, while in animal tissues its expression is relatively weak. SREBP-1c, highly expressed in liver, is the key regulator of lipogenesis and enhances transcription of genes encoding acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD-1) and glycerol-3-phosphate acyltransferase (GPAT), all important enzymes in the lipogenic pathway. SREBP-2, predominantly expressed in cell lines, is also present in the liver and adipose tissue, but overall has a rather weak expression in animal tissues. It activates cholesterol synthesis by inducing expression of genes encoding enzymes that catalyze the various steps in cholesterol synthesis, including HMG-CoA reductase (Desvergne et al., 2006).

SREBPs are synthesized as inactive precursors of ~125-kDa, bound to the endoplasmic reticulum (Hua et al., 1995). In order to reach the nucleus and act as transcription factors, their NH2-domains must be cleaved. One protein required for the transfer of SREBP to the nucleus is an escort protein designated SREBP cleavage activating protein (SCAP). A schematic representation of SREBP activation is shown in Figure 3.

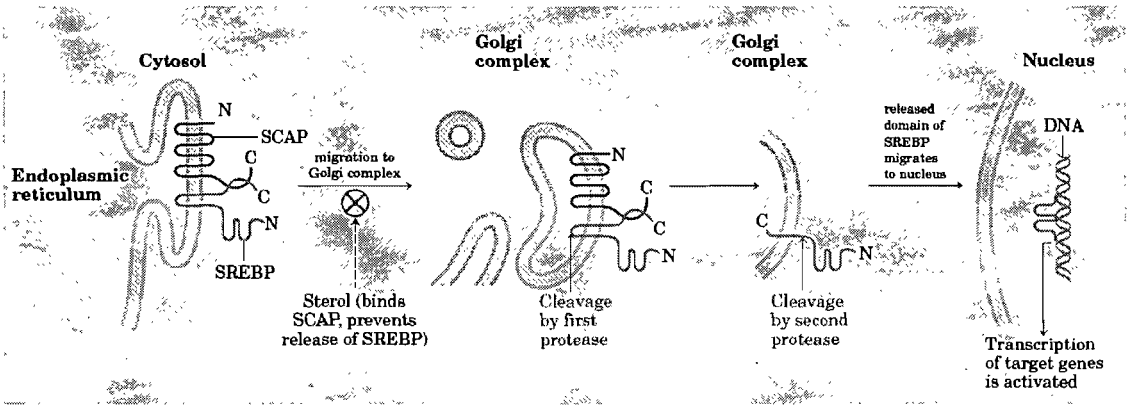

Figure 3. SREBP activation. Sterol regulatory element-binding proteins are embedded in the ER when first synthesized, in a complex with the protein SREBP cleavage-activating protein (N and C represent the amino and carboxyl termini of the proteins). When bound to SCAP, SREBPs are inactive. When sterol levels decline, the complex migrates to the Golgi complex, and SREBP is cleaved by two different proteases in succession. The liberated amino-terminal domain of SREBP migrates to the nucleus, where it activates transcription of sterol-regulated genes. (Adapted from Lehninger et al., 2001)

Low membrane cholesterol levels lead to the transport of SCAP/SREBP to the Golgi membrane where activation of the site 1 serine protease results in a first cleavage. A second enzyme, the site 2 metalloproteinase, completes the maturation of SREBPs and releases the 68-kDa NH2-terminal domain of SREBP from the membrane (Edwards et al., 2000). This fragment contains a basic helix loop helix (HLH) leucine zipper domain, which functions as a transcription factor upon translocation into the nucleus. The mature forms of SREBPs bind to elements initially characterized as featuring an enhancer sequence called E-box that is recognized by members of the HLH transcription factor family. SREBPs also bind to sites related to the direct repeat TCANCCAC (Horton et al., 2002).

Deletion of SREBP-1 (eliminating both SREBP-1a and SREBP-1c) or SREBP-2 leads to partial or fully embryonic lethality, respectively. In contrast, specific deletion of the SREBP-1c transcript is not lethal, suggesting an important role of SREBP-1a and SREBP-2 in embryonic development. The SREBP maturation

process via membrane cholesterol sensing is consistent with their important role in cholesterol homeostasis (Sundqvist and Ericsson, 2003). SREBP-2 is mainly involved in cholesterol metabolism. SREBP-1c has an important implication in fatty acid synthesis, whereas SREBP-1a is involved in cholesterol and fatty acid synthesis (Desvergne et al., 2006).

3. Intestinal Cholesterol Transport

Next to endogenous synthesis, dietary intake provides the other main source of cholesterol in mammals. Only a fraction of dietary cholesterol is absorbed in the small intestine, with large interindividual variations in humans. Reported values range from 25% to 85% (Sehayek et al., 1998; Bosner et al., 1999). In contrast, only small amounts of plant sterols are absorbed (Lu et al., 2001b; Turley and Dietschy, 2003).

Recent studies suggest that cholesterol absorption is a protein-mediated process. In support to this hypothesis, cholesterol uptake by apical membranes *in vitro* follows a second-order reaction kinetics changing to a low-affinity first-order kinetics mechanism upon proteolytic digestion of proteins on the surface of the brush-border membranes (Thurnhofer and Hauser, 1990). The recent discovery of inhibitors (that selectively block cholesterol absorption at very low doses) and their binding to intestinal mucosa in a specific and saturable manner, supports the protein-mediated cholesterol absorption hypothesis (Hernandez et al., 2000).

Various transporters, including fatty acid translocase/cluster determinant 36 (FAT/CD36), scavenger receptor class B type I (SR-BI) and Niemann Pick C1-Like1 (NPC1L1) may be involved in cholesterol uptake, while the ATP Binding Cassette transporter family, including several cholesterol carriers (ABCA1, ABCB1, ABCG5/G8), may act as efflux pumps favoring cholesterol export out of absorptive cells into the lumen or basolateral compartments (Levy et al., 2007).

Intestinal cholesterol absorption consists on a multistep process that can be controlled at several levels. A schematic overview of the absorption process is shown in Figure 4.

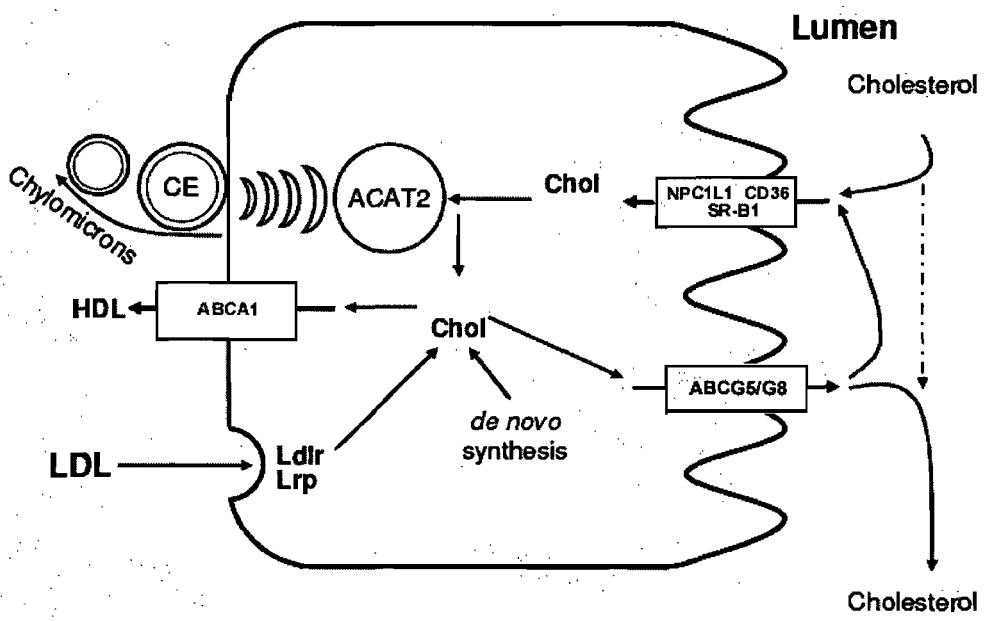

Figure 4. Schematic overview of cholesterol transport in enterocytes. ABCA1/G5/G8, ABC-transporters A1,G5 and G8; ACAT2, acyl-coenzyme A:cholesterol acyltransferase-2; CE, cholesteryl ester; Chol, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLr, LDL-receptor; LRP, LDL-receptor related protein; NPC1L1, Niemann-Pick C1 like 1, CD36, cluster determinant 36; SR-BI, scavenger receptor class B type I. (Adapted from (Levy et al., 2007)

After cholesterol is taken up by the enterocyte, it is esterified by the action of acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) (Lee et al., 2000). Cholesteryl esters are packed into chylomicrons and subsequently secreted via the lymph to the circulation (Wang and Carey, 2003).

In addition, studies have shown that enterocytic cholesterol can also be transferred to lipid-poor apoA-I through the action of intestinal ABCA1 (Brunham et al., 2006). The HDL particles are directly excreted into the circulation and contribute for approximately 30% to the steady-state plasma HDL pool in mice (Brunham et al.,

2006). However, *abca1*$^{-/-}$ mice do not show reduced cholesterol absorption (McNeish et al., 2000; Drobnik et al., 2001), indicating that this process is not of regulatory relevance for mass cholesterol absorption. Non esterified cholesterol and plant sterols can be excreted from the enterocyte back into the intestinal lumen. This process is facilitated by the ABC half-transporters ABCG5 and ABCG8 (Berge et al., 2000; Igel et al., 2003). Overexpression of these transporters in mice increases excretion of cholesterol into the lumen and limits net cholesterol absorption (Yu et al., 2002b).

3.1. Cholesterol Transporters

Considerable effort has been spent over the past several years on identifying the cholesterol transporter(s) in enterocyte membranes. This section focuses on recent progress and explanatory findings associated to various proteins that are potentially involved in cholesterol transport.

3.1.1. Scavenger Receptors CD36 and SR-BI

Several cell surface glycoproteins, including CD36 and SR-BI are designated as scavenger receptors and contribute to the uptake of modified lipoproteins (Ades et al., 1992; Calvo et al., 1995; Acton et al., 1999; Nakata et al., 1999; Husemann et al., 2001).

3.1.1.1. CD36

FAT/CD36, (fatty acid translocase/cluster determinant 36) an 88-kDa membrane glycoprotein, is found in several cell types, including platelets, monocytes, macrophages and endothelial cells where it facilitates cellular uptake of long-chain fatty acids. In humans and mice, CD36 is also expressed in intestinal epithelial cells. (Ohgami et al., 2001; Nicholson and Hajjar, 2004).

CD36 has been reported to be a multifunctional receptor recognizing several ligands including OxLDL (Nakata et al., 1999; Nozaki, 1999), thrombospondin (Simantov and Silverstein, 2003), collagen (Fernandez-Ruiz et al., 1993; Kopprasch et al., 2004), *Plasmodium falciparum*-infected erythrocytes (Beeson and Brown, 2004) and anionic phospholipids (Ryeom et al., 1996; Bottcher et al., 2006). The importance of CD36 in fatty acid absorption is well established, since CD36 deficiency leads to abnormal lipid processing in enterocytes.

The potential role of CD36 in cholesterol absorption is reported in studies showing enhanced cholesterol uptake from micellar substrates by CD36-transfected COS-7 cells compared to mock-transfected cells (van Bennekum et al., 2005). The CD36-mediated cholesterol uptake properties in the transfected cells are similar to that observed with SR-BI-transfected cells as well as those observed with brush-border membrane vesicles prepared from wild-type mice (van Bennekum et al., 2005). The importance of CD36 in mediating cholesterol absorption was demonstrated in a study that showed a significant reduction of cholesterol transport from the intestinal lumen to the lymph in CD36-null mice (Nauli et al., 2006). Interestingly, the CD36-facilitated cholesterol uptake process is similar to that observed for SR-BI-mediated cholesterol uptake in their sensitivity to ezetimibe inhibition (van Bennekum et al., 2005).

3.1.1.2. SR-BI

SR-BI, the scavenger receptor class B type I, an 82-kDa protein, is highly expressed in the small-intestine brush border membrane where it facilitates the uptake of dietary cholesterol from either bile salt micelles or phospholipid vesicles (Hauser et al., 1998). SR-BI is able to bind with fair but different affinities to HDL, LDL, and modified (acetylated or oxidized) LDL. On the other hand, HDL and LDL appear not to share the same binding sites (Gu et al., 2000).

The participation of SR-BI in cholesterol absorption was suggested by studies that showed that SR-BI cDNA-transfected cells displayed increased cholesterol uptake from micellar substrates compared with mock-transfected cells with low SR-BI expression (Altmann et al., 2002; van Bennekum et al., 2005). Moreover, the increase in cholesterol uptake by SR-BI-transfected cells showed a sensitive effect to ezetimibe inhibition in both of these studies.

Despite the strong evidences suggesting that SR-BI may participate in cholesterol absorption on the surface of brush-border membranes, its role in cholesterol absorption in a physiological environment remains controversial. Since *scarbi*$^{-/-}$ mice, with a disruption of the *sr-bi* gene, efficiently absorbed cholesterol, in similar fashion to wild-type mice (Altmann et al., 2004), whereas cholesterol absorption was increased in transgenic mice with SR-BI-specific overexpression in the intestine (Bietrix et al., 2006).

3.1.2. NPC1L1

Niemann-Pick C1-like 1 protein (NPC1L1), a 151-kDa protein, is expressed predominantly in the gastrointestinal tract with peak expression in the proximal jejunum (Altmann et al., 2004; Davis et al., 2004). *In situ* hybridization and immunohistochemistry analysis of the jejunum revealed discrete NPC1L1 localization to the epithelial layer lining the luminal space along the crypt-villus axis (Altmann et al., 2004). Furthermore, our group (Sane et al., 2006) was able to assign NPC1L1 to the brush-border membrane with the use of cell fractionation and high-resolution immunoelectron microscopy. The protein was also found to be located in subcellular compartments of the human enterocyte, including lysosomes and mitochondria (Sane et al., 2006).

NPC1L1 has 50% amino acid homology to NPC1, which is defective in the cholesterol storage disease Niemann-Pick type C (Carstea et al., 1997). A strong support for the essential role that NPC1L1 plays in intestinal cholesterol absorption

lies in the following examples. Mice deficient in NPC1L1 lack the ability to absorb cholesterol and exhibit prevailing protection against the rise in plasma and hepatic cholesterol associated with the administration of high cholesterol diets (Altmann et al., 2004; Davis et al., 2004; Davis et al., 2007). Genetic modifications of NPC1L1 in cultured intestinal cells alter cholesterol uptake (Palmer et al., 1995; Yu et al., 2006; Yamanashi et al., 2007). Reduced expression in NPC1L1 was found associated with reduced sterol absorption, low-density lipoprotein-cholesterol (LDL-C) levels (Cohen et al., 2006) and LDL-C response to ezetimibe therapy (Hegele et al., 2005; Simon et al., 2005; Wang et al., 2005). Inactivation of NPC1L1 led to defects in cholesterol transport, variations in key regulatory sterol enzymes (HMG-CoA reductase and ACAT), and high gene expression of SREBP, which suggest key roles for NPC1L1 in cholesterol homeostasis (Sane et al., 2006).

3.1.3. ATP-Binding-Cassette (ABC) Transporters

ABC-transporters comprise a large family of membrane proteins that mediate transport of a variety of compounds across cellular membranes against concentration gradients at the cost of ATP. Most ABC-transporters contain two transmembrane and two ATP-binding domains. Genes that encode for the ABC transporters are conserved from bacteria to mammals. They play critical roles in the active transport of a wide range of molecules across cellular membranes. Each ATP-binding domain contains the conserved Walker A and Walker B motifs that are involved in ATP binding and hydrolysis. Each transmembrane domain consists of six membrane-spanning α-helices. Some ABC-transporters contain only a single transmembrane and a single ATP-binding domain and are therefore called "half-transporters". These half-transporters assemble either as homodimers or heterodimers to create a functional transporter (Dean et al., 2001; Borst and Elferink, 2002; Tusnady et al., 2006). Three specific ABC transporters of relevance for this study are discussed below.

3.1.3.1. ABCA1

ABCA1, a full-sized transporter, is probably the most extensively studied transporter of the ABC superfamily. ABCA1 is expressed in virtually all organs and tissues, with a high expression in hepatocytes, enterocytes and macrophages (Luciani et al., 1994; Wellington et al., 2002). The ABCA1 protein is located on the basolateral surface of intestinal cells, indicating that this protein does not have a direct role in cholesterol absorption from the intestinal lumen. Current studies indicate that ABCA1 expression in the basolateral membrane is crucial for intestinal secretion of HDL, which accounts for ~30% of HDL production in the body (Attie, 2007). ABCA1 mediates the efflux of cholesterol and phospholipids ABCG5/G8 to lipid-poor apoA-I and to pre-β-HDL. Therefore, ABCA1 has a crucial role in HDL formation, as evidenced from the discovery of mutations in the ABCA1 gene in Tangier disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). Tangier patients, as well as mice lacking ABCA1, are characterized by an almost complete absence of HDL and accumulation of cholesteryl esters in various tissues.

However, it is important to note that ABCA1, present in other tissues promotes reverse cholesterol transport to the liver for biliary excretion (Vaisman et al., 2001). Therefore, ABCA1 may indirectly impact on cholesterol absorption through the modulation of lipid composition in bile and the intestinal lumen, as evidenced by the moderately lower cholesterol absorption in *abca1*$^{(-/-)}$ mice.

3.1.3.2. ABCG5 and ABCG8

Two groups (Berge et al., 2000; Lee et al., 2001) independently identified the two adjacent genes ABCG5 and ABCG8, in a head-to-head configuration that encode transporters, highly expressed in the liver and intestine. Unlike other ABC transporter genes that encode proteins with 12 transmembrane domains, ABCG5 and ABCG8 separetely encode a protein with 6 transmembrane domains, and the

heterodimerization of the two encoded proteins is required for transport activity (Graf et al., 2002).

ABCG5 and ABCG8 are localized at the apical brush border membranes of enterocytes and the canalicular membranes of hepatocytes. These transporters constitute an efficient efflux pump system for cholesterol and plant sterols transporting out of intestinal cells back into the intestinal lumen and from hepatocytes into the bile. These efflux processes thus contribute to the regulation of intestinal absorption and biliary secretion of cholesterol and plant sterols (Yu et al., 2002a; Yu et al., 2002b; Yu et al., 2003; Klett et al., 2004).

Mutations in either one of these genes cause sitosterolemia, an error of metabolism characterized by the accumulation of plant sterols in the body due to a decreased ability for their hepatobiliary secretion (Berge et al., 2000; Lee et al., 2001; Lu et al., 2001a). Accordingly, mice deficient for ABCG5 and/or ABCG8 are characterized by accumulations of plant sterols in blood and organs. These mice have a reduced secretion and a clearly enhanced intestinal absorption of plant sterols, whereas absorption of cholesterol is not affected (Yu et al., 2002a; Klett et al., 2004; Plosch et al., 2004).

However, overexpression of ABCG5 and ABCG8 in transgenic mice causes a poor fractional intestinal cholesterol absorption besides the increased hepatobiliary cholesterol secretion (Yu et al., 2002b). These findings have established ABCG5 and ABCG8 as key transporters that regulate excretion of cholesterol and plant sterols from the body.

4. Transcription Factors

One type of metabolic regulation relies on the transcriptional regulation mechanism, which affects the level of expression of key enzymes and/or proteins and is effective on a long time scale. Transcriptional control requires specific signals, called

transcriptional factors, to be transduced to the cell nucleus where defined sets of genes are targeted.

Transcription factors are soluble proteins that are able to bind to DNA. Their binding to promoter sites of genes influences the transcription of these genes, leading to an up- or down-regulation of their expression. Some transcription factors need to be activated by ligands before they are targeted to the nucleus (Desvergne et al., 2006). A schematic model of this type of transcriptional regulation is given in Figure 5.

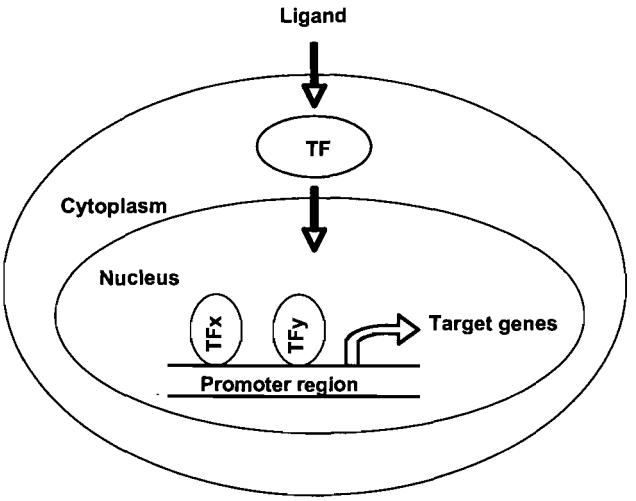

Figure 5. General schema of gene regulation by transcription factors. TF, transcription factor. (Adapted from Bandsma et al., 2004)

Several food components and derivatives thereof may act as ligands for specific transcriptional factors, providing means to adapt gene expression patterns in response to environmental (i.e., dietary) signals. The distinct roles of certain factors important in transcriptional control of genes involved in cholesterol, lipid and bile acid metabolism made them highly interesting as potential pharmacological targets for prevention or treatment of certain diseases.

Transcriptional factor proteins exhibit a characteristic structure with regions of conserved sequence and function (Figure 6A) (Francis et al., 2003). The proteins contain an NH2-terminal region that harbors a ligand-independent transcriptional activation function (AF-1). Adjacent is the DNA binding domain (DBD), containing two highly conserved zinc finger motifs that target the receptor to specific DNA sequences. This is followed by a region that permits protein flexibility to allow simultaneous receptor dimerization and DNA binding. Close to the COOH-terminal region is the usually highly conserved ligand–binding domain, and the last part of the receptor contains a ligand-dependent activation function (AF-2) (Chawla et al., 2001; Germain et al., 2006). Transcriptional factors can bind to DNA response elements in the promotor regions of their target genes as monomers, homodimers or as heterodimers with the Retinoid X Receptor (RXR) (Figure 6B).

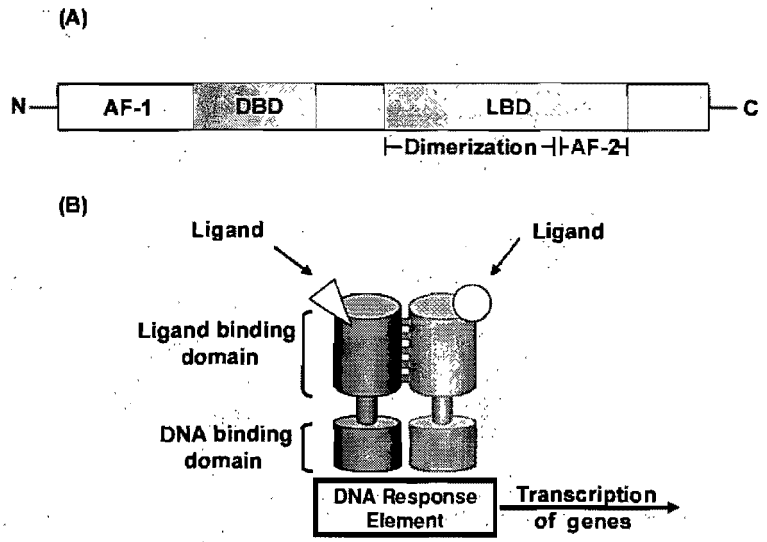

Figure 6. Schematic structure of transcriptional factors. (A) Organisation of a transcriptional factor. AF, activation function; DBD, DNA-binding domain; LBD, Ligand-binding domain. (B) Heterdimerization and DNA-binding of transcriptional factors. (Adapted from Edwards et al., 2000)

4.1. The Liver X Receptor (LXR)

The liver X receptor is a major player in regulating cholesterol metabolism. Two LXR isotypes have been identified in mammals, i.e., LXRα (NR1H3), which is predominantly expressed in the liver, kidney, intestine, adipose tissue, and macrophages, and LXRβ (NR1H2) with a more ubiquitous expression pattern (Willy et al., 1995; Peet et al., 1998). These two isotypes, LXRα and LXRβ, share 77% amino acid identity in their DBD and LBD and are highly conserved between rodents and human.

LXR endogenous activators are oxysterols, i.e., cholesterol metabolites (Janowski et al., 1996; Forman et al., 1997; Lehmann et al., 1997; Janowski et al., 1999). As such, they participate in the cholesterol sensing processes and regulate important aspects of cholesterol and fatty acid metabolism (Tontonoz and Mangelsdorf, 2003).

LXRs heterodimerize with RXR to bind to their DNA response element, formed from a direct repeat of two hexamers related to the sequence AGTTCA, separated by four nucleotides. Mono-oxidized derivatives of cholesterol are strong LXR ligands. The most potent are 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol, which activate both LXRα and LXRβ. Little is known about the sterol hydroxylases that produce these metabolites, but it is assumed that oxysterol concentrations parallel those of cholesterol (Desvergne et al., 2006). Importantly, oxysterols are found at micromolar concentrations in tissues that express high levels of LXRα or LXRβ. Activation of the heterodimer can also be triggered by RXR ligands (Field et al., 2004). LXRα and LXRβ null mutant mice have been generated and confirm the important role of these receptors, and more particularly that of LXRα in cholesterol homeostasis.

When LXRα becomes activated, the LXRα/RXR complex induces transcription of target genes amongst which are ABCA1, ABCG5/G8. Activation of LXRα thus results in elevated levels of HDL-cholesterol, reduced intestinal cholesterol

absorption, increased hepatobiliary cholesterol secretion and increased neutral sterol loss via the feces. In this way, LXR activation has anti-atherogenic actions, as has been substantiated in a mouse model that is susceptible to develop atherosclerosis (ldl-r $^{-/-}$) treated with a synthetic LXR agonist, T090131773 (Goodwin et al., 2008). However, besides stimulation of cholesterol disposal, activation of LXR by synthetic ligands like T0901317 also leads to increased lipogenesis, hypertriglyceridemia through the production of larger VLDL particles and hepatic steatosis in rodents (Grefhorst et al., 2002). Therefore, general LXRα activation can only be used as an atheroprotective therapy if the undesirable effects on lipogenesis can be eliminated by the development of selective LXR modulators.

4.2. Peroxisome Proliferator-Activated Receptors (PPAR)

PPARs were the first nuclear receptors identified as "sensors" rather than classic hormone receptors. They are molecules that control a variety of genes in several pathways of lipid metabolism (De Vos et al., 1995). Three mammalian peroxisome proliferator-activated receptors (PPARs) have been cloned in Xenopus, rodents, and humans: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). Two PPARγ isoforms, PPARγ1 and PPARγ2, are splice variants in their NH2-terminal domain. They are all activated by polyunsaturated fatty acids and eicosanoids and are therefore considered to be fatty acid sensors (Willson et al., 2000). All PPARs play important roles in the control of lipid and glucose metabolism and have been involved in obesity-related metabolic diseases, such as hyperlipidemia, insulin resistance, and coronary artery disease. PPARs, which recognize and bind a variety of fatty acids, regulate most of the pathways linked to lipid metabolism (Michalik et al., 2003).

PPARα is most highly expressed in tissue with high activity levels of lipid catabolism, e.g., liver, brown adipose tissue, and skeletal and heart muscle and was recognized to be responsive to fibrates, which are widely used drugs for treatment of hyperlipidemia (Issemann et al., 1993; Schonfeld, 1994). Activated PPARα

increases β-oxidation of fatty acids, thereby stimulating energy production and preventing lipid accumulation. Under the physiological fasting condition, PPARα becomes activated by the free fatty acids released from adipose tissue, after which hepatic β-oxidation is induced in order to provide energy to peripheral tissues (Kersten et al., 1999).

PPARγ is mainly expressed in the adipose tissue and is involved in adipocyte proliferation. PPARγ1 is mainly expressed in adipose tissues but is also detected in the colon, spleen, retina, hematopoeitic cells, and skeletal muscle. PPARγ2 has been found mainly in the brown and white adipose tissue. PPARγ is critical for the maintenance of glucose homeostasis and is a molecular target of thiazolidinediones (TZDs), a class of insulin-sensitizing drugs (Rosen and Spiegelman, 2001).

PPARβ has an ubiquitous expression pattern and appears to serve several functions. Besides its role in lipid metabolism, it is involved in skin biology, energy homeostasis and inflammatory processes (Michalik et al., 2003). PPARβ was reported as an important factor in the regulation of glucose metabolism and insulin sensitivity (Lee et al., 2006).

The ligand binding pocket of PPARs is much larger than that of the other nuclear receptors and relatively easily accessible. The DNA binding domain is extremely well conserved. The less conserved NH2-terminal region bears a ligand-independent activation domain, at least in PPARα and PPARγ (Xu et al., 2001a; Xu et al., 2001b; Xu et al., 2002; Xu et al., 2004).

PPARs bind to DNA as heterodimers with RXR, on PPAR response elements (PPRE) comprising direct repeats of two hexamers closely related to the sequence AGGTCA and separated by one nucleotide. The five nucleotides that flank the 5'-end of this core sequence are also important for the efficiency of PPARα:RXR binding (Desvergne et al., 2006).

The first molecules to be recognized as PPARα activators, and later characterized as ligands, belong to a group of molecules that induce peroxisome proliferation in rodents, thus explaining the name of peroxisome proliferator activated receptor given to this receptor. This diverse group of substances includes, for example, some plasticizers and herbicides. More interestingly, various fatty acids, more particularly unsaturated fatty acids, and some eicosanoids mainly derived from arachidonic acid and linoleic acid, bind to PPARa, -β, and -γ with varying affinities (Desvergne et al., 2006). In addition to being activated by fatty acids, PPARα responds to fibrates that are hypolipidemic drugs, and PPARγ responds to thiazolidinediones that are insulin sensitizers, demonstrating their potential as drug targets. In the process of transcriptional regulation, ligand-bound PPARs recruit coactivators, most likely organized in large complexes (Surapureddi et al., 2002). Cofactor recruitment may be PPAR isotype specific and may ensure the specificity of target gene activation. In addition to PPAR ligand binding, PPARs can also be activated by phosphorylation of serines located in the A/B domain, and the PPAR:RXR heterodimer can be activated by RXR ligands (Desvergne et al., 2006).

4.3. The Retinoid X Receptor

Retinoid X receptors (RXRs; NR2B1) play an important role in nuclear receptor signalling, as they function as general partners for a variety of nuclear hormone receptors that bind as heterodimers to DNA. There are three isotypes of RXR, α, β, and γ, and several isoforms for each of them (Chambon, 1996). Each isotype and isoform has its specific expression pattern. However, each single tissue contains one or several forms of RXR.

Almost all the nuclear receptors are active as heterodimers with RXR. The important part that RXR may play is further emphasized by the fact that RXR is itself a nuclear receptor that can be activated by specific ligands. PPAR:RXR and LXR:RXR are permissive heterodimers: RXR can bind its own ligand, in the

absence of a ligand for its partner and can thereby activate the transcription of the heterodimer target genes (Desvergne et al., 2006).

In addition to the various heterodimers for which RXR is an obligatory partner, RXR can form homodimers. The *in vivo* relevance of these homodimers is still under study. RXR can be activated by 9-cis-retinoic acid, an isomer of all-trans-retinoic acid (Heyman et al., 1992). Retinoids are used for treatment of dermatological disorders and certain cancers and a common side-effect of this treatment is dyslipidemia (Farol and Hymes, 2004), indicating that retinoids and RXR are involved in the regulation of lipid metabolism.

Deletion of the RXRα gene in the liver allowed the identification of the most affected pathways (Wan et al., 2000). As expected, many PPARα-mediated functions were altered and the activity of LXR and FXR was also compromised, suggesting that the absence of RXRα cannot be compensated by RXRβ and RXRγ in the liver.

5. Glucose metabolism

Carbohydrates are a main source of energy and can be stored in the form of starch in plants and glycogen in animals. Carbohydrates are also part of the structural framework of both DNA and RNA and form structural elements in cell walls of bacteria and plants. An important group of carbohydrates comprises the monosaccharides of which glucose is an example. Glucose is primordial for the generation of energy. Monosaccharides are aldehydes or ketones with two or more hydroxyl groups, that can be described by the formula $(CH_2O)n$. Glucose metabolism is tightly regulated in humans and animals to guarantee a sufficient glucose supply to glucose-dependent organs. The brain is the organ that is most dependent on an adequate supply of glucose, since it can only use ketone bodies as an alternative energy source and this only to a limited extent. Carbohydrates are transported to and from various tissues through the blood compartment. Glucose can

enter the blood via two routes, i.e., dietary glucose derived from the intestine and glucose production by the liver and the kidney. During fasting, the organism will solely depend on the production of glucose, mainly by the liver. Glucose can be produced directly through gluconeogenesis from various substrates, such as certain amino acids, lactate and glycerol. The liver is also able to produce glucose indirectly through phosphorylation of glycogen, the storage form of glucose. This process is called glycogenolysis (Rothman et al., 1991; Hellerstein et al., 1997). Glucose can also be taken up first by the blood, phosphorylated by glucokinase to form glucose-6-phosphate (G6P) and then be secreted again after dephosphorylation by glucose-6-phosphatase (G6Pase). This process is called glucose cycling (Jenssen et al., 1990).

5.1. Physiological interactions between carbohydrates and lipid metabolism

Carbohydrate metabolism and lipid metabolism are linked in many ways. First of all, mammals are capable of turning glucose into fat. Glucose is degraded, through glycolysis, into acetyl-CoA, which is the precursor for fatty acid synthesis. On the other hand, fat cannot be turned into glucose by mammals, because the enzyme system for this conversion is lacking. Evidence was generated in the sixties by the group of Dr. Randle evidencing that fat oxidation inhibits glucose oxidation, by interference at multiple levels (Randle et al., 1963). The key enzyme in this inhibitory process is pyruvate dehydrogenase, which catalyzes the oxidative decarboxylation of pyruvate leading to the formation of acetyl-CoA. It was found that free fatty acids (FFA) increase concentrations of acetyl-CoA as well as of citrate, important in the citric acid cycle (Randle et al., 1963). Acetyl-CoA was found to decrease pyruvate dehydrogenase allosterically and citrate was found to inhibit phosphofructokinase, an enzyme involved in glycolysis. This whole process came to be known as the glucose-fatty acid cycle or Randle cycle. More recently, the group of Dr. Robert Wolfe provided data to indicate the opposite phenomenon (Sidossis and Wolfe, 1996). Using a hyperinsulinemic-hyperglycemic clamp technique they found that elevated glucose concentrations inhibited fatty acid

oxidation. This effect might be due to increased intracellular malonyl-CoA levels. Malonyl-CoA is produced from acetyl-CoA and is the first step in fatty acid synthesis, i.e. de novo lipogenesis. Increased glycolysis produces more pyruvate leading to increased acetyl-CoA production, which in turn will lead to more malonyl-CoA. Malonyl-CoA is known for its inhibitory effect on carnitine-palmitoyl transferase 1, an enzyme catalyzing the binding of carnitine to long-chain fatty acids, a necessary step for entry into mitochondria and subsequent oxidation. Lipids and carbohydrates do not only influence each other in terms of oxidation but also in their synthetic processes. It has been known for some time that glucose is capable of promoting de novo lipogenesis (Groen et al., 2001). However, a high glucose intake probably does not promote hepatic synthesis of quantitatively important amounts of fatty acids in humans with a western dietary lifestyle (Hellerstein et al., 1996). It was found that the regulation of hepatic de novo lipogenesis is, at least partly, controlled by specific transcription factors. Evidence however shows that SREBP-1 and 2 can partially compensate each other, as SREBP-1 knockout mice showed elevated levels of SREBP-2 and increased cholesterol synthesis rates (Shimano et al., 1997). Glucose is able to induce lipogenesis indirectly by inducing insulin secretion. Insulin has long been known for its lipogenic activity (Beynen et al., 1979). Two groups separately found that insulin has an additional effect by enhancing SREBP-1c gene expression and the abundance of the protein in the endoplasmic reticulum (Moon et al., 1999; Shimomura et al., 1999; Azzout-Marniche et al., 2000). The carbohydrate responsive element binding protein (ChREBP) (Foufelle et al., 1998; Koo et al., 2001), is also involved in transcriptional regulation of lipogenesis. ChREBP is induced in situations characterized by high glucose concentrations (Koo et al., 2001; O'Callaghan et al., 2001; Yamashita et al., 2001). ChREBP itself was found to activate gene expression of both pyruvate kinase and acetyl-CoA carboxylase (Kawaguchi et al., 2001; Koo et al., 2001; O'Callaghan et al., 2001; Yamashita et al., 2001).

Hepatic very-low density lipoprotein (VLDL) secretion to plasma is also a process in which insulin is a primary factor. Insulin, after secretion in response to a rise in

plasma glucose concentration, regulates VLDL-triglyceride secretion, either directly by influencing the rate of apoB synthesis, or indirectly via its effect on the supply of FFA to the liver (Sparks and Sparks, 1994b; Aarsland et al., 1996; Sidossis et al., 1998). The acute effects of insulin on regulation of VLDL secretion differ from its chronic effects. Acutely, insulin inhibits hepatic VLDL secretion (Sparks and Sparks, 1994b), whereas chronic exposure to insulin has an stimulatory effect (Zammit et al., 1999). In addition to the regulation of lipid synthesis and secretion by carbohydrates and insulin, lipids might also promote gluconeogenesis. FFA stimulate hepatic glucose production. However, fasting, a situation with increased FFA availability, is well-known to inhibit hepatic glucose production (HGP) (Rothman et al., 1991; Neese et al., 1995).

Another level of metabolic regulation by FFA might be related to the transcription factor PPARα. PPARα has also been suggested to induce phosphoenolpyruvate carboxykinase PEPCK gene expression (Kersten et al., 1999). PPARα knockout mice suffer from fasting induced hypoglycemia, indicating a possible role in control of hepatic glucose production (Kersten et al., 1999). Apart from PPARα, evidence exists that other transcription factors are involved in regulation of glucose metabolism. Glucokinase expression is activated by hepatic nuclear factor-4α (HNF-4α) (Roth et al., 2002). Glucose, through activation phosphorylation/ dephosphorylation of ChREBP, influences transcription of pyruvate kinase (Kawaguchi et al., 2001). Glucose-6-phosphatase expression is also found to be mediated by transcriptional mechanisms as well as by breakdown of mRNA (Massillon, 2001).

In summary, transcriptional regulation is a form of metabolic regulation that is important for all metabolic routes of glucose. One must realize that it is likely that more transcription factors playing an important role in carbohydrate metabolism will be found in the future.

5.2. Pathophysiology of carbohydrate and lipid metabolism in diabetes

Diabetes means "excessive urination". The name diabetes mellitus was given to patients with excessive urine production in combination with a honey-flavored taste of the urine, caused by urinary glucose excretion. Diabetes mellitus today comprises a group of metabolic disorders characterized by chronic hyperglycemia. Currently, three types of diabetes mellitus are known: diabetes mellitus type 1, caused by an autoimmune-driven destruction of pancreatic β-cells; diabetes mellitus type 2 (DM2), or non-insulin dependent diabetes mellitus as it mistakenly is also known. The third group is called maturity-onset diabetes of the young (MODY), which is a group of genetic diseases caused by mutations in numerous genes such as glucokinase and insulin promoter factor 1 (McGarry, 2002). DM2 is the most common disorder, accounting for more than 90 percent of cases, whose incidence is still growing in the western world even in children. The development of DM2 is in almost all cases caused by an overconsumption of food in relation to the energy expenditure and has become an epidemic disease in western societies. The primary event leading to full-blown DM2 is the development of insulin resistance, although discussion remains. Fat accumulation in muscle, liver and other tissues have been thought to induce insulin resistance (McGarry, 2002). Some researchers consider defective insulin secretion by the pancreas, instead of insulin resistance, to be primary in the development of DM2 (Ferrannini, 1998). It is, however, clear that insulin resistance can precede clinically detectable DM2 by more than ten years (Lillioja et al., 1988), underscoring the importance of insulin resistance in the etiology of this disease. DM2 is associated with hyperglycemia and hyperlipidemia. Hyperinsulinemia occurs in the early stages of the disease when the pancreatic β-cells try to compensate for the insulin resistance by increasing insulin secretion. As the disease progresses, pancreatic β-cell failure develops giving rise to the full-blown DM2 phenotype.

DM2 is also characterized by hyperlipidemia, including hypercholesterolemia and

Hypertriglyceridemia (Yoshino et al., 1996). Increased levels of VLDL particles and small, dense LDL particles and decreased levels of HDL particles are commonly found (Reaven et al., 1993), giving rise to an atherogenic lipid profile.

Increased VLDL secretion might result from the decreased sensitivity to the inhibitory effects on this process of insulin directly as studies in animal models of diabetes and diabetic humans have shown (Lewis et al., 1993; Sparks and Sparks, 1994a; Bourgeois et al., 1995). Increased VLDL secretion in DM2 might also be caused by insulin indirectly through modulation of the supply of FFA to the liver. Increased FFA flux by modulation of hormone sensitive lipase, which is observed in insulin resistant states, has been suggested to enhance VLDL secretion by the liver. A number of studies have shown a diminished ability of insulin to suppress FFA rate of appearance in DM2 patients (Lewis et al., 2002). There is strong evidence that elevated FFA levels are associated with increased VLDL production in healthy humans (Lewis et al., 1995; Lewis, 1997). Overall, consensus practically exists that increased FFA flux to the liver is an important cause of overproduction of VLDL triglycerides by the liver in DM2. Decreased clearance of triglycerides from the blood in DM2 patients is related to impaired lipolysis of VLDL-triglycerides. Since this process is mediated by lipoprotein lipase, which is an insulin-senstivie enzyme, insulin resistance can lead to decreased levels of lipoprotein lipase. Multiple studies have shown decreased triglyceride clearance (Kissebah et al., 1982; Howard et al., 1983), although this has not been conclusive (Blades and Garg, 1995; Yost et al., 1995).

Objective of Study

Whole body cholesterol balance is regulated by the net effects of dietary cholesterol absorption, *de novo* cholesterol biosynthesis and biliary excretion from the liver (Davies and Ioannou, 2006; Kruit et al., 2006).

During the last decade, it has become clear that cellular cholesterol transport is a protein-mediated process, which in turn is regulated by certain nuclear receptors. Several transporters, including fatty acid translocase/cluster determinant 36 (FAT/CD36), scavenger receptor class B type I (SR-BI) and Niemann Pick C1-Like 1 (NPC1L1) influence cholesterol uptake. ATP Binding Cassette transporter family, including some cholesterol carriers such as ABCA1 and ABCG5/G8 act as efflux pumps facilitating cholesterol export out of absorptive cells.

In vitro studies undertaken to characterize intestinal lipid absorption have revealed relationships between glucose levels and lipid uptake. High extracellular glucose concentrations significantly increase brush border membrane fluidity and permeability at tight junctions in human intestinal mucosa (Komissarchik et al., 1993; D'Souza et al., 2003b). Those studies demonstrated that glucose may affect the transepithelial transport of nutrients (D'Souza et al., 2003a; D'Souza et al., 2003b).

Furthermore, *in vivo* studies confirm the hypothesis that there may be a regulation of intestinal lipid uptake by dietary glucose. An inverse relationship between glycemic load and HDL cholesterol was described (Liu et al., 2001; Slyper et al., 2005). It was reported that women who consume cholesterol with a low carbohydrate intake have lower concentrations of low density lipoprotein (LDL) than those with a high carbohydrate intake (Lofgren et al., 2005).

Increased cholesterol absorption has also been described in patients with diabetes mellitus. The prevalence of diabetes is increasing worldwide and coronary heart

disease (CHD) is the leading cause of death in type 2 diabetes mellitus (T2DM) (Gu et al., 1998). Patients with T2DM are two to three times more likely to die from CHD than non-diabetic individuals (Garcia et al., 1974; Stamler et al., 1993). Therefore, considerable attention has been focused on the dyslipidemia accompanying diabetes and metabolic syndrome. Exaggerated very-low density lipoprotein production in the liver represents a major pathway of the hypertriglyceridemia that characterizes the diabetic condition. On the other hand, a significant relationship exists between intestinally-derived triacylglycol (TG)-rich lipoproteins and the progression of atherosclerosis (Gylling et al., 2004; Lally et al., 2006).

Although numerous investigations have attempted to elucidate the abnormal mechanisms of intestinal cholesterol absorption process in diabetic dyslipidemia, they have not given full consideration to nutrients other than TG and cholesterol. However, changing the carbohydrate content of a mixed meal altered the postprandial accumulation of chylomicrons (Harbis et al., 2001). Furthermore, high glucose levels alter the expression of various genes involved in high-density lipoprotein (HDL) metabolism (Tu and Albers, 2001).

Accordingly, the main objective of this study was to evaluate the effect of glucose on the transport and metabolism of cholesterol in intestinal cells. The specific aims were to evaluate the effect of a high glucose level on i) cholesterol absorption processes; ii) the expression of proteins that may influence cholesterol uptake (SR-BI, NPC1L1, CD36) and those favoring cholesterol export (ABCA1, ABCG5/G8); iii) enzymes that control cholesterol homeostasis; and iv) the status of transcriptional factors involved in cholesterol regulation.

Establishing the relation between glucose levels and cholesterol transport and metabolism, may contribute to find new alternative therapeutic treatments improving the conditions of glucose and lipid related diseases such as obesity, coronary heart disease and diabetes mellitus among others.

Article

Modulation of intestinal cholesterol absorption by high glucose levels: impact on cholesterol transporters, regulatory enzymes and transcription factors

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Running Head: Regulation of intestinal cholesterol absorption by glucose

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ABBREVIATION LIST

ABC;	ATP Binding Cassette transporter
CE;	cholesteryl ester
CHD;	coronary heart disease
DMEM;	Dulbecco's Modified Eagle Medium
ER;	endoplasmic reticulum
FA;	fatty acid
FAT/CD36;	fatty acid translocase/cluster determinant 36
FBS;	fetal bovine serum
FC;	free cholesterol
HDL;	high-density lipoprotein
LDL-C;	low-density lipoprotein-cholesterol
LXR;	liver X receptors
NPC1L1;	Niemann Pick C1-Like1
PBS;	phosphate buffered saline
PPAR;	peroxisome proliferator-activated receptors
PPRE;	peroxisome proliferators response element
RXR;	retinoid X receptors
SR-BI;	scavenger receptor class B type I
SREBP-2;	sterol regulatory element binding protein-2
T2DM;	type 2 diabetes mellitus
TG;	triacylglycol

ABSTRACT

Growing evidence suggests that the small intestine may contribute to excessive postprandial lipemia, which is highly prevalent in insulin-resistant/type 2 diabetic individuals and substantially increases the risk of cardiovascular disease. The aim of the present study was to determine the role of high glucose levels on intestinal cholesterol absorption, cholesterol transporter expression, enzymes controlling cholesterol homeostasis and the status of transcription factors. To this end, we employed highly differentiated and polarized cells (20 days of culture), plated on permeable polycarbonate filters. In the presence of [^{14}C]-cholesterol, glucose at 25 mM stimulated cholesterol uptake compared to Caco-2/15 cells supplemented with 5 mM glucose ($p<0.04$). Because combination of 5 mM glucose with 20 mM of the structurally related mannitol or sorbitol did not change cholesterol uptake, we conclude that extracellular glucose concentration is uniquely involved in the regulation of intestinal cholesterol transport. The high concentration of glucose enhanced the protein expression of the critical cholesterol transporter NPC1L1 and that of CD36 ($p<0.02$) and concomitantly decreased SR-BI protein mass ($p<0.02$). No significant changes were observed in the protein expression of ABCA1 and ABCG8, which act as efflux pumps favoring cholesterol export out of absorptive cells. At the same time, HMG-CoA reductase activity was decreased ($p<0.007$), whereas ACAT activity remained unchanged. Finally, increases were noted in the transcription factors LXRα, LXRβ, PPARβ and PPARγ along with a drop in the protein expression of SREBP-2. Collectively, our data indicate that glucose at high concentrations may regulate intestinal cholesterol transport and metabolism in Caco-2/15 cells, thus suggesting a potential influence on the cholesterol absorption process in type 2 diabetes.

Key words: ABCA1, ABCG5/G8, SR-BI, CD36, NPC1L1, PPAR, LXR, SREBP, ACAT and HMG-CoA reductase

INTRODUCTION

Elevated plasma cholesterol levels constitute a major risk factor for atherosclerosis and coronary heart diseases (CHD) (60). Whole body cholesterol balance is regulated by the net effects of dietary cholesterol absorption, *de novo* cholesterol biosynthesis and biliary excretion from the liver (21, 49). Available evidence supports the concept that several proteins are involved in mediating intestinal cholesterol transport. While various transporters, including fatty acid translocase/cluster determinant 36 (FAT/CD36), scavenger receptor class B type I (SR-BI) and Niemann Pick C1-Like1 (NPC1L1) may influence cholesterol uptake, the ATP Binding Cassette transporter family, including several cholesterol carriers (ABCA1, ABCB1, ABCG5/G8), act as efflux pumps favouring cholesterol export out of absorptive cells into the lumen or basolateral compartment. Among all the cholesterol transporters, the enriched NPC1L1 protein in the apical membrane of polarized cells is considered essential for intestinal cholesterol absorption. To provide only a few examples, (i) mice deficient in NPC1L1 lack the ability to absorb cholesterol and exhibit prevailing protection against the rise in plasma and hepatic cholesterol associated with feeding mice high cholesterol diets (5, 22, 23); (ii) genetic modifications of NPC1L1 in cultured intestinal cells alter cholesterol uptake (73, 88, 90); and (iii) variations in NPC1L1 were found associated with reduced sterol absorption, low-density lipoprotein-cholesterol (LDL-C) levels (17) and LDL-C response to ezetimibe therapy (41, 75, 84). Although, various aspects of NPC1L1, as a cell surface transporter or an intracellular cholesterol transport protein needs clarification (43), intensive research is focused on drugs that interact with NPC1L1 given their potential to treat individuals with hypercholesterolemia and to reduce their risk of developing CHD.

The prevalence of diabetes is increasing worldwide and CHD is the leading cause of death in type 2 diabetes mellitus (T2DM) (35). Patients with T2DM are two to three times more likely to die from CHD than non-diabetic individuals (31, 77). Therefore, considerable attention has been focused on the dyslipidemia

accompanying diabetes and metabolic syndrome. Elevated liver very-low density lipoprotein production represents a major pathway of the hypertriglyceridemia that characterizes the diabetic condition. On the other hand, a significant relationship has been shown between intestinally-derived triacylglycol (TG)-rich lipoproteins and the progression of atherosclerosis. Increased cholesterol absorption has also been described in patients with T2DM (36, 52). Although numerous investigations have attempted to elucidate the abnormal mechanisms of intestinal cholesterol absorption process in diabetic dyslipidemia, they have not given full consideration of nutrients other than TG and cholesterol. However, it has been reported that changing the carbohydrate content of a mixed meal altered the postprandial accumulation of chylomicrons (37). Furthermore, a high glucose level alters the genetic expression of various genes involved in high-density lipoprotein (HDL) metabolism in HepG2 cells, including human ABCA1, SR-BI and hepatic lipase (79). The aims of the present study were 1) to evaluate the effect of a high glucose concentration on cholesterol absorption and 2) to explore the influence of an elevated glucose level on the expression of genes regulating cholesterol synthesis and absorption in a cell culture system.

MATERIALS AND METHODS

Cell culture

The Caco-2/15 cell line was obtained from Dr. JF Beaulieu (Department of Cellular Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada). This clone of the parent Caco-2 cell line (HTB37; American Type Culture Collection, Manassas, VA) has been extensively characterized (3, 6, 80) and was originally selected for expressing the highest level of sucrase-isomaltase among 16 clones obtained by random cloning. Caco-2/15 cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% Glutamax (GIBCO-BRL) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Flow, McLean, VA). Caco-2/15 cells (passages 40–60) were maintained in T-75-cm^2 flasks (Corning Glass Works, Corning, NY). Cultures were split (1:6) when they reached 70-90% confluence, by use of 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). For individual experiments, cells were plated at a density of 1 x 10^6 cells/well on 24.5-mm polycarbonate Transwell filter inserts with 0.4-μm pores (Costar, Cambridge, MA), in DMEM (as described above) supplemented with 5% FBS. The inserts were placed into six-well culture plates, permitting separate access to the upper and lower compartments of the monolayers. Cells were cultured for various periods, including 21 days, at which the Caco-2 cells are highly differentiated and appropriate for lipid metabolism (34, 54, 55, 61, 73). The medium was refreshed every second day. At day 21, Caco-2/15 cells were washed twice with phosphate buffered saline (PBS) (Invitrogen) and incubated in a serum-free supplemented DMEM (Invitrogen) (5 mM or 25 mM glucose), added to the apical compartment, for 24 h.

Cholesterol absorption by Caco-2/15 cells

To study cholesterol uptake by the cells, a solution containing 0.113 μCi [^{14}C]-cholesterol and 100 μM cholesterol bound to albumin was prepared. The differentiated cells were incubated at 37°C for 30 min and 4 h in DMEM containing 5 or 25 mM glucose, as well as cholesterol solution. At the end of the treatment,

cells were washed twice with PBS, scrapped in 1 ml lysis buffer (5mM Tris, 15 mM NaCl, EDTA 5 mM, 0.1% SDS, 1% Triton X 100, 0.5% sodium deoxycholate) and homogenized by sonication followed by a 5-min at 13800 g centrifugation to remove cell debris. An aliquot of 0.1 ml was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was measured by scintillation counting (LS 5000 TD, Beckman). Cell protein was quantified by the Bradford method (BioRad).

Acyl-Coenzyme A: cholesterol acyltransferase (ACAT) activity assay

The activity of ACAT was determined at initial rates by adding 5 nmol of [^{14}C]-oleoyl-CoA (specific activity ~167 Bq/nmol) to the mixture containing 190 μg of cellular protein to initiate the reaction in a buffer solution (pH 7.5) consisting of cholesterol, 0.04 M KH_2PO_4, 50 mM NaF, 0.25 M sucrose, and 1 mM EDTA (73). After incubation for 10 min at 37°C, the reaction was stopped by adding chloroform-methanol (2:1, v/v) followed by free cholesterol (FC) and cholesteryl ester (CE) as carriers. The FC and CE formed were isolated by TLC and counted.

HMG-CoA reductase activity assay

Enzymatic activity was assayed as described previously (19, 73). The reaction mixture contained 100 mM potassium phosphate (pH 7.4), 200 μg of cellular protein, 20 mM glucose-6-phosphate, 12.5 mM dithiothreitol, 2.5 M NADP, and 1.2 units of glucose-6-phosphate dehydrogenase. Initiation of the reaction was done by the addition of [^{14}C]-HMG-CoA (200 Bq/nmol) for 30 min at 37°C. The [^{14}C]-mevalonate formed was converted into lactone by the addition of 10 N HCl, isolated by TLC, and counted using an internal standard to correct for incomplete recovery.

Western Blot

To assess the presence of NPC1L1, SR-B1, CD36, ABCA1, ABCG8, ACAT, and HMG-CoA reductase, Caco-2/15 cells were homogenized and prepared for Western blotting as described previously (56). The Bradford assay (Bio-Rad) was used to determine protein concentration. Proteins were denatured in sample buffer

containing SDS and β-mercaptoethanol, separated on a 7.5% SDS-PAGE gel, and blotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked using 5% defatted milk proteins. Reactions took place by the addition of primary antibodies directed against targeted proteins. Reaction was revealed with species-specific horseradish peroxidase-conjugated secondary antibody. β-actin was used as an internal control to confirm equal loading protein on SDS-PAGE. Blots were developed and proteins were quantified using a Hewlett-Packard scanner equipped with a transparency adaptor and UN-SCAN-IT (Silk Scientific Corporation) software.

Immunocytochemical analysis

Caco-2/15 cells grown for 21 days and exposed to either low (5 mM) or high (25 mM) glucose medium were fixed with 1% glutaraldehyde in 0,1 M phosphate buffer for 2 hours and processed for embedding in Lowicryl at -30°C as described in details previously (7, 55, 58, 76). Thin sections were mounted on Parlodion-carbon coated grids and processed for the immunogold labeling. Various proteins were studied, namely SR-B1, NPC1L1, ABCA1, ABCG8 and CD36. The thin sections of the cells were first treated with a saturated solution of sodium metaperiodate for 10 min, followed by 1 % ovalbumin and then incubated overnight at 4°C with the corresponding antibody. Grids were thoroughly rinsed with PBS and incubated with the protein A-gold or an anti-rabbit IgG-gold complex for 30 min at room temperature. Upon counterstaining with uranyl acetate, the sections were examined with a Philips 410 electron microscope. The antibodies were used at the following dilutions NPC1L1 at 1:10; SRB1 at 1:50; CD36 at 1:10; ABCA1 at 1:10 and ABCG8 at 1:10. In order to assess specificity on the labeling, control experiments were performed omitting the incubation with the primary antibody. Grids were only exposed to the protein A-gold or the anti-rabbit IgG-gold complex for 30 minutes. For morphometrical evaluations, a large number of photographs were recorded at the original magnification of x14000; they were scanned and printed to the final magnification of x28000. The specific membrane domain was selected for morphometrical evaluation according to the specific localization of the transporters,

i.e. the apical membrane with its large number of microvilli or the basolateral membrane with its deep invaginations. First, the length of the membrane was measured and then the number of gold particles delineating the same membranes was counted. Results are expressed in number of gold particles per μm (mean values ± SD). An image processing system (Videoplan 2, Carl Zeiss Inc. Toronto) was used. For each of the experiments and for each protein studied, the length of apical membrane evaluated was in the range of ~800 μm, while that of the basolateral membrane was in the range of ~250 μm. The major difference in membrane length evaluated between apical and basolateral membranes is due to the presence of the large number of microvilli in the apical membrane. Morphometrical evaluations were also performed on the control experiments.

RNA isolation

Total RNA was isolated from Caco-2/15 cells using the TRIzol reagent according to the manufacturer's instructions (Sigma Chemical Co.). Concentrations of RNA were determined by spectrophotometer analysis and the integrity of total RNA was assessed by electrophoresis.

RT-PCR

PCR experiments for transcription factors (LXRs, RXRs, PPARs, SREBP-2) genes, ACAT, and HMG-CoA reductase, as well as GAPDH (as a control gene) were performed using the mastercycler gradient (EPPENDORF®). Specific primers were designed to bind to regions with minimal homology, to span at least 1 intron for distinction from genomic DNA and to avoid nonspecific annealing (Table 1). All primers were Blast searched to confirm specificity for each individual isoform. Approximately 30-40 cycles of amplification were used at 95°C for 30 s, 53-62°C for 30 s, and 72°C for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. For all RT-PCRs, analysis of mRNA expression was carried out during the exponential phase of the amplification, which was assessed in preliminary experiments for each pair of primers.

Statistical analysis

Unless otherwise stated, all values are given as mean values ± SD. Data were assessed by Student's two-tailed *t*-test. A p value <0.05 was considered statistically significant.

RESULTS

Cholesterol absorption

Following pre-incubation (24 h) of Caco-2/15 cells with medium containing 5 or 25 mM glucose, cholesterol uptake was determined at short-and long-term incubation times. As illustrated by Figure 1, cells exposed to 25 mM glucose displayed a higher capacity to incorporate cholesterol compared with cells treated with 5 mM glucose ($p<0.04$). Furthermore, compared to control cells, the output of cholesterol at 6 h (23%, $p<0.04$) was also augmented at the long term incubation time (data not shown).

To determine whether the influence of glucose on cholesterol transport could possibly be explained by the difference in the osmolarity of the 5 and 25 mM glucose solutions, we incubated Caco-2/15 cells with mannitol and sorbitol combined to 5 mM glucose. The cholesterol uptake of cell monolayers maintained in 5 mM glucose plus 20 mM mannitol or sorbitol did not differ from cells maintained in physiological glucose (5 mM) media (Figure 2). We, therefore, conclude that extracellular glucose concentration is uniquely involved in the regulation of intestinal cholesterol transport.

Protein expression of cholesterol transporters assessed by Western blot

The enhanced cholesterol uptake exhibited by Caco-2/15 cells incubated with the high concentration (25 mM) of glucose may be due to differences in the expression of cholesterol transporters. To test this hypothesis, the protein expression of cholesterol transporters present in intestinal epithelial cells was examined. We assessed NPC1L1, CD36 and SR-B1 that transport cholesterol into the enterocyte, as well as ABCA1 and ABCG8 that are presumed to be involved in cholesterol efflux from the enterocyte toward plasma HDL or back into the intestinal lumen, respectively. Exposure to 25 mM glucose as compared to 5 mM glucose resulted in a significant increase in the protein expression of NPC1L1 and CD36 along with a decrease in the protein expression of SR-B1 (Figure 3). On the other hand, the

protein expression of both ABCA1 and ABCG8 was not affected by the different glucose concentrations (Figure 4).

Protein levels of cholesterol transporters assessed by high-resolution quantitative immunogold approach.

Since Western blotting measures the total protein mass without being able to distinguish the cellular localization of cholesterol transporters, we employed the Protein A-gold immunocytochemical technique, to determine whether alterations in cholesterol transporters, as a function of glucose concentrations, were associated with their specific membrane domain. Electron microscopic immunocytochemical experiments mostly confirmed the findings obtained by Western blot. They revealed significant increases in immunogold labellings for NPC1L1 and CD36 in the luminal region of enterocytes, particularly associated with the apical plasma membrane lined by the microvilli (Figure 5). A representative illustration documents the immunochemical detection of CD36 in Caco-2/15 cells following exposure to different glucose levels (panel A: 5mM and panel B: 25 mM) (Figure 6). However, the labelling of ABCA1 by gold particles was decreased in the basolateral membrane following the addition of 25 mM glucose (Figure 5). Furthermore, no significant alterations were noted in the intensity of labelling of SR-BI and ABCG8 in the apical membrane (Figure 5). Importantly, under control conditions, the labeling was negligible and the few gold particles present over the cells were rather randomly distributed.

Involvement of NPC1L1 in cholesterol uptake

In order to elucidate the specific contribution of NPC1L1 to cholesterol uptake in the presence of glucose, we employed ezetimibe, a selective hypocholesterolemic drug, which has been reported to bind NPC1L1 and substantially block cholesterol absorption. We could observe a lessened action of glucose when ezetimibe was added to the culture medium (Figure 7), highlighting the input of NPC1L1.

Regulatory enzymes of cholesterol metabolism

Next, we determined the impact of glucose on the regulatory sterol enzymes: HMG-CoA reductase (EC 1.1.1.34), the rate-limiting step in cholesterol synthesis, and ACAT (EC 2.3.1.26), an integral protein present in the rough endoplasmic reticulum (ER) that catalyzes the formation of CE from FC and fatty acyl-CoA. HMG-CoA reductase mRNA and protein expression remained unchanged following increase of glucose to 25 mM glucose to Caco-2/15 cells (Figure 8). On the other hand, the higher glucose concentration led to a significant reduction in the enzymatic activity of HMG-CoA reductase (Figure 8). As to ACAT, no significant changes were recorded in the gene expression and activity after the exposure to 25 mM glucose (Figure 9).

Transcription factors

To approach the mechanisms triggered by glucose, we assessed the gene expression of several factors that affect the transcription of a variety of genes associated with lipid and cholesterol metabolism, including liver X receptors (LXRα, β), peroxisome proliferator-activated receptors (PPARα, β, γ), retinoid X receptors (RXRα, β), and protein and gene expression of sterol regulatory element binding protein-2 (SREBP-2). Data on Figures 10 to 12 illustrate how glucose at the high concentration of 25 mM impacted on the expression of the different nuclear and transcription factors in Caco-2/15 cells. It did not cause any significant variation on the mRNA levels of RXRα (Figure 10C), RXRβ (Figure 10D), PPARα (Figure 11A), and SREBP-2 (Figure 12A) gene expression, whereas it produced a significant enhancement in gene expression of LXRα (Figure 10A) and LXRβ (Figure 10B), as well as PPARβ (Figure 11B) and PPARγ (Figure 11C). Finally, when we explored the effect of glucose on SREBP-2 protein expression, we detected a significant reduction upon exposure to 25 mM glucose (Figure 12B).

DISCUSSION

Numerous studies have dealt with the regulation of intestinal fat absorption by lipid components (27, 57, 61, 70). However, the role of carbohydrates has barely been investigated. In the present paper, we showed that high glucose concentrations (i) enhance cholesterol transport in Caco-2/15 cells by upregulating the protein expression of NPC1L1 and CD36; and (ii) reduce SR-BI protein mass and HMG-CoA reductase activity without altering ABCA1 and ABCG8, involved in cholesterol efflux. A schematic diagram (Figure 13) depicts the major players in cholesterol transport and metabolism. Moreover, our studies document that particular transcription factors are glucose sensors, which may explain the impact of glucose on cholesterol absorption via its action on specific cholesterol transporters.
In the present study, we have found a relationship between glucose and cholesterol assimilation. In order to clarify whether this observation could be explained by differences in the osmolarity of the 5 and 25 mM glucose solutions, we incubated Caco-2/15 cells with 20 mM mannitol or sorbitol combined to 5 mM glucose. Our results indicate that the exposure of Caco-2/15 cells to high concentrations of glucose, but not to structurally related compounds such as mannitol and sorbitol, increased cholesterol transport capacity.

NPC1L1 is a critical protein for cholesterol absorption by the small intestine, since NPC1L1 knockout mice exhibited a reduction in intestinal cholesterol absorption and are insensitive to ezetimibe (5, 23), a drug that lowers serum cholesterol by reducing cholesterol absorption. In addition, the use of genetically-modified intestinal epithelial cells and ezetimibe support the central role for NPC1L1 in intestinal cholesterol absorption (28, 31, 73, 90). The high glucose level used in our investigation raised not only cholesterol uptake but also the protein expression of NPC1L1. Our results are in line with the findings in diabetic patients who displayed increased levels of NPC1L1 mRNA in intestinal tissue (52). Increased cholesterol absorption has also been shown in streptozotocin diabetic rats (89), in which NPC1L1 mRNA was found to be increased (51). Altogether, these findings suggest

an important role for intestinal NPC1L1 in the delivery of cholesterol to the blood circulation in the presence of high glucose levels. However, additional studies are needed to examine whether glucose may influence the absorption of cholesterol via non-mediated passive uptake of cholesterol or other intestinal transporters since residual cholesterol absorption persisted in NPC1L1-deficient mice (5).

In the present investigation, ,the protein CD36 was highly expressed in intestinal luminal surface of enterocytes (82) and was found to be raised upon exposure to high glucose levels. Undoubtedly, CD36 does contribute to the intestinal transport of cholesterol since enterocytes isolated from *Cd36*$^{-/-}$ mice exhibit reduced uptake of cholesterol (60%) (68). From the present experiments, we can deduce that elevated glucose-mediated cholesterol uptake is likely related to the up-regulation of NPC1L1 and CD36. Interestingly, the participation of NPC1L1 and CD36 was reinforced by the experiments with ezetimibe, although the former displayed more sensitivity to ezetimibe inhibition (32) than the latter (82). Of note is the modest decrease in cholesterol uptake from the apical side, in line with the studies of Field et al. (23) probably because the glucuronidated form of ezetimibe is more potent than the native unmodified drug in inhibiting cholesterol absorption by binding more avidly to enterocyte brush-border membranes (83).

SR-BI was originally identified as a novel scavenger receptor that mediates endocytosis of acetylated LDL (2). Subsequent studies revealed that SR-BI is a cell surface receptor that binds HDL with high affinity and mediates the selective uptake by liver and steroidogenic tissues of cholesterol esters without endocytic uptake of HDL apolipoproteins (1). Efflux of radiolabeled cholesterol on the cell surface to HDL particles is also promoted by SR-BI (45). Together, SR-BI accelerates reverse cholesterol transport by promoting cholesterol efflux from peripheral cells, including macrophages in vascular walls (16), and selective uptake of HDL-C by hepatocytes for excretion of cholesterol as bile acids. Therefore, SR-BI plays crucial roles in the atheroprotective functions of HDL (48). Additional investigations have reported that SR-BI is highly expressed in the luminal side of proximal small

intestine villi where the bulk of cholesterol absorption takes place and may be responsible for the cholesterol uptake by enterocytes (4, 39, 55). Nevertheless, the involvement of SR-BI in cholesterol absorption has been questioned since variable results were obtained with genetically-modified mice (8, 62). In the present investigation, glucose in high concentrations downregulates SR-BI protein expression. This suppressive effect has also been reported in hepatocytes HepG2 cells following exposure to high glucose concentrations (67). The use of inhibitors for select signal transduction pathways in HepG2 cells indicated that glucose suppression of SR-BI expression is partially mediated by the activation of the p38 MAPK-Sp1 pathway (67). Further studies are needed to determine the detailed regulatory mechanisms of intestinal SR-BI expression.

We reasonably hypothesized that increased NPC1L1- and CD36-mediated cholesterol uptake would lead to reduced HMG-CoA reductase activity. Based on the data in Figure 8, this assumption turned out to be true. However, ACAT was insensitive to the accumulation of intracellular cholesterol, probably because the latter did not expand the finite cholesterol substrate pool for ACAT and manifested a high-order dependence on ER cholesterol concentration (11, 14).

The coordinated regulation of genes implicated in cholesterol homeostasis is governed by the actions of several transcription factors, such as LXRs and PPARs. Also, SREBPs are transcription factors and crucial regulators of cholesterol synthesis and metabolism. In response to specific effectors, LXRs form heterodimers with RXRs that regulate an integrated network of genes that control whole body cholesterol and lipid homeostasis assays (44, 53). In particular, LXR appears to serve as a safety valve to limit free cholesterol in tissues that are experiencing high cholesterol flux (20). Moreover, glucose activates LXR at physiological concentrations expected in the liver and induces expression of LXR target genes with efficacy similar to that of oxysterols, the known LXR ligands (66).

Therefore, since these nuclear factors act as glucose sensors (66) and exhibit anti-diabetic effects (13, 50), we first measured their gene expression. LXRα and LXRβ mRNA were increased by the presence of 25 mM glucose in Caco-2/15 cells, but were not accompanied with the expected induction of ABCA1 and ABCG8 protein expression. This may be due to the irresponsiveness of RXRs that work as partners with LXRs.

The family of SREBP regulates the coordinated expression of genes involved in lipid synthesis and uptake (10). Three SREBP isoforms are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1c preferentially activates genes required for fatty acid (FA) synthesis and their incorporation into TGs and phospholipids, SREBP-2 preferentially activates the LDL- receptor gene and various genes required for cholesterol synthesis such as HMG-CoA reductase (EC1.1.1.34) (42). SREBP-1a is an activator of both, the cholesterol and FA biosynthetic pathway, but it is present in much lower amounts (74). Since SREBP-2 plays a more important role in the regulation of cholesterol synthesis in the intestine (25, 26), we measured the gene and protein expression of SREBP-2. Our results highlighted a decline in the protein expression of SREBP-2 without any alteration of SREBP-2 mRNA. Similarly, the gene and protein expression of HMG-CoA reductase were not changed, but the activity was decreased, suggesting post-transcriptional mechanisms for SREBP-2 and HMG-CoA reductase.

PPARs have been shown to regulate the expression of genes involved in a variety of biological processes, including lipid metabolism and insulin sensitivity (18, 81). PPARα regulates numerous aspects of FA catabolism, whereas PPARγ controls adipocyte differentiation, systemic glucose levels and lipid homeostasis (65, 87). PPARδ is also involved in development, lipid metabolism, and epidermal cell proliferation (59). The PPARs are ligand-dependent transcription factors that regulate target genes expression by binding to characteristic DNA sequences termed peroxisome proliferators response element (PPREs) located in the 5′ -flanking region of target genes (33, 69). Each receptor binds to its PPRE as a heterodimer

with the receptor for 9-cis retinoic acid, the RXR. Upon binding a ligand, the conformation of a PPAR is altered and stabilized so that a binding cleft is created, and recruitment of transcriptional coactivators occurs. In the present study, treatment of Caco-2/15 cells with 25 mM glucose enhanced the gene expression of PPARβ and PPARγ and concomitantly decreased the protein concentration of SREBP-2. Similarly, PPARγ activation by troglitazone downregulated cholesterol synthesis in Caco-2 cells by a reduced concentration of SREBP-2 protein (47).

Glucose absorption in the small intestine is mediated by the combined action of two glucose transporters in the enterocytes: the sodium-dependent D-glucose co transporter SGLT1 in the brush-border membrane and the sodium-independent glucose transporter GLUT2 in the basolateral membrane (40, 46). Dietary glucose significantly influences blood glucose concentration (63), as well as glucose and fat metabolism in the liver (15, 78). Apparently mammals developed a highly complex regulatory network in which small intestinal sugar absorption steers the regulation of gastric and intestinal mobility (30, 71, 72), liver metabolism (9, 29) and insulin secretion (64, 86). Both, the glucose concentration in the small intestinal lumen and the activity of glucose uptake systems SGLT1 and GLUT2, determine the D-glucose concentration in the small intestinal submucosa and the portal blood. In the present study, glucose was effective in regulating cholesterol uptake and intracellular processing. Since the excessive consumption of diets containing high levels of carbohydrates enhances the absorption of monosaccharides and influences the risk of developing insulin resistance and T2DM (24), we reasonably propose that glucose-mediated intestinal cholesterol may contribute to increasing circulating cholesterol and, consequently, the risk of developing CHD, a feature of T2DM.

In conclusion, our experiments provide evidence that the process of intestinal cholesterol uptake is regulated by glucose concentrations, which modify important cholesterol transporters and transcription factors. In fact, high glucose concentrations may presumably modify the transcription factors, which in turn altered the cholesterol transporters, and therefore cholesterol uptake.

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TABLE 1. Sequences of the specific primers, as confirmed by BLAST sequence analysis, designed to amplify human HMG-CoA reductase, ACAT-2, LXRs, PPARs, RXRs and SREBP-2 isoforms by RT-PCR.

Isoform	Direction	5'-3' Primer sequence	Product size (bp)	Reference
hHMGCoA-R	forward	ACC CTT AGT GGC TGA AAC AGA TAC CC	291	designed
	reverse	AAC TGT CGG CGA ATA GAT ACA CCA CG		
hACAT-2	forward	CTC TAC TTC CTC TTC TGC CC	128	designed
	reverse	GAT GAA GCA GGC ATA GAG CA		
hLXRα	forward	GCT GCA AGT GGA ATT CAT CAA CC	166	(12)
	reverse	ATA TGT GTG CTG CAG CCT CTC CA		
hLXRβ	forward	GGA GCT GGC CAT CAT CTC A	132	designed
	reverse	GTC TCT AGC AGC ATG ATC TCG ATA GT		
hPPARα	forward	CCA GGC TTC GCA AAC TT	591	designed
	reverse	CCC GTC TCC TTT GTA GTG CT		
hPPARβ	forward	GCT TTG TCA CCC GTG AGT T	354	designed
	reverse	AGG TCT CGG TTT CGG TCT TC		
hPPARγ	forward	AGA CAA CAG ACA AAT CAC CAT	400	(91)
	reverse	CTT CAC AGC AAA CTC AAA CTT		
hRXRα	forward	ATA AGC ATC ACA TTT TGG GG	417	(85)
	reverse	GAC ATG CAG ATG GAC AAG T		
hRXRβ	forward	ATT AAC TCA ACA GTG TCA CTC CC	503	(85)
	reverse	TTA GTC ACA GGG TCA TTT GG		
hSREBP-2	forward	ATG GTG TGA TTG TCC TGA GCG TCT	341	designed
	reverse	TTC GTC TTC AAA GCC TGC CTC AGT		
hGAPDH	forward	GTC CAC TGG CGT GTT CAC CA	260	(38)
	reverse	GTG GCA GTG ATG GCA TGG AC		

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FIGURE LEGENDS

Figure 1 Effect of glucose concentrations on cholesterol uptake in Caco-2/15 cells. Differentiated Caco 2/15 cells were cultured for 24 h in medium DMEM containing 5 or 25 mM glucose. At the end of this pre-incubation, cells were exposed to 100 μM cholesterol containing 250000 DPM [^{14}C]-cholesterol in the presence of the same concentrations of glucose for 30 min. Results are expressed as nmol/mg cell protein. Mean values ± SD. n=4. *p<0.04 versus 5 mM glucose condition.

Figure 2 Combinatory effect of glucose and mannitol or sorbitol on cholesterol uptake in Caco-2/15 cells. Differentiated Caco 2/15 cells were cultured for 24 h in medium DMEM containing 5 mM, 5 mM glucose + 20 mM mannitol, 5 mM glucose + 20 mM sorbitol, or 25 mM glucose. At the end of this pre-incubation, cells were exposed to 100 μM cholesterol containing 250000 DPM [^{14}C]-cholesterol in the presence of the same concentrations of sugars for 30 min. Results are expressed as nmol/mg cell protein. Mean values ± SD. n=4. *p<0.04 versus 5 mM glucose condition.

Figure 3 Effect of glucose concentrations on the protein expression of transporters mediating cholesterol influx. Caco 2/15 cells were cultured for 24 h in DMEM containing 5 or 25 mM glucose. Western blot was used to analyze the protein expression of NPC1L1 (A), CD36 (B) and SR-B1 (C). Mean values ± SD. n=4. *p<0.02 versus 5 mM glucose condition.

Figure 4 Effect of glucose concentrations on the protein expression of transporters mediating cholesterol efflux. Caco 2/15 cells were cultured for 24 h in DMEM containing 5 or 25 mM glucose. Western blot was used to analyze the protein expression of ABCA1 (A) and ABCG8 (B). Mean values± SD. n=4.

Figure 5 Immunocytochemical evaluation of cholesterol transporters following incubation of Caco 2/15 cells were cultured for 24 h in DMEM containing 5 or 25mM. Thereafter, cells were fixed with 1% glutaraldehyde and embedded in Lowicryl. Immunogold labelings were carried out on thin sections. Immunogold labeling for NPC1L1 (A), CD36 (B), SR-B1 (C), ABCA1 (D) and ABCG8 (E) were quantified. For each of the proteins, the total length of apical and basolateral membranes evaluated was in the range of 800 μm and 250 μm, respectively. Under control conditions, the labeling was negligible with few gold particles randomly distributed over the cells. For the 5 and 25 mM glucose experiments, the control protein A-gold density (gold particles/μm) on apical membrane was 0.037± 0.006 and 0.023 ± 0.012, respectively, and on basolateral region: 0.076 ± 0.048 and 0.063 ± 0.012, respectively. For the control IgG gold, the density (gold particles/μm) on the apical membrane was 0.021 ± 0.005 and 0.015 ± 0.007 for 5 and 25 mM glucose, respectively, and on basolateral membrane 0.037 ± 0.018 and 0.050 ± 0.015, respectively. Mean values ± SEM. *p<0.05

Figure 6 CD36 detection in Caco-2/15 cells as a representative illustration of the immunocytochemical detection. Protein A-gold immunocytochemical technique was applied with the specific polyclonal antibody directed against CD36 to reveal it in the apical membrane. Panel A represents Caco-2/15 cells cultured with 5 mM glucose, whereas Panel B corresponds to Caco-2/15 cells cultured with 25 mM glucose. MV, microvilli. Bars= 0.5μm.

Figure 7 Interference of Ezetimibe with the uptake of cholesterol in the presence of glucose. In order to delineate the role of NPC1L1 in the increased glucose-mediated cholesterol absorption, ezetimibe (100 μM) was added to the medium of Caco-2/15 cells cultured at the experimental conditions described in the legend of Figure 3. Results

are expressed as % of controls. Mean values ± SD. n=3. *p<0.05 versus 5 mM glucose condition.

Figure 8 Effect of glucose concentrations on HMG-CoA reductase gene expression, protein mass and activity. Caco 2/15 cells were treated as described in the legend of Figure 3 and tested for transcript levels (A) and protein mass (B) by RT-PCR and Western blotting, respectively. Cell homogenates were assayed for HMG-CoA reductase activity (C). Mean values ± SD. n=4.

*p<0.007 versus 5 mM glucose condition.

Figure 9 Effect of glucose concentrations on ACAT gene expression and activity. Caco 2/15 cells were treated as described in the legend of Figure 3 and tested for transcript levels (A) by RT-PCR. Cell homogenates were assayed for ACAT activity (B). Mean values ± SD. n=4.

Figure 10 Effect of glucose concentrations on the gene expression of the nuclear receptors LXR and RXR. Caco 2/15 cells were treated as described in the legend of Figure 3. The transcript levels of LXRα (A), LXRβ (B), RXRα (C) and RXRβ (D) were assessed by RT-PCR. Representative autoradiograms of the different amplicons are shown. Mean ± SD. n=5. *p<0.004 and **p<0.001 versus 5 mM glucose condition.

Figure 11 Effect of glucose concentrations on the gene expression of the nuclear receptors PPAR. Caco 2/15 cells were treated as described in the legend of Figure 3. The transcripts of PPARα (A), PPARβ (B) and PPARγ (C) were assessed by RT-PCR. Representative autoradiograms of the different amplicons are shown. Mean ± SD. n=5.

*p<0.003 and **p<0.002 versus 5 mM glucose condition.

Figure 12 Effect of glucose concentrations on the gene and protein expression of SREBP-2. Caco 2/15 cells were treated as described in the legend of Figure 3. The levels of transcripts (A) and protein expression (B)

were determined by RT-PCR and Western blotting, respectively. Mean ± SD. n=5. *p<0.04 versus 5 mM glucose condition.

Figure 13 Diagram of the main players influencing cholesterol transport in intestinal epithelial cells. Uptake of alimentary or biliary cholesterol is mediated by putative sterol transporters such as NPC1L1, SR-BI and FAT/CD36. Excessive cholesterol is secreted back to the intestinal lumen by the ABCG5/ABCG8 heterodimer localized at the apical membrane of the enterocyte. Similarly, ABCA1 promotes cholesterol efflux (through the basolateral membrane) to plasma apolipoprotein A-I, thereby enhancing the formation of nascent HDL. Key enzymes such as HMG-CoA reductase and ACAT contribute to cholesterol homeostasis by synthesizing and esterifying, respectively, intracellular cholesterol. Most of the processes, involved in cholesterol metabolism, are controlled by transcription factors (RXR, LXR, SREBP-2 and PPAR. According to our data, glucose-mediated cholesterol uptake may be sensed by transcription factors, which in turn altered the cholesterol transporters and downregulated cholesterol biosynthesis.

Figure 1

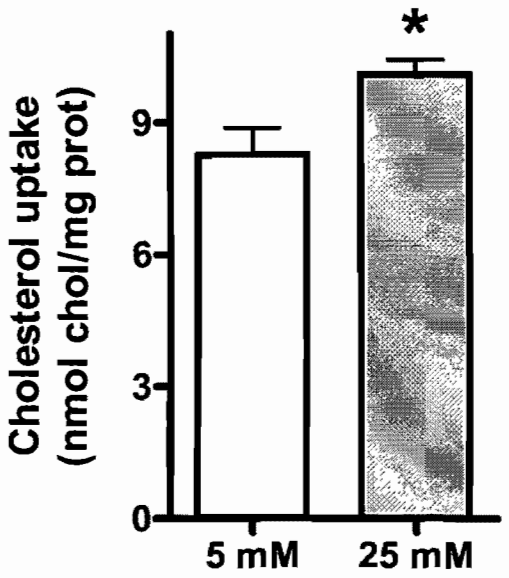


Figure 2

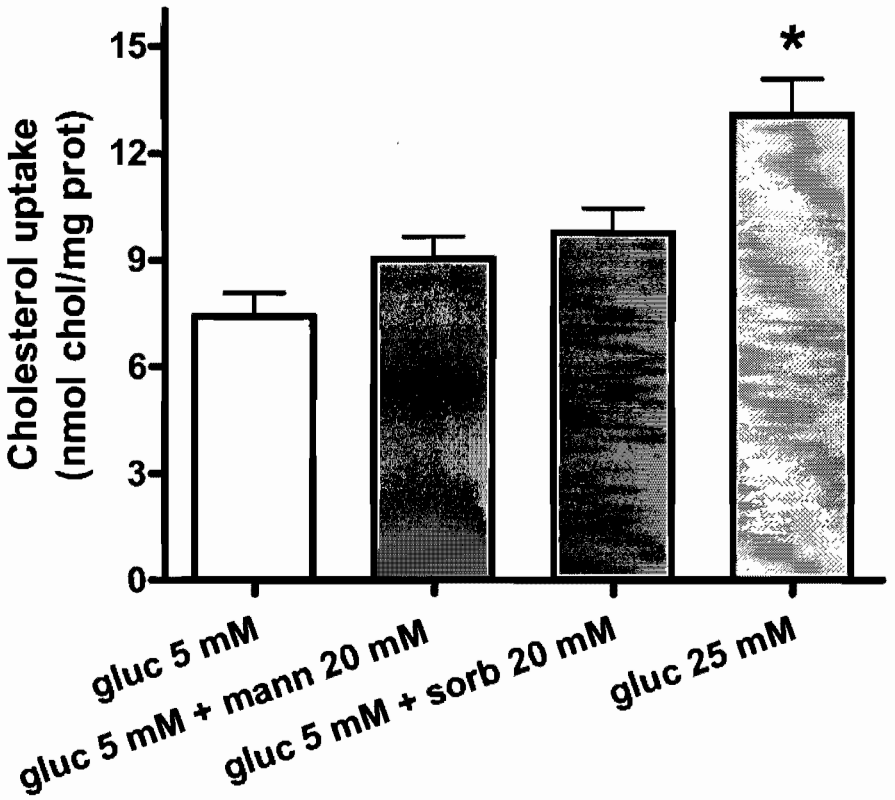
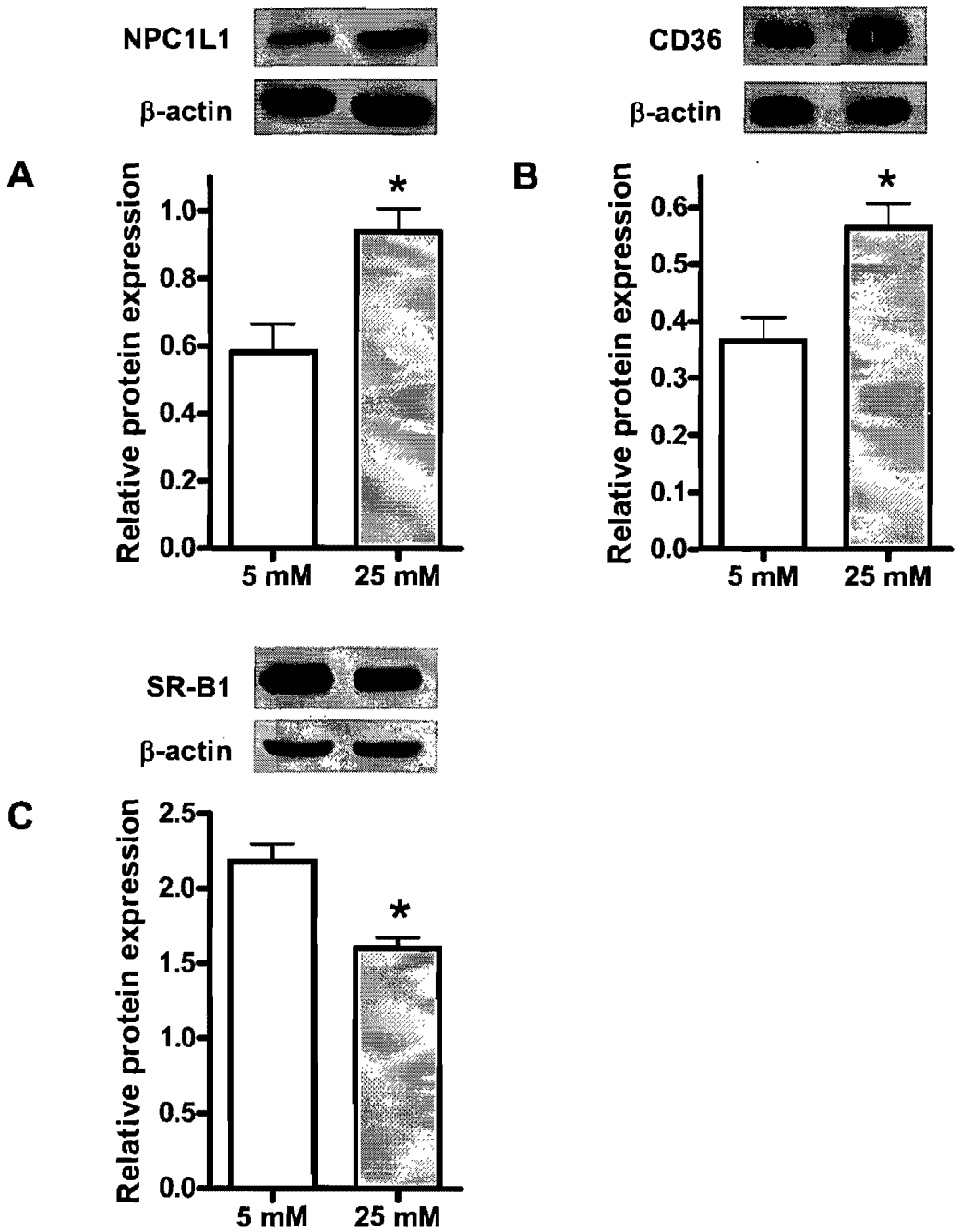

Cholesterol uptake
(nmol chol/mg prot)
15
12
9
6
3
0
*
gluc 5 mM
gluc 5 mM + mann 20 mM
gluc 5 mM + sorb 20 mM
gluc 25 mM

Figure 3


NPC1L1
β-actin
A
Relative protein expression
1.0
0.8
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0.2
0.0
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CD36
β-actin
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Relative protein expression
0.6
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0.3
0.2
0.1
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5 mM
25 mM
SR-B1
β-actin
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Relative protein expression
2.5
2.0
1.5
1.0
0.5
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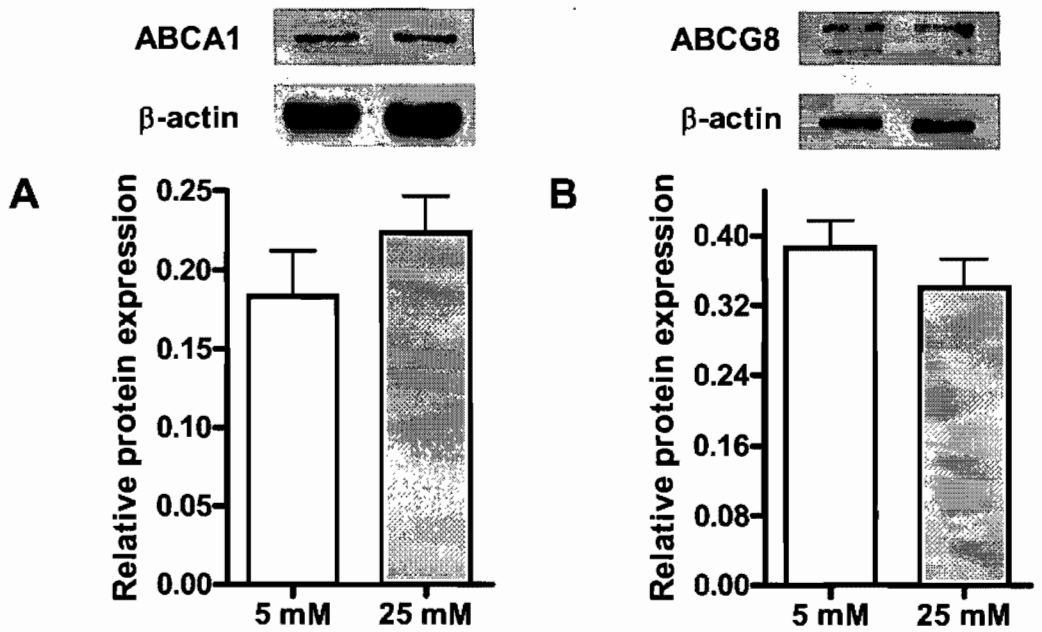
*
5 mM
25 mM

Figure 4


ABCA1
β-actin
A
Relative protein expression
0.25
0.20
0.15
0.10
0.05
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5 mM
25 mM
ABCG8
β-actin
B
Relative protein expression
0.40
0.32
0.24
0.16
0.08
0.00
5 mM
25 mM

Figure 5

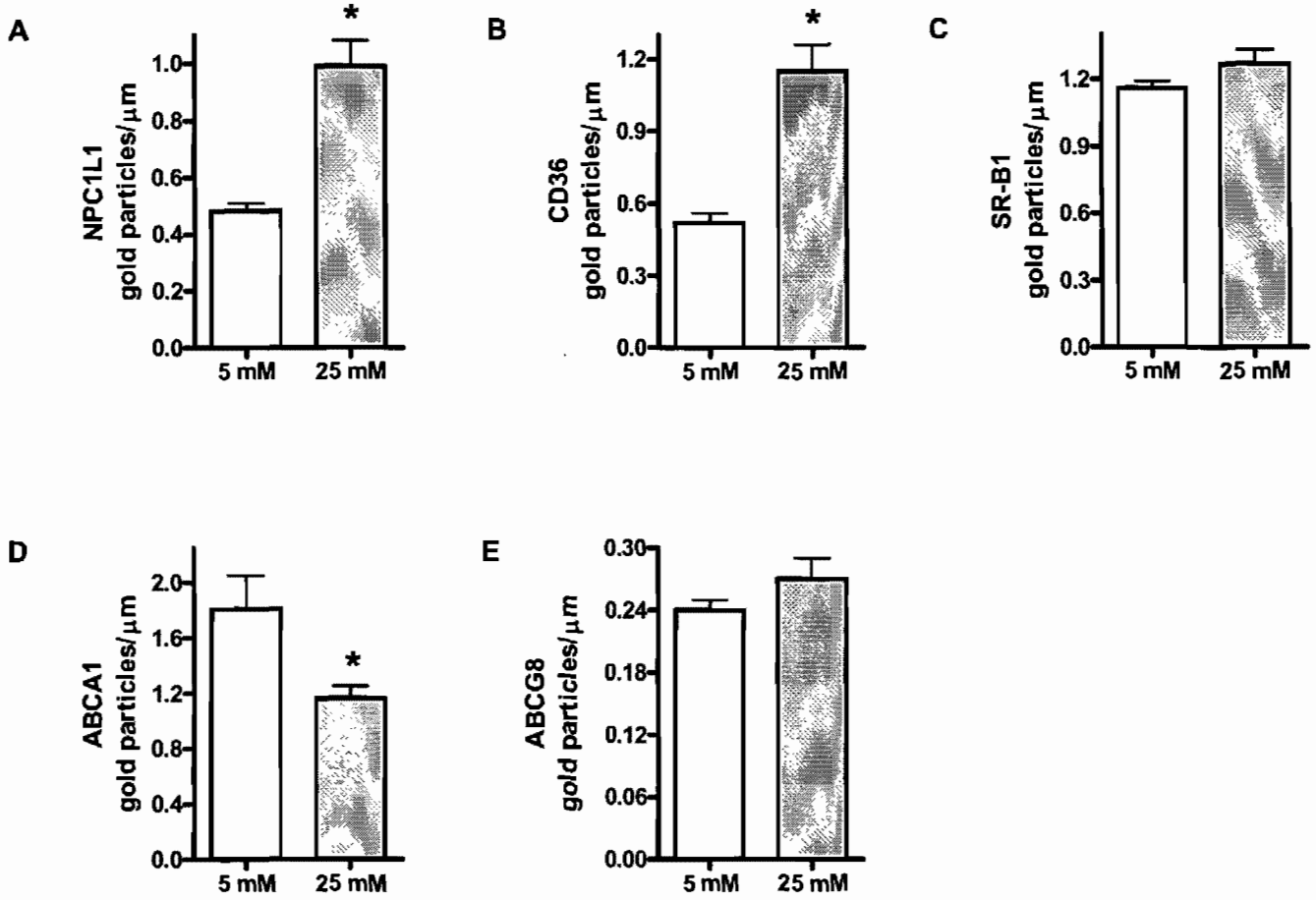

A
NPC1L1
gold particles/µm
1.0
0.8
0.6
0.4
0.2
0.0
5 mM
25 mM
*
B
CD36
gold particles/µm
1.2
0.9
0.6
0.3
0.0
5 mM
25 mM
*
C
SR-B1
gold particles/µm
1.2
0.9
0.6
0.3
0.0
5 mM
25 mM
D
ABCA1
gold particles/µm
2.0
1.6
1.2
0.8
0.4
0.0
5 mM
25 mM
*
E
ABCG8
gold particles/µm
0.30
0.24
0.18
0.12
0.06
0.00
5 mM
25 mM

Figure 6

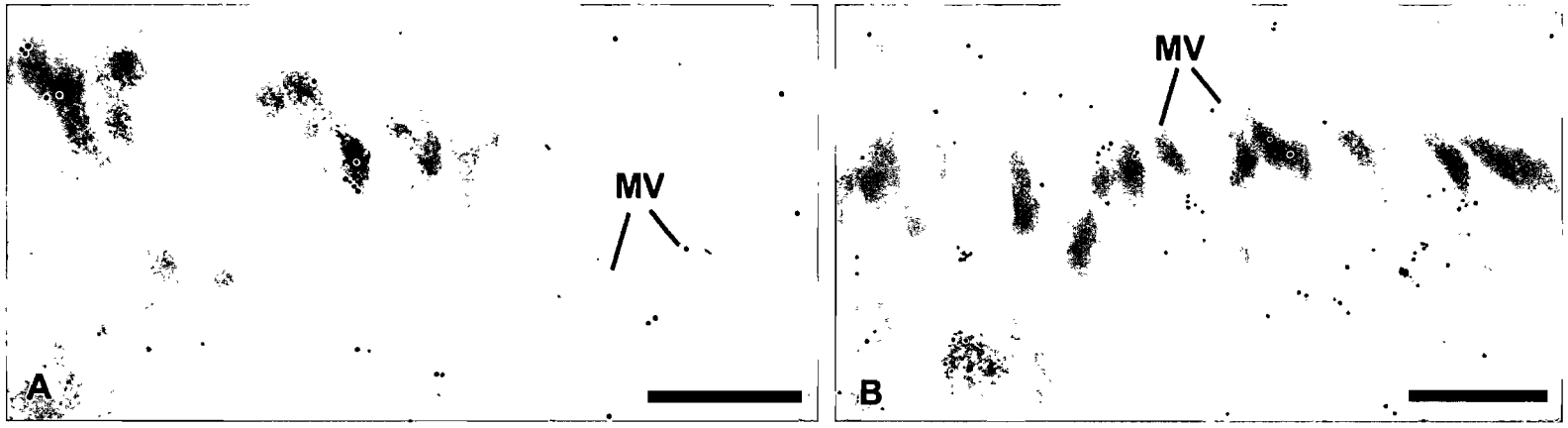


Figure 7

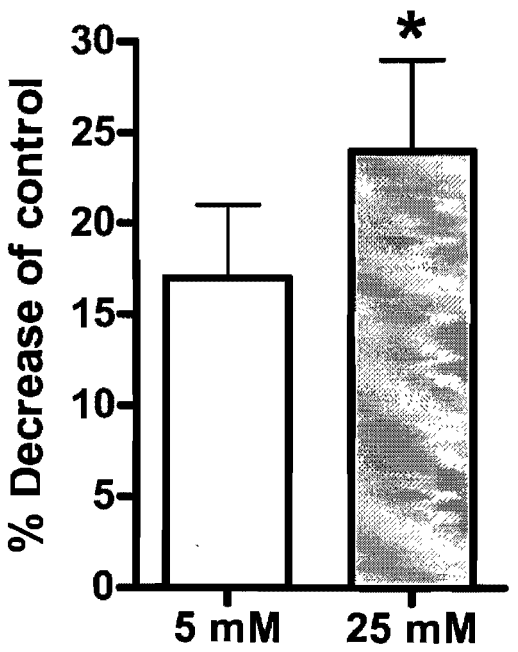


Figure 8

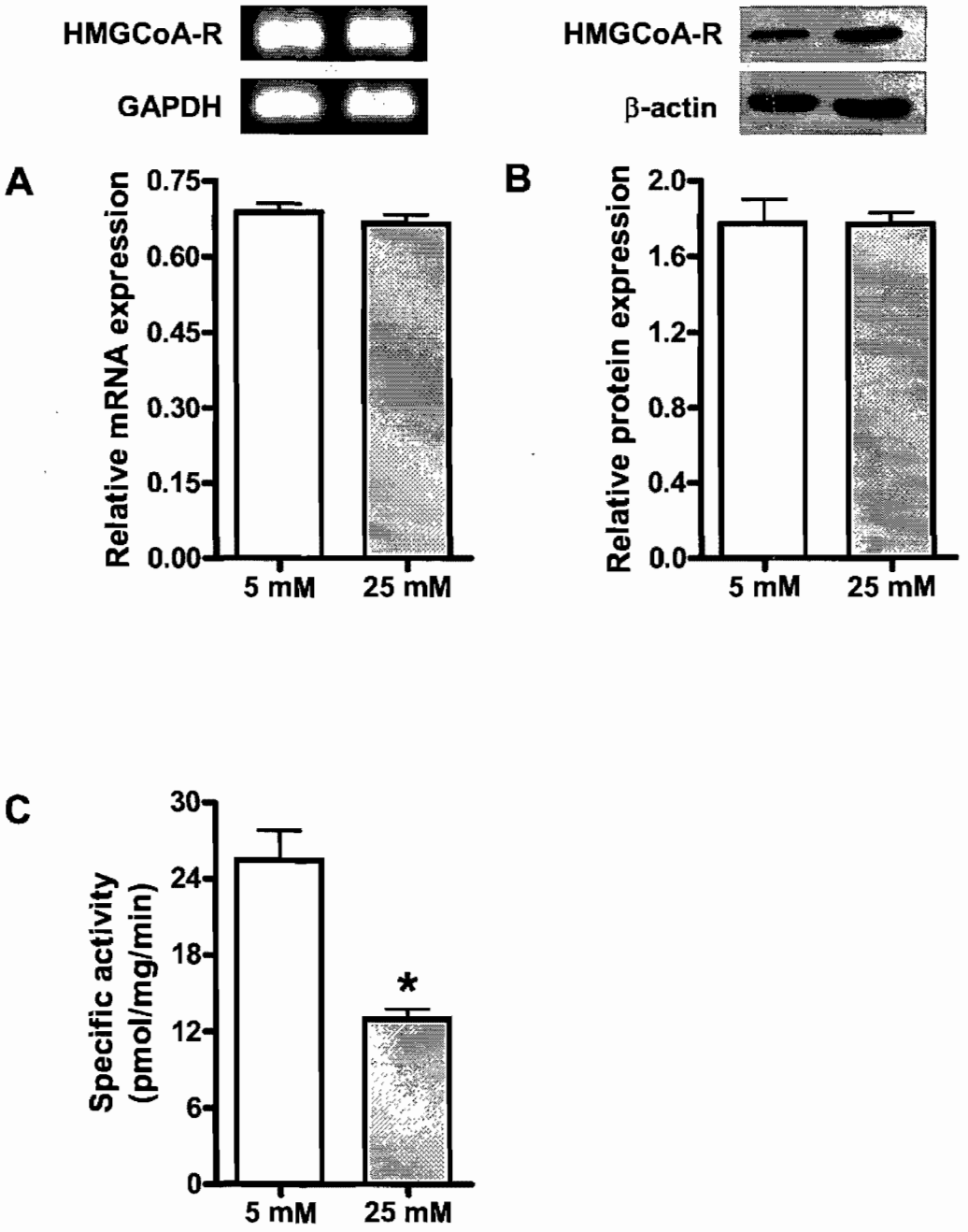

HMGCoA-R
GAPDH
HMGCoA-R
β-actin
A
Relative mRNA expression
0.75
0.60
0.45
0.30
0.15
0.00
5 mM
25 mM
B
Relative protein expression
2.0
1.6
1.2
0.8
0.4
0.0
5 mM
25 mM
C
Specific activity (pmol/mg/min)
30
24
18
12
6
0
*
5 mM
25 mM

Figure 9

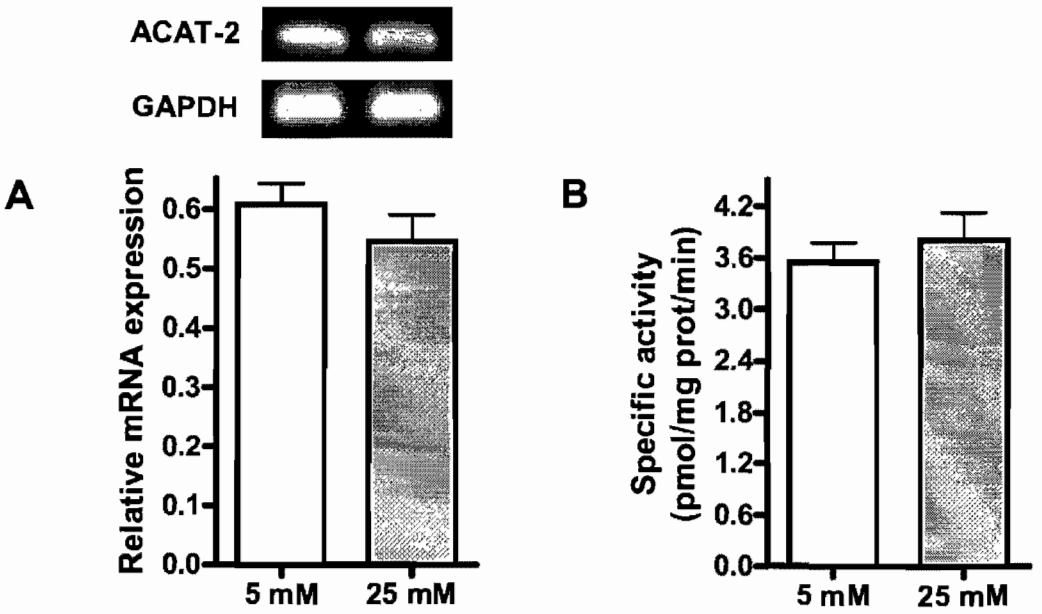

ACAT-2
GAPDH
A
Relative mRNA expression
0.0
0.1
0.2
0.3
0.4
0.5
0.6
5 mM
25 mM
B
Specific activity
(pmol/mg prot/min)
0.0
0.6
1.2
1.8
2.4
3.0
3.6
4.2
5 mM
25 mM

Figure 10

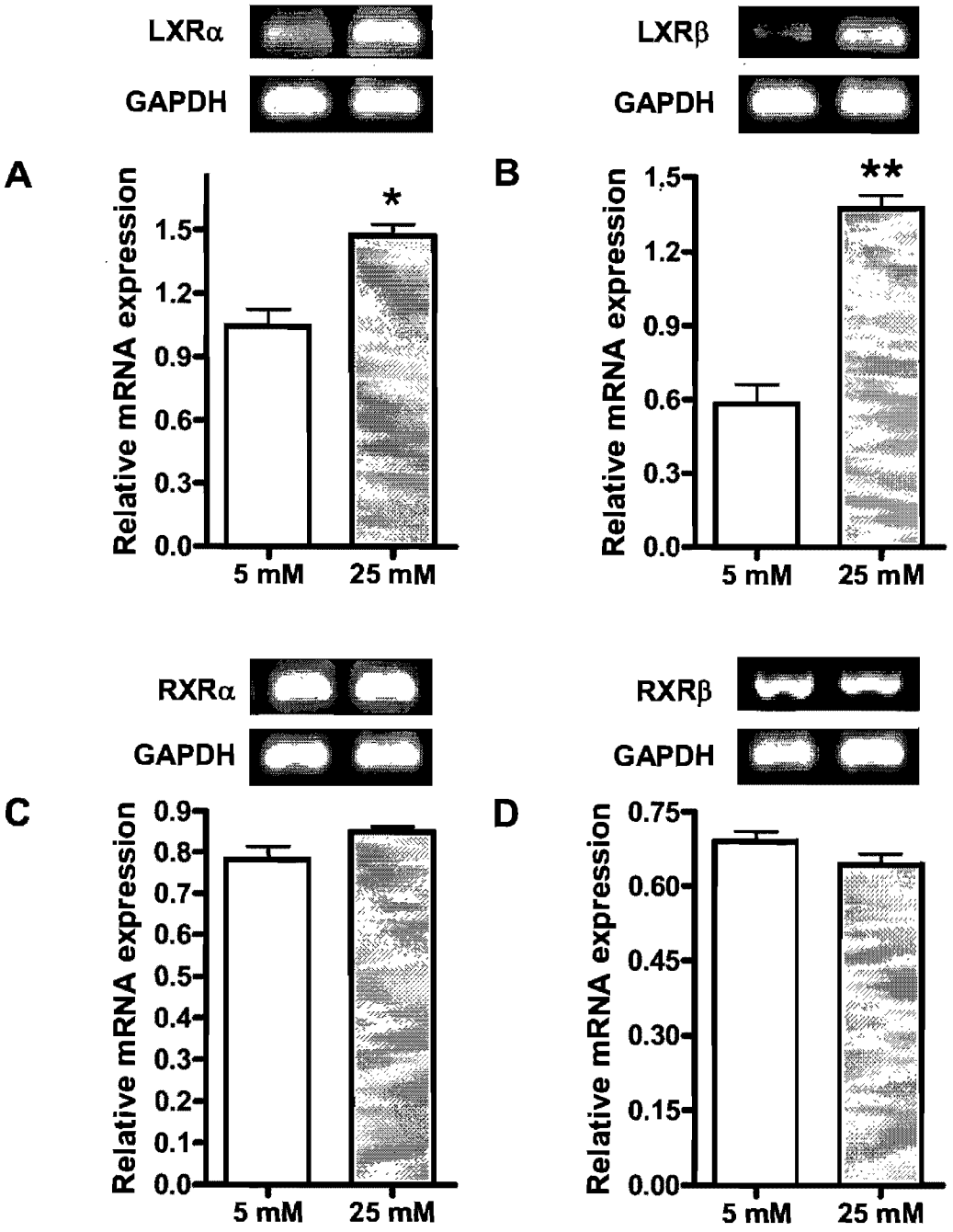

LXRα
GAPDH
A
Relative mRNA expression
1.5
1.2
0.9
0.6
0.3
0.0
*
5 mM
25 mM
LXRβ
GAPDH
B
Relative mRNA expression
1.5
1.2
0.9
0.6
0.3
0.0
**
5 mM
25 mM
RXRα
GAPDH
C
Relative mRNA expression
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5 mM
25 mM
RXRβ
GAPDH
D
Relative mRNA expression
0.75
0.60
0.45
0.30
0.15
0.00
5 mM
25 mM

Figure 11

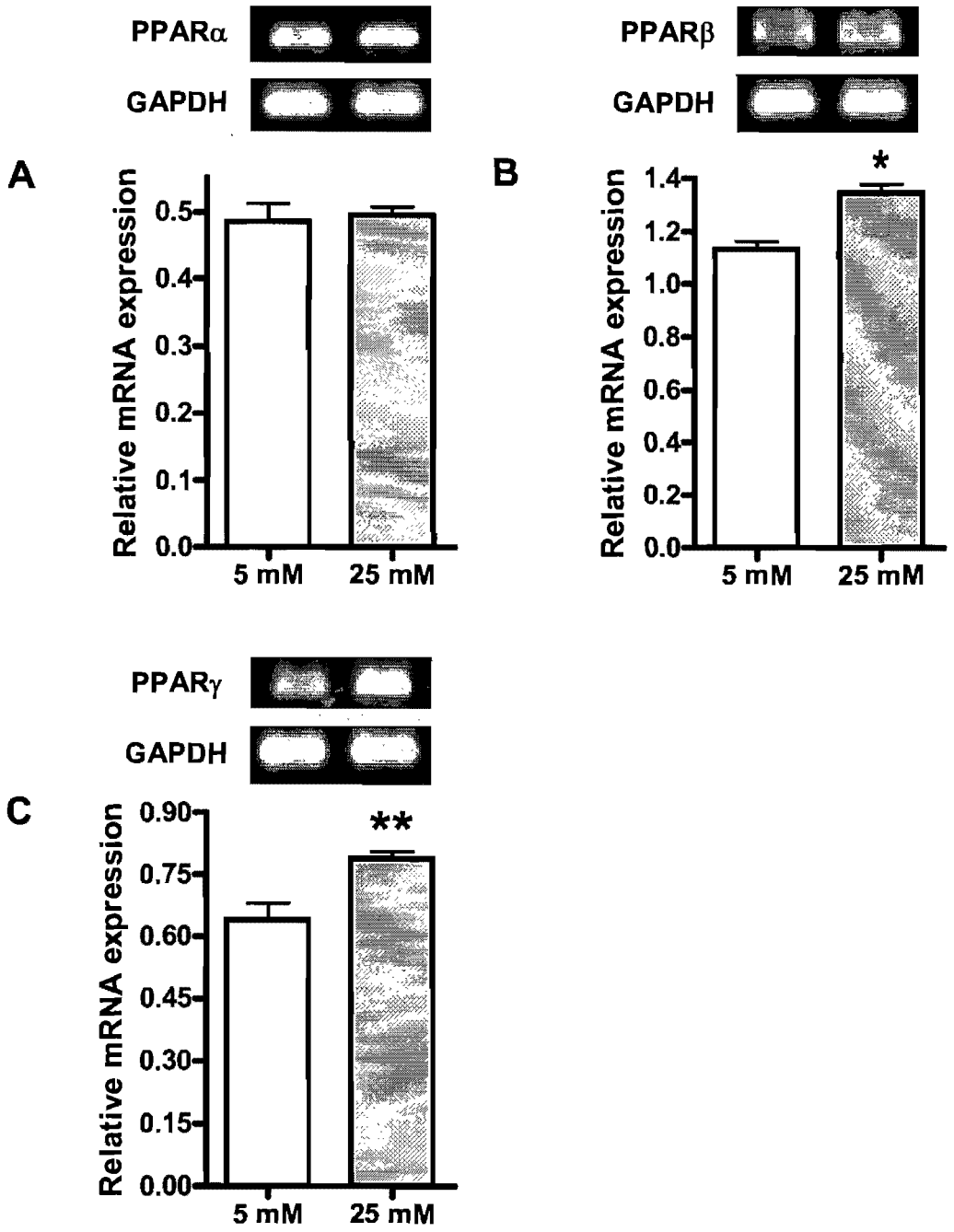

PPARα
GAPDH
A
Relative mRNA expression
0.5
0.4
0.3
0.2
0.1
0.0
5 mM
25 mM
PPARβ
GAPDH
B
*
Relative mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5 mM
25 mM
PPARγ
GAPDH
C
**
Relative mRNA expression
0.90
0.75
0.60
0.45
0.30
0.15
0.00
5 mM
25 mM

Figure 12

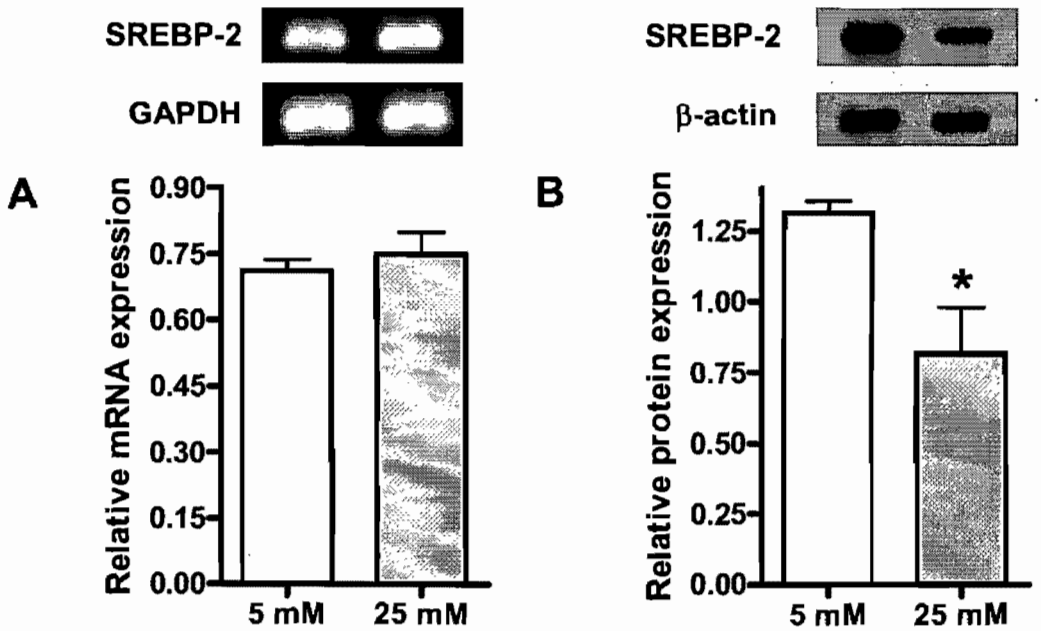
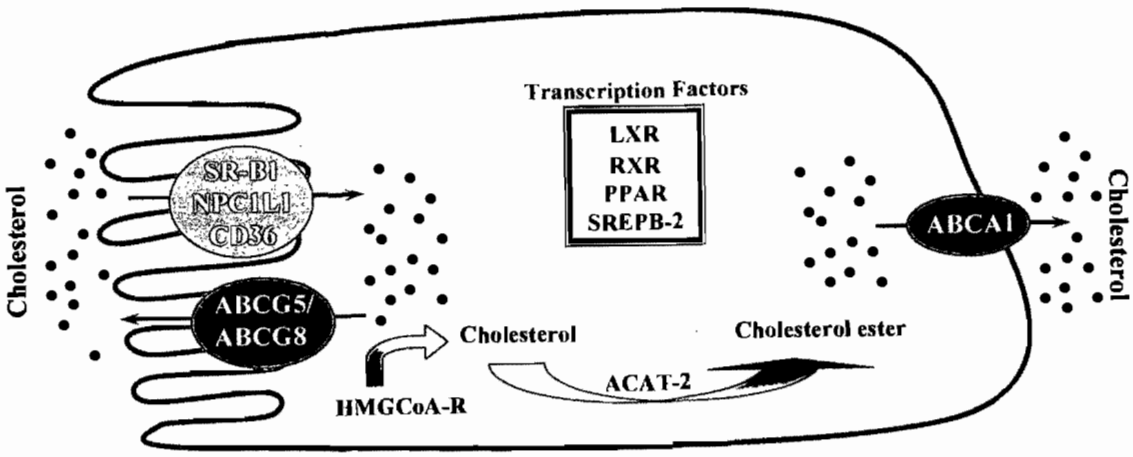

SREBP-2
GAPDH
A
Relative mRNA expression
0.90
0.75
0.60
0.45
0.30
0.15
0.00
5 mM
25 mM
SREBP-2
β-actin
B
Relative protein expression
1.25
1.00
0.75
0.50
0.25
0.00
*
5 mM
25 mM

Figure 13


Transcription Factors
LXR
RXR
PPAR
SREPB-2
Cholesterol
SR-B1
NPC1L1
CD36
ABCG5/
ABCG8
Cholesterol
HMGCoA-R
Cholesterol ester
ACAT-2
ABCA1
Cholesterol

Discussion

Numerous studies have dealt with the regulation of intestinal fat absorption by lipid components. However, the role of carbohydrates has barely been investigated. In the present study, we showed that high glucose concentrations (i) enhanced cholesterol transport in Caco-2/15 cells by upregulating the protein expression of NPC1L1 and CD36; and (ii) reduced SR-BI protein expression and HMG-CoA reductase activity without altering ABCA1 and ABCG8, involved in cholesterol efflux. Moreover, our studies documented that particular transcription factors are glucose sensors, which may explain the impact of glucose on cholesterol absorption via its action on specific cholesterol transporters.

Intestinal cholesterol absorption is a multistep process that is regulated by multiple genes, and the absorption efficiency of cholesterol is most likely determined by the net effect between influx and efflux of intraluminal cholesterol molecules.

NPC1L1 is an essential and a critical protein for cholesterol absorption by the small intestine, since NPC1L1 knockout mice exhibited a reduction in intestinal cholesterol absorption and are insensitive to ezetimibe (Altmann et al., 2002; Davis et al., 2004), a potent drug that lowers serum cholesterol by reducing cholesterol absorption. In addition, the use of genetically-modified intestinal epithelial cells and ezetimibe support the central role for NPC1L1 in intestinal cholesterol absorption (Garcia et al., 1974; Sane et al., 2006; Yu et al., 2006; Field et al., 2007). We found that the high glucose level employed in our investigation raises not only cholesterol uptake but also the expression of NPC1L1. Our results are in line with the findings in diabetic patients who displayed increased levels of NPC1L1 mRNA (Lally et al., 2006). Increased cholesterol absorption has also been shown in streptozotocin diabetic rats (Young et al., 1988), in which NPC1L1 mRNA was found to be increased (Lally et al., 2007). Altogether, these findings indicate an important role

for intestinal NPC1L1 in the delivery of cholesterol to the blood circulation in the presence of high glucose levels.

CD36, a protein highly expressed on the luminal surface of enterocytes (van Bennekum et al., 2005) was found to increase following the administration of high glucose levels. Undoubtedly, CD36 must contribute to the intestinal transport of cholesterol since enterocytes isolated from the small intestines of *cd36*$^{-/-}$ mice, when compared with wild type counterparts, exhibited reduced uptake of cholesterol (60%) (Nassir et al., 2007).

From the present experiments, we can deduce that the elevated glucose-mediated cholesterol uptake is likely related to the up-regulation of NPC1L1 and CD36. Interestingly, the participation of NPC1L1 and CD36 was more reinforced by the experiments with ezetimibe, although the former displayed more sensitivity to ezetimibe inhibition (Garcia-Calvo et al., 2005) than the latter (van Bennekum et al., 2005). Of note is the modest decrease in cholesterol uptake from the apical side, in line with the studies of Field et al. (Field et al., 2001a) probably because the glucuronidated form of ezetimibe is more potent than the native unmodified drug in inhibiting cholesterol absorption by binding more avidly to enterocyte brush-border membranes (van Heek et al., 2000).

SR-BI was originally identified as a novel scavenger receptor that mediates endocytosis of acetylated LDL (Acton et al., 1994). Subsequent studies revealed that SR-BI is a cell surface receptor that binds HDL with a high affinity and mediates the selective uptake by the liver and steroidogenic tissues of cholesterol esters without the endocytic uptake of HDL apolipoproteins (Acton et al., 1996). In addition, SR-BI may facilitate the initial steps of HDL-mediated cholesterol efflux in the arterial wall (Ji et al., 1997). Together, SR-BI accelerates reverse cholesterol transport by promoting cholesterol efflux from macrophages in vascular walls (Chinetti et al., 2000) and selective uptake of HDL-C by hepatocytes for excretion

of cholesterol as bile acids. Therefore, SR-BI plays crucial roles in atheroprotective functions of HDL (Krieger, 2001).

Additional studies, employing the high-resolution immunogold technique revealed that SR-BI may be responsible for the cholesterol uptake by enterocytes; they found SR-BI labeling mainly over microvilli of the enterocyte, where the bulk of cholesterol absorption takes place (Hauser et al., 1998; Altmann et al., 2002; Levy et al., 2004). Despite these results, the involvement of SR-BI in cholesterol absorption has been questioned since inconsistent results were obtained with genetically-modified mice (Mardones et al., 2001; Bietrix et al., 2006).

In the present study, we showed that an increase in the concentration of glucose downregulates the protein expression of SR-BI in Caco-2/15 cells. Such suppressive effect of glucose has also just been reported in hepatocytes HepG2 cells (Murao et al., 2008). The use of inhibitors for select signal transduction pathways in HepG2 cells indicated that glucose suppression of SR-BI expression is partially mediated by the activation of the p38 MAPK-Sp1 pathway (Murao et al., 2008). Further studies are needed to determine the detailed regulatory mechanisms of intestinal SR-BI expression.

Our hypothesis was that if NPC1L1 and CD36 mediate an increased cholesterol uptake, it is conceivable that the highly available cholesterol in Caco-2/15 cells would lead to reduced HMG-CoA reductase activity. Based on the data in Figure 7, this assumption turned out to be true. However, ACAT was insensitive to the accumulation of intracellular cholesterol, probably because the latter did not expand the limited cholesterol substrate pool for ACAT and manifested a high-order dependence on ER cholesterol concentration (Chang et al., 1997; Buhman et al., 2000).

The coordinated regulation of genes implicated in cholesterol homeostasis is governed by the actions of several transcription factors, such as LXRs and PPARs.

Also, SREBPs are transcription factors that are crucial regulators of cholesterol synthesis and metabolism. In response to specific effectors, LXRs form a heterodimer with the RXRs and regulate an integrated network of genes that control whole body cholesterol and lipid homeostasis assays (Lehmann et al., 1997; Janowski et al., 1999).

In particular, LXR appears to serve as a safety valve to limit free cholesterol in tissues that are experiencing high cholesterol flux (Cummins and Mangelsdorf, 2006). Since these nuclear factors act as glucose sensors (Mitro et al., 2007) and exhibit anti-diabetic effects (Cao et al., 2003; Laffitte et al., 2003), we first measured their gene expression. LXRα and LXRβ mRNA was increased by the presence of 25 mM glucose in Caco-2/15 cells, but it was not accompanied with the expected induction of ABCA1 and ABCG8 protein expression. This may be due to the irresponsiveness of RXRs that work as partners with LXRs.

The family of SREBP regulates the coordinated expression of genes involved in lipid synthesis and uptake (Brown and Goldstein, 1999). Three SREBP isoforms are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1c preferentially activates genes required for fatty acid (FA) synthesis and their incorporation into TGs and phospholipids, SREBP-2 preferentially activates the LDL- receptor gene and various genes required for cholesterol synthesis such as HMG-CoA reductase (EC1.1.1.34) (Horton et al., 2002). SREBP-1a is an activator of both the cholesterol and FA biosynthetic pathways, but it is present in much lower amounts (Shimomura et al., 1997). Since SREBP-2 plays a more important role in the regulation of cholesterol synthesis in the intestine (Field et al., 2001a, b), we therefore measured the gene and protein expression of SREBP-2. Our results highlighted a decline in the protein expression of SREBP-2 without any alteration of SREBP-2 mRNA. Similarly, the gene and protein expression of HMG-CoA reductase were not changed, but its activity was decreased in a significant manner, suggesting post-transcriptional mechanisms for SREBP-2 and HMG-CoA reductase regulation.

PPARs have been shown to regulate the expression of genes involved in a variety of biological processes, including lipid metabolism and insulin sensitivity (Vamecq and Latruffe, 1999; Corton et al., 2000). PPARα regulates numerous aspects of FA catabolism, whereas PPARγ controls adipocyte differentiation, systemic glucose levels and lipid homeostasis (Michalik and Wahli, 1999; Willson et al., 2000). PPARδ is also involved in development, lipid metabolism and epidermal cell proliferation (Lim et al., 1999). The PPARs are ligand-dependent transcription factors that regulate target genes expression by binding to characteristic DNA sequences termed peroxisome proliferators response element (PPREs) located in the 5′ -flanking region of target genes (Gearing et al., 1993; Palmer et al., 1995). Each receptor binds to its PPRE as a heterodimer with the receptor for 9-cis retinoic acid, the RXR. Upon binding a ligand, the conformation of a PPAR is altered and stabilized so that a binding cleft is created, and recruitment of transcriptional coactivators occurs. In the present study, treatment of Caco-2/15 cells with 25 mM glucose enhanced the gene expression of PPARβ and PPARγ and concomitantly decreased the protein concentration of SREBP-2. Similarly, PPAR activation by troglitazone downregulated cholesterol synthesis in Caco-2 cells by reducing the expression of SREBP-2 protein (Klopotek et al., 2006).

All these data add new key elements to the complex relationship linking carbohydrate and cholesterol intestinal assimilation and metabolism. The complete mechanism involved in the glucose-induced regulation of uptake process is not yet fully identified and/or understood. An alteration of the physical properties of the enterocyte brush border, which in turn regulates the activity of membrane transporters, is probable, but other regulation pathways should also be considered.

In conclusion, glucose at 25 mM stimulated cholesterol uptake by Caco-2 cells. The high concentration of glucose enhanced the protein expression of the critical cholesterol transporters NPC1L1 and CD36, and concomitantly decreased SR-BI protein expression. No significant alterations were observed in the protein

expression of ABCA1 and ABCG8, which act as efflux pumps favoring cholesterol export out of absorptive cells. At the same time, HMG-CoA reductase activity was significantly decreased, whereas ACAT activity remained unchanged. Finally, an increase was noted in the transcription factors LXRα, LXRβ, PPARβ and PPARγ along with a drop in the protein expression of SREBP-2.

Collectively, our experiments indicate that glucose at high concentrations may regulate intestinal cholesterol transport and metabolism in Caco-2 cells, modifying important cholesterol transporters and transcription factors, thus suggesting a potential influence on the cholesterol absorption process in type 2 diabetes.

In our society, morbidity and mortality from cardiovascular diseases is becoming an increasingly severe problem. Overnutrition and a sedentary lifestyle lead to obesity and hypercholesterolemia already in a high percentage of children, who will be future patients with atherosclerosis-associated diseases (Ernst and Obarzanek, 1994; Gidding, 1999; Belay et al., 2004; Cruz and Goran, 2004). Certainly, preventive educational programs have to be developed to adjust and prevent this process. However, also therapeutic options have to be improved to ameliorate hypercholesterolemia.

Clearly, intracellular handling of cholesterol needs more emphasis, as well as the regulation of cholesterol homeostasis during development. Evidently, the current situation allows to identify promising targets for cholesterol-lowering therapies at a molecular level and to develop effective means to prevent cardiovascular disease in the future.

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Annexe

Reactives used:

Antibodies	**Company**	**Dilution**
SR-B1	Novus	1/15000
β-actine	Sigma	1/40000
NPC1L1	Novus	1/500
CD36	Santacruz	1/1000
ABCA1	Novus	1/500
ABCG8	Santacruz	1/500
SREBP-2	Cayman	1/1000
HMG CoA	Upstate	1/1000
Ezetimibe (Ezetrol)	Schering-Plough	