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Red Wine Polyphenolics Suppress the Secretion of ApoB48 from Human Intestinal CaCo-2 Cells

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Epidemiological studies suggest that the consumption of red wine lowers the risk of cardiovascular disease. Although the cardioprotective effect of red wine has been attributed to its polyphenolic content, presently, very little is known about the mechanisms by which these compounds benefit the cardiovascular system. Therefore, the aim of this study was to elucidate whether red wine polyphenolics attenuate the synthesis and secretion of proatherogenic chylomicrons from intestinal cells. Apolipoprotein B48 levels (marker of intestinal chylomicrons), quantitated by western blotting, were significantly reduced by 30% in cultured CaCo-2 cells and medium when cells were incubated with either dealcoholized red wine, alcoholized red wine, or atorvastatin compared with controls. Intracellular cholesterol availability was also attenuated in cells incubated with dealcoholized red wine (72.5%), alcoholized red wine (81.5%), and atorvastatin (83.5%) compared to control cells. Collectively, this study suggests that red wine polyphenolics downregulate the production of atherogenic chylomicrons from intestinal cells, which may explain the reduced CVD mortality rates following its consumption.

KEYWORDS: Red wine polyphenolics; apoB48; cholesterol; CaCo-2

INTRODUCTION

Coronary heart disease (CHD) is a main cause of premature death and disability in most developed countries. In France, however, the mortality of CHD is low despite a high intake of dietary fat (*1*); this is called the "French paradox". Epidemiological studies have shown an association between the French paradox and red wine consumption (*2*, *3*). It is postulated that the polyphenolic components in red wine may be responsible for some of the beneficial effects in reducing risk for CHD (*4*). Hertog et al. (*5*) demonstrated that populations consuming polyphenolic-rich diets (from fruits, vegetables, and wine) have low risk of developing cardiovascular disease (CVD). Furthermore, some animal studies have shown a positive association between consumption of red wine polyphenolics and CHD (*6*−*8*). Currently, the mechanisms by which red wine polyphenolic compounds lower CVD risk are inconclusive.

Atherosclerosis is the buildup of plaque in the subendothelial space of the artery wall. Initiation of plaque formation begins when the lipids cholesterol and triglyceride (TG) are deposited by circulating lipoproteins into the arterial wall. These atherogenic lipoproteins are called low-density lipoprotein (LDL) and chylomicron remnants (*9*) and are derived from very low-density lipoprotein (VLDL) and chylomicrons, respectively. Strategies used to decrease the risk of developing cardiovascular disease aim to reduce circulating levels of these lipoproteins by decreasing their production and increasing their clearance (via the hepatic LDL receptor) out of the blood. These strategies include the use of lipid-lowering drugs such as statins, as well as changes to lifestyle and diet.

Apolipoprotein B (apoB) is a principal structural protein necessary for the synthesis and secretion of lipoprotein. In humans, apoB100 is associated with LDL and VLDL and is exclusively synthesized and secreted from the liver. Once in circulation, VLDL breaks down into LDL, which can then deposit its lipids (triglyceride and cholesterol) in the arterial wall. Cell culture studies in our laboratory have shown significant reductions in VLDL secretion in liver cells (with apolipoprotein B100 as a biological marker) and increased expression of LDL receptors after incubation with red wine (RW + OH) and dealcoholized red wine (RW − OH) (*9*). Consistent with these findings, Vinson et al. (*6*) found dramatic reductions in LDL cholesterol of 46% and 13.3%, following the regular consumption of RW + OH and RW − OH, respectively, in dyslipidemic hamsters.

The effect of red wine polyphenolics on apoB48 production and secretion from intestinal cells is presently unknown. ApoB48 is associated with chylomicrons and chylomicron remnants, which also transport dietary fat such as cholesterol in circulation and deposit them in the arterial wall, and are synthesized by

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the intestine. Regulatory mechanisms for chylomicron metabolism in the intestine are thought to be similar to VLDL metabolism in the liver. We hypothesize that red wine polyphenolics may also inhibit the production and secretion of chylomicron from the intestine via the same mechanism as in the liver. A decrease in chylomicron from the intestine may lead to a reduction in the risk of CHD.

In the present study, we examined the effects of red wine polyphenolics on chylomicron synthesis and secretion from human colonic epithelial CaCo-2 cells compared with a lipid-lowering drug, atorvastatin, which reduces hepatic production of lipoproteins by limiting the availability of newly synthesized cholesterol in liver cells and increases clearance of proatherogenic lipoproteins by the hepatic LDL receptor. CaCo-2 is an intestinal cell line derived from a human colorectal carcinoma and is well investigated as a model for intestinal lipoprotein synthesis and secretion. CaCo-2 cells show morphological and biochemical properties consistent with spontaneously differentiated small intestinal enterocytes under standard culture conditions. Therefore, CaCo-2 cells are a suitable model to study the formation of chylomicrons in the intestine. The intracellular production and secretion of apoB48 was measured as a marker of chylomicron metabolism by use of western blotting and the enhanced chemiluminescence (ECL) detection system.

MATERIALS AND METHODS

Human transformed colonic epithelial CaCo-2 cells were purchased from ATCC (American Type Culture Collection, Rockville, MD). Cell culture media, reagents, and fetal bovine serum (FBS) (certified grade) were obtained from Gibco BRL Life Technologies (Gaithersburg, MD). Costar transwell, polycarbonate microporous cell culture inserts and tissue culture flasks were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland). Bicinchoninic acid protein assay reagents were obtained from Pierce (Rockford, IL). 5α-Cholestane, Triton X-100, oleic acid, essentially fatty acid-free bovine serum albumin (BSA), sodium pyruvate, and other common laboratory reagents were from Sigma Chemical Co. (St. Louis, MO). Poly(vinylidene fluoride) (PVDF) membrane was from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence detection reagents, hyperfilm ECL, and anti-goat IgG linked to horseradish peroxidase were purchased from Amersham International, England. Rabbit anti-human apo B antibody was from DAKO A/S, Denmark. Atorvastatin was kindly donated by Parke-Davis, ANN Arbor, MI.

Cell Culture Conditions. The CaCo-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 20% (v/v) fetal bovine serum (FBS), 2% penicillin−streptomycin, and 1% nonessential amino acid (NEAA) at 37 °C under 5% carbon dioxide. The cells were seeded at a density of 1×10^6 in 75 cm^2 tissue culture flasks, and the medium was changed every 2 or 3 days or when the color of the medium changed from red/pink to yellow until the cells were 80−90% confluent.

Subculturing Cells for Treatment. The cells were subcultured on polycarbonate microporous membranes (0.4 μm pore size, inserts of 24.5 mm diameter) by plating at a density of 1.3×10^4/apical well (13 days postsubculturing). Each basolateral well contained 2 mL of DMEM medium. The medium in both apical and basolateral wells was then replaced with 2 mL of serum-free medium and incubated for 24 h. Serum-free medium was made by supplementing DMEM with 0.08% fatty acid-free BSA, 50 μM oleic acid, 22.2 mM glucose, 45.5 mM sodium bicarbonate, 1 mM sodium pyruvate, 2% penicillin−streptomycin, and 1% NEAA.

Treatment of Cells. The subcultured cells were incubated with 5 μM dealcoholized red wine (RW − OH) or 5 μM alcoholized red wine (RW + OH) in 2 mL of serum-free medium for 24 h. The concentration of wine used was based on tannic acid equivalence and was selected as it has been used in a previous study examining the effects of red wine polyphenols on apoB100 in HepG2 cells (*9*). Cells were also treated with 13% absolute ethanol or 10 μM atorvastatin in 2 mL of serum-free medium. The concentration 10 μM has been used previously in a number of cell culture experiments examining the effect of statins on cholesterol metabolism in HepG2 cells (*10*−*12*), and 10 μM supplemented to cells is thought to be representative of the physiological dose in humans after taking 20−40 mg of atorvastatin (*13*). Atorvastatin solution was made by dissolving 2.928 mg of atorvastatin in 5 mL of dimethyl sulfoxide and stored at 4 °C until subsequent use. The basolateral wells contained 1 mL of serum-free medium each.

Table 1. Total Polyphenol Content of Red Wine[a]

polyphenol	amount (mg/L)
total polyphenols	2000
caffeic acid	11.0
gallic acid	9.5

[a] Values of specific phenolic acids are the mean of duplicate determinations that were <5% different from each other.

Measurement of Total Polyphenols in Wine. The total polyphenol in red wine (Cabernet Shiraz 1994, 1.3 mg/mL alcohol; Hardy's Nottage Hill, McLaren, South Australia) was 2.0 mg/mL and was quantified by a colorimetric assay with Folin-Denis reagent according to the method published by the Association of Official Analytical Chemists (*14*). This method estimates polyphenols at 760 nm in relation to a standard curve for tannic acid. Table 1 shows the phenolic composition of the red wine.

Dealcoholization of Red Wine. Red wine was dealcoholized by vacuum distillation with gentle heating to 50 °C.

Cell Isolation, The volume of medium in the basolateral well was measured and transferred into microfuge tubes and stored at −80 °C following incubation with media containing the different treatments (alcoholized red wine, dealcoholized red wine, ethanol, or atorvastatin) in apical wells. The medium in the apical well was discarded, as apoB48 is not secreted into this compartment of the transwell. The cells were washed, harvested, and resuspended in phosphate-buffered saline and pelleted at 250*g* at 4 °C for 5 min. The supernatant was carefully removed. The pelleted cells were resuspended in 90 μL of solubilization buffer (500:1 3% Triton X-100:protease inhibitor cocktail solution) for apoB quantification and in 400 μL of SDS (sodium dodecyl sulfate) buffer for cholesterol analysis by gas chromatography (GC). Solubilized cell protein was separated from cell debris by centrifuging at high speed for more than 5 min, after shaking on a low-speed wheel spinner at 4 °C for 2 h. The supernatant was collected and stored at −80 °C.

Cholesterol Analysis. Lipid was extracted from solubilized CaCo-2 cells to measure free cholesterol by a modified method of Folch et al. (*15*). Briefly, 1 mL of chloroform ($CHCl_3$) was added in 200 μL of cells along with 100 μL of internal standard (5α-cholestane) and 25 μL of saturated salt solution to prevent the formation of a fatty acid emulsion. Samples were mixed to extract lipid and centrifuged at 2060*g* for 10 min. The top aqueous layer was aspirated and discarded and the lower chloroform layer was evaporated under a stream of nitrogen. Samples were stored at −20 °C and reconstituted in 50 μL of toluene before GC analysis.

To measure total cholesterol in solubilized cells, 2 mL of 1 M KOH in methanol (CH_3OH) was added to 200 μL of cells. Samples were then incubated at 45 °C for 1 h after being flushed with nitrogen. After hydrolysis was complete, 2 mL of water and 100 μL of internal standard were added, and lipid was extracted in 2 mL of chloroform. Samples were then centrifuged at 515*g* for 5 min and the top layer was carefully removed. The lower layer was transferred to microfuge tube and evaporated under a stream of nitrogen. Samples were stored at −20 °C and reconstituted in 50 μL of toluene before GC analysis.

Both free and total cholesterol were determined by comparison of peak area with a five-point external calibration curve, and an internal standard was used to correct for injection volume variability. Calibration was performed daily. A Perkin-Elmer Autosystem XL gas chromatograph fitted with a ZB-1 dimethyl polysiloxane column (30 m × 530 μm, 1.5 μm film thickness, Phenomenex: Phenomenex, Torrance, CA) and splitless injector was used with helium as the carrier gas at a flow rate of 6.0 mL/min. An estimate of esterified cholesterol was derived as the difference between total and free cholesterol.

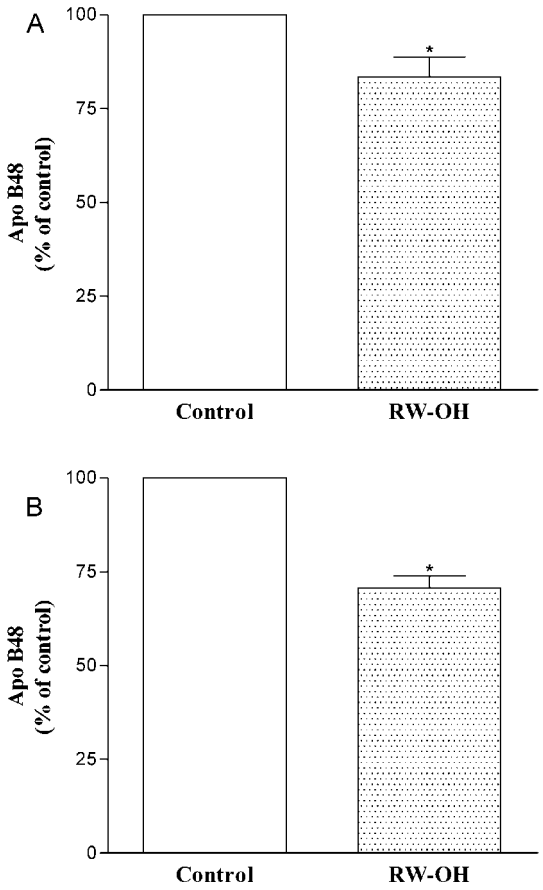

Figure 1. Effect of dealcoholized red wine (RW − OH) on apoB48 production (A) and secretion (B) from CaCo-2 cells. Concentrations of apoB48 are expressed as a percentage of control, mean ± SEM (*p < 0.05, n = 4 by independent t-test).

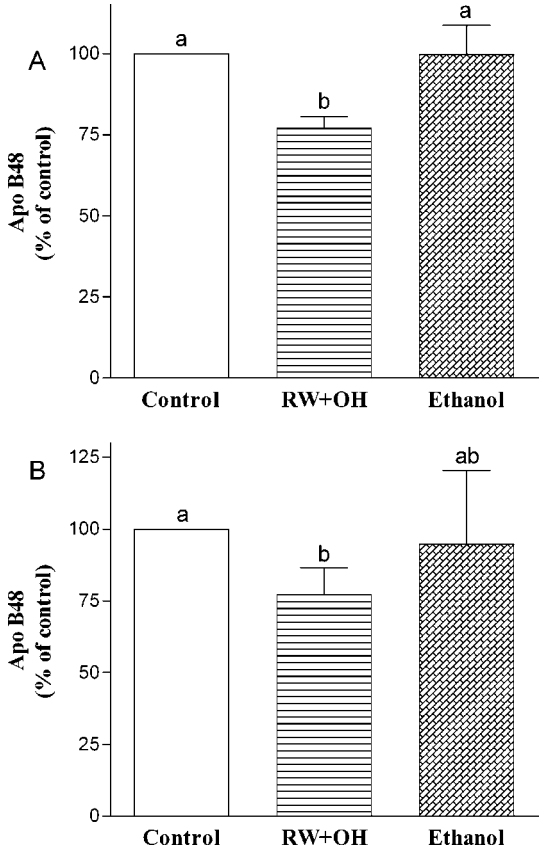

Figure 2. Effect of alcoholized red wine (RW + OH) and ethanol on apoB48 production (A) and secretion (B) from CaCo-2 cells. ApoB48 concentrations are expressed as a percentage of control, mean ± SEM (different letters above bar graphs indicate significant difference at p < 0.05, n = 4 by one-way ANOVA).

ApoB48 Quantitation. Solubilized cell protein (150 μg) and purified apoB48 standards were separated by SDS−PAGE on NuPAGE 3−8% gradient gels with a Novex Mini-Cell (Novex Instruments, CA) at 150 V for 1 h. Separated samples were electrotransferred onto 0.45 μm poly(vinylidene fluoride) (PVDF) membrane at 30 V for 1.5 h. The membrane was blocked in skim milk solution (10% skim milk powder in Tris-buffered saline with Tween-20, TBST: 19 μM Tris-HCl buffer, 150 mM NaCl, and 0.1% Tween-20 at pH 7.4). The membrane was then incubated in 5.0 μg/mL rabbit anti-human apoB in TBST for 1 h and incubated in 0.5 μg/mL anti-goat IgG linked to horseradish peroxidase in TBST for another 1 h. The membrane was exposed onto hyperfilm ECL (enhanced chemiluminescence) following incubation in ECL detection reagent for 60 s. Films were scanned with UMAX Vista S6E flatbed scanner and the intensities of the apoB48 protein bands were quantitated by use of the computer program Scion Image (Scion Inc.).

Statistical Analysis. Statistical analysis was carried out by using SPSS for Windows Student Edition Release 10.0.7 (SPSS Inc.). Means were compared by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and independent t-test. All means were compared to each other; means with different letters above bar graphs indicate significant differences at p < 0.05.

RESULTS

Effect of Dealcoholized Red Wine on ApoB48 Production and Secretion from CaCo-2 Cells. Cells were incubated with 5 μM dealcoholized red wine for 24 h, and apoB48 content in the cells and in culture medium was determined. The apoB48 production in the cells was significantly decreased by 17% (Figure 1A) and secretion into medium was significantly decreased by 30% (Figure 1B) when cells were incubated with dealcoholized red wine compared to control cells, respectively.

Effect of Alcoholized Red Wine and Ethanol on ApoB48 Production and Secretion from CaCo-2 Cells. Cells were incubated with 5 μM alcoholized red wine and 13% (v/v) ethanol for 24 h, and apoB48 content in cells and in culture medium was determined. There was no significant change in apoB48 production and secretion in cells incubated with ethanol compared to control cells. The apoB48 production in the cells incubated with alcoholized wine was significantly decreased by 23% compared to control cells and cells incubated with ethanol (Figure 2A). The apoB48 secretion in the medium was significantly decreased by 23% when cells were incubated with alcoholized red wine compared to control. However, there was no significant difference in apoB48 secretion between cells incubated with alcoholized red wine and those incubated with ethanol (Figure 2B).

Effect of Atorvastatin on ApoB48 Production and Secretion from CaCo-2 Cells. Cells were incubated with 10 μM atorvastatin, and apoB48 content in the cells and in culture medium was determined. The apoB48 secretion into medium was significantly decreased by 18% when cells were incubated with atorvastatin compared to control cells (Figure 3).

Effect of Red Wine on Intracellular Cholesterol Levels. The effect of red wine on cholesterol content was determined by GC (Table 2). Total and free cholesterol in the cells were significantly decreased when incubated with dealcoholized and alcoholized red wine compared to control. Total and free cholesterol and cholesterol ester in the cells was significantly

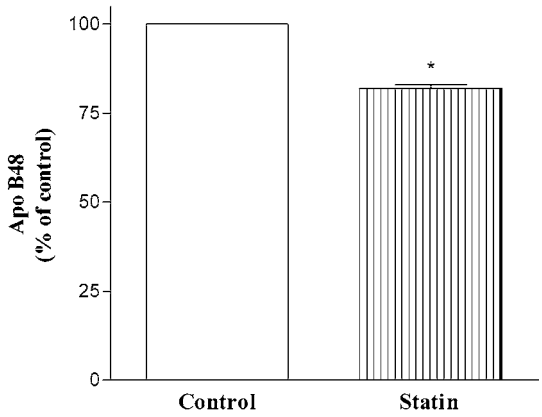

Figure 3. Effect of atorvastatin on apoB48 production in CaCo-2 cells. ApoB48 concentrations are expressed as a percentage of control, mean ± SEM (*$p < 0.05$, $n = 4$).

Table 2. Effect of Dealcoholized and Alcoholized Red Wine, Ethanol, and Atorvastatin on Intracellular Total, Free, and Esterified Cholesterol Concentrations in CaCo-2 Cells[a]

treatment	free cholesterol	cholesterol ester	total cholesterol
control	100 ± 0	100 ± 0	100 ± 0
RW − OH	72.5 ± 2.5*	114.5 ± 7.5	87.5 ± 1.5*
RW + OH	81.5 ± 18.5*	103.5 ± 35.5	93.0 ± 3.0*
ethanol	99.0 ± 4.0	99.0 ± 9.0	91.5 ± 4.5
atorvastatin	83.5 ± 0.5*	86.5 ± 8.5*	89.0 ± 4.0*

[a] Results are expressed as a percentage of control, mean ± SEM (*$p < 0.05$, $n = 3$ by independent t-test).

decreased with atorvastatin compared to control. There were no significant differences in the cells incubated with ethanol compared to control cells.

DISCUSSION

Human apolipoproteins B100 and B48 are the major structural components of VLDL and LDL and of chylomicrons, respectively, which are considerable risk factors for cardiovascular disease. A previous study in our laboratory has demonstrated that red wine polyphenolics suppress the secretion of apoB100 in human HepG2 liver cells (*9*). The results herein show that red wine polyphenolics suppressed the production and secretion of apoB48 in human intestinal CaCo-2 cells by regulating cholesterol production within the cell. The decrease in secretion of atherogenic chylomicrons from intestinal cells incubated with red wine polyphenolics may explain the reduction in CVD mortality rates following its consumption.

Our results suggest that decreased cholesterol availability in the presence of dealcoholized red wine, alcoholized red wine, and atorvastatin may decrease the secretion of apoB48 from intestinal cells. ApoB48 is mainly synthesized and secreted by intestinal cells as chylomicrons before it is converted to chylomicron remnants in circulation. The formation of intestinal apoB48-containing lipoprotein particles is a complex process that requires the coordinated synthesis and assembly of apoB48, triglyceride (TG), cholesterol esters, phospholipids, and other components (*16*). Regulatory mechanisms for chylomicron metabolism in the intestine are thought to be similar to VLDL metabolism in the liver. We believe it is lipid synthesis and not protein synthesis that is affected. Previous studies have demonstrated that apoB secretion is primarily regulated posttranslationally as apoB production rates are directly correlated with the fraction of newly synthesized apoB that escapes intracellular degradation during translocation across the endoplasmic reticulum (*17*). It is the availability of lipid that seems to influence the proportion of newly synthesized apoB that is degraded (*18*). In the endoplasmic reticulum, microsomal triglyceride transfer protein (MTP) catalyzes the transfer of the lipids cholesterol ester and triglyceride (*19*) to the apoB molecule (*20*). If this step does not occur, the apoB molecule is degraded by proteases and a lipoprotein molecule is not produced. However it is unclear whether the regulation exclusively involves triglyceride, cholesterol, cholesterol ester, phospholipids, or a combination of these. Many in vitro (*21*, *22*) and in vivo (*23*, *24*) studies have reported the importance of cholesterol and cholesterol ester in the control of apoB secretion. Cell cultures studies have shown that if cholesterol availability is limited by introducing atorvastatin, an HMG-CoA reductase inhibitor (HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis), then apoB secretion will also be attenuated (*10*). Previous studies in our laboratory have demonstrated that red wine polyphenolics suppress the secretion of proatherogenic lipoproteins (very low density lipoproteins) from human hepatic HepG2 cells (*9*) by limiting cholesterol availability. Previous studies have also suggested that flavonoids decrease apoB100 secretion due to increased degradation of apoB100 in the cell. Collectively, these studies suggest that apoB in cells is degraded due to the limited availability of cholesterol (*25*−*27*).

The effect of polyphenolics on the synthesis and secretion of apoB48 has not previously been investigated in intestinal CaCo-2 cells. However, the effect of other phenolic compounds on apoB100 synthesis and secretion has been investigated in HepG2 liver cells (*26*, *27*). Previous studies have shown that citrus and soy flavonoids decrease apoB100 secretion from liver cells due to increased degradation of apoB100. In these studies, increased degradation of apoB100 secretion in the presence of these phenols was also associated with reduced cholesterol mass in the cells. Similarly, our findings in intestinal cells suggest that dealcoholized and alcoholized red wine and atorvastatin all significantly suppressed intracellular total and free cholesterol levels (Table 2). We suggest that polyphenolics decrease apoB48 levels in the medium due to increased degradation, which in turn is due to limited availability of lipids. Therefore, dealcoholized and alcoholized red wine possibly attenuates the secretion of apoB48 from intestinal cells by limiting the availability of intracellular cholesterol. Atorvastatin inhibits cholesterol synthesis, and hence the availability of cholesterol within the cell, by competitively inhibiting HMG-CoA reductase (rate-limiting enzyme in the cholesterol synthesis pathway). The decrease in cellular cholesterol in the presence of statins thereby reduces apoB secretion from the cell (*28*). We believe the same mechanism may be occurring in our cells in the presence of polyphenols, on the basis of our previous studies. Data from HepG2 cells suggest that polyphenolics were similar to statins in decreasing cholesterol availability in the cells, which resulted in a suppression of apoB100 secretion and an enhancement of LDL receptor and HMG-CoA reductase mRNA levels (*9*). This effect of the polyphenolic compounds is just as powerful as the lipid-lowering drug atorvastatin.

In a 10-week trial, Vinson et al. (*6*) showed significant reductions in total and LDL cholesterol levels after chronic consumption of red wine and dealcoholized wine in hamsters. Given the marked improvement shown in these potentially atherogenic lipids over an extended period of time, similar reductions in other lipid and lipoprotein parameters, specifically chylomicrons, may occur. Acute alcoholic red wine consumption has been shown to decrease the number of apoB48 particles

present in plasma after a meal, therefore demonstrating that red wine helps to regulate lipid and lipoprotein metabolism (*29*). This is consistent with our findings in CaCo-2 cells where particle number was also decreased as assessed by apoB levels. Further investigation of the effect of long-term consumption of red wine polyphenols on chylomicron metabolism, specifically in humans, is required.

In conclusion we have shown that red wine polyphenolics may confer cardioprotective effects by modulating postprandial lipoproteins. Our studies suggest that CVD benefits may be observed with chronic consumption of red wine polyphenols by reducing basal secretion of intestinal lipoproteins.

ABBREVIATIONS

Apo, apolipoprotein; BSA, bovine serum albumin; CHD, coronary heart disease; CVD, cardiovascular disease; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NEAA, non-essential amino acid; PVDF, poly(vinylidene difluoride); RW + OH, red wine; RW − OH, dealcoholized red wine; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween-20; TG, triglyceride.

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