ABSTRACT  Epidemiologic studies suggest that the consumption of red wine may lower the risk of cardiovascular disease. The cardioprotective effect of red wine has been attributed to the polyphenols present in red wine, particularly resveratrol (a stilbene, with estrogen-like activity), and the flavonoids, catechin, epicatechin, quercetin and phenolic acids such as gallic acid. At present, very little is known about the mechanisms by which red wine polyphenols may benefit the cardiovascular system. Therefore, the aim of this study was to elucidate whether red wine polyphenolics reduce lipoprotein production and clearance by the liver. Cultured HepG2 cells were incubated in the presence of dealcoholized red wine, alcohol-containing red wine and atorvastatin for 24 h. The apolipoprotein B100 (apoB100) protein (marker of hepatic lipoproteins) was quantified on Western blots with an anti-apoB100 antibody and the enhanced chemiluminescence detection system. Apolipoprotein B100 levels in the cells and that secreted into the media were significantly reduced by 50% in liver cells incubated with alcohol-stripped red wine compared with control cells. This effect of dealcoholized red wine on apoB100 production in HepG2 cells was similar to the effect of atorvastatin. Apo B100 production was significantly attenuated by 30% in cells incubated with alcoholized red wine, suggesting that the alcohol was masking the effect of red wine polyphenols. Apo B100 production was significantly attenuated by 45% with the polyphenolic compounds resveratrol and quercetin. In addition, dealcoholized and alcoholized red wine and atorvastatin significantly increased 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA and LDL receptor binding activity relative to controls. Dealcoholized red wine also increased LDL receptor gene expression. Collectively, this study suggests that red wine polyphenols regulate major pathways involved in lipoprotein metabolism.  


KEY WORDS: • apoB100 • red wine polyphenolics • cholesterol • LDL receptor • lipoproteins

Studies on the Mediterranean and French diets show that low rates of cardiovascular disease (CVD) occur despite diets rich in saturated fat and cholesterol (1). The Lyon heart study showed a 76% reduction in clinical events in subjects consuming a Mediterranean diet (2). The Mediterranean diet is rich in polyphenolics from fruits, vegetables and wine, and these components are likely to confer some cardioprotective effects (3,4). Epidemiologic studies indicate that moderate consumption of red wine (30 g alcohol/d) lowers the risk of CVD (5–7), with alcohol drunk as wine having a stronger inverse relationship with CVD incidence than alcohol intake alone [correlation coefficient, r = −0.66 (red wine) and −0.39 (alcohol)]. However, the mechanisms by which red wine polyphenolic compounds benefit the cardiovascular system remain unclear.

The polyphenolics in red wine have been shown to have antiatherogenic properties (8–12). Those which are thought to be protective against the development of CVD are resveratrol (a stilbene, with estrogen-like activity), the flavonoids, catechin, epicatechin, quercetin and phenolic acids such as gallic acid. Some of these phenolic compounds are also present in other beverages, fruits and vegetables. The aim of this study was to elucidate the mechanisms by which polyphenolics reduce the risk of developing CVD. Central to the pathology of coronary heart disease is the accumulation of cholesterol within the intima of arterial blood vessels. Several lipoprotein types probably contribute to the initiation and progression of atherosclerosis, including remnant lipoproteins of hepatic and intestinal origin, LDL and chylomicron remnants, respectively. Arterial retention of proatherogenic lipoproteins is dependent on the level and duration of exposure and is inversely related to the size of the lipoproteins (13). Smaller more dense lipoproteins penetrate arterial tissue more readily, are preferentially retained and may be more susceptible to oxidative modification (14). On the basis of this concept, the

Nutrient-Gene Interactions

Red Wine Polyphenolics Increase LDL Receptor Expression and Activity and Suppress the Secretion of ApoB100 from Human HepG2 Cells

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POLYPHENOLS MODULATE ApoB100 SECRETION

**MATERIALS AND METHODS**

*Materials.* The human transformed hepatic HepG2 cells were purchased from ATCC (American Type Culture Collection, Rockville, MD). Cell culture media, reagents and fetal bovine serum (certified grade) were from Gibco BRL Life Technologies, Gaithersburg, MD. Flasks for tissues culture were obtained from TPP Techno plastic products (AG, Switzerland). Bicinchoninic acid protein assay reagents were obtained from Pierce, Rockford, IL. Benzamidine, phenylmethylsulfonyl fluoride, 5α-cholestone, Triton X-100, oleic acid, fatty acid-free bovine serum albumin (BSA), gallic acid, resveratrol, quercetin and other common laboratory reagents were from Sigma Chemical, St. Louis, MO. The polyvinylidene fluoride (PVDF) membrane was from Millipore, Bedford MA. Enhanced chemiluminescence (ECL) detection reagents, hyperfilm ECL, and rabbit anti-donkey immunoglobulin (IgG) were purchased from Amersham International, London, UK. Rabbit anti-human apoB antibody was purchased from DAKO A/S, Denmark. Atorvastatin was kindly donated by Parke-Davis, Ann Arbor, MI.

*Cell culture.* The HepG2 cells were grown at 37°C under 5% CO2, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 12 mg/L penicillin, 16 mg/L gentamycin, 20 mol/L HEPES, 10 mol/L NaOH, 2 mol/L L-glutamine, and 1 g/L fetal calf serum (FCS; CSL, Melbourne, Australia). Cells were grown in 175 cm2 flasks containing DMEM supplemented with 1 g/L FCS until confluent.

**Preparation of cells for treatment.** Cells were grown under the same conditions as described above. They were then subcultured from 175 cm2 flasks into 25 cm2 flasks and allowed to grow without disruption for 24 h in a medium of DMEM supplemented with FCS. The medium was replaced with fresh, prewarmed serum-free medium and cells were incubated for another 24 h. Serum-free DMEM was made by supplementing DMEM with 80 g/L BSA (fatty acid free) complexed to 50 μmol/L oleic acid (sodium salt), 22.2 mol/L glucose, 45.5 mol/L Na2CO3 and 1 mol/L sodium pyruvate. The cells were then incubated with treatment media for 24 h. The treatment media were created by adding 5 μmol/L dealcoholized red wine, 5 μmol/L alcohol containing wine, 6 mg/L ethanol, 10 μmol/L atorvastatin or specific polyphenolic compounds, 25 μmol/L gallic acid, 5 μmol/L resveratrol and 10 μmol/L quercetin to assess the effect of these compounds on apoB100 production and secretion from cells. LDL receptor expression and HMG-CoA reductase mRNA levels were measured in cells incubated with atorvastatin and deacoholized red wine.

*Cell viability test using trypan blue.* The viability of cells incubated with polyphenolic compounds (catechin, resveratrol, gallic acid, quercetin and atorvastatin), and red wine was tested using trypan blue. Morphological changes associated with apoptosis and the number of apoptotic cells were recorded.

*Measurement of total polyphenols in wine.* The total polyphenols in red wine (Cabernet Shiraz 1994, 1.3 g/L alcohol; Hardy’s Nottage Hill, McLaren, South Australia, Australia) were quantified using a colorimetric assay with Folin-Denis reagent according to the method published by the AOAC (24). This method estimates polyphenols at 760 nm in relation to a standard curve for tannic acid.

*LDL receptor binding assay.* The HepG2 cells were assayed for LDL receptor binding activity as previously described (25).

*Sterol quantitation.* Lipid was extracted from solubilized HepG2 cells for the analysis of free cholesterol using a modified method of Folch et al. (27). Briefly, 1 mL of chloroform (CHCl3) was added to cells along with internal standard (5α-cholestanol) and 25 μL of saturated salt solution to prevent the formation of a fatty acid emulsion. Samples were mixed thoroughly and centrifuged at 200 × g for 10 min. The top aqueous layer was aspirated and discarded and the lower chloroform layer dried under a stream of N2. Samples were reconstituted in hexane before gas chromatographic analysis.

To measure total cholesterol in the cells, 1 mL of 1 mol/L KOH in methanol was added to an aliquot of cells to saponify cholesterol esters. The tubes were then flushed with N2 and heated at 45°C for 1 h. After hydrolysis was complete, the solution was diluted with 2 mL of water, internal standard was added and the lipids were extracted twice with hexane (1 mL).

Free and total cholesterol were determined by comparison of peak areas with a five-point external calibration curve. An internal standard was used to correct for injection volume (1 μL) variability. Calibration was performed daily. A Perkin Elmer Autosystem XL gas chromatograph fitted with a ZB-1 dimethyl polysiloxane column (30 m × 0.25 mm, 0.5 μm film thickness, Phenomenex) 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 calculated as the difference between total cholesterol and free cholesterol.

*ApoB100 quantification in HepG2 cells and media.* Solubilized cell protein (100 μg) and purified apoB100 standards [prepared according to Zsilversmit and Shea (28)] were separated by SDS-PAGE...
using precast NuPAGE 3–8% gradient gels in a Novex Mini-Cell (Novex Instruments, Los Angeles, CA) at 150 V for 1 h. Separated proteins were electrotransferred at 30 V for 90 min onto 0.45-µm PVDF membrane. Western blotting was performed as described previously (25). Membranes were incubated with ECL substrate solution for detection of horseradish and exposed to hyperfilm ECL. Films were scanned to determine the intensity of the apoB100 or LDL receptor protein bands using a UMAX Vista S6E Flatbed Scanner. Films were then scanned using the LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, Australia) to determine the intensity of the two bands corresponding to cellular HMG-CoA reductase mRNA at 258 bp and synthetic AW109 internal standard RNA at 301 bp. The amount of LDL receptor or HMG-CoA reductase in the HepG2 cells was calculated relative to the intensity of the band for the known amount of AW109 RNA added as internal standard and was expressed as copies/mg of cellular RNA originally reverse transcribed.

**RESULTS**

**Intracellular cholesterol levels.** Alcoholic and dealcoholized red wine as well as atorvastatin all significantly decreased free, esterified and total cholesterol in cells compared with control cells (Fig. 1). Cells incubated with ethanol did not differ from control cells in free and total cholesterol (Fig 1B and C). Dealcoholized red wine decreased free cholesterol and total cholesterol levels more in cells than did atorvastatin (Fig. 1A and C).

**ApoB100 production and secretion from liver cells.** ApoB100 concentrations in cells and that secreted into the culture media were significantly reduced up to 50% in cells and media incubated with alcohol-stripped wine compared with control cells (Fig 2A and B). The effect of dealcoholized red wine on apoB100 production and secretion was similar to that of the lipid-lowering drug, atorvastatin. When cells were incubated with alcohol-stripped red wine, apoB100 production in cells and secretion into the media were attenuated by 30% compared with control cells. However, the degree of reduction was not as great with alcoholic wine compared with alcohol-stripped wine. When cells were incubated with ethanol alone, apoB100 production and secretion into the media were not different from that of control cells.

Cell viability was determined using the trypan blue exclusion test. There was no difference in cell viability between control cells and cells treated with dealcoholized or alcoholized red wine polyphenolics, atorvastatin or ethanol (data not shown).

**LDL receptor activity.** LDL receptor binding activity was significantly enhanced in cells incubated with alcohol-stripped red wine compared with control cells (Fig 3A). The effect of dealcoholized red wine on LDL receptor binding activity was similar to that of atorvastatin. Cells incubated with alcoholic red wine also had increased LDL receptor activity compared with control cells but activity was not as great as in cells incubated with dealcoholized red wine. Cells incubated with alcohol alone did not differ from control cells.

The LDL receptor mRNA levels in the cells in the presence of dealcoholized red wine was measured (Fig. 3B). LDL receptor mRNA levels increased up to 350% when cells were incubated with increasing concentrations from 0 to 10 µmol/L of dealcoholized red wine (gallic acid equivalents).

**HMG-CoA reductase expression.** Atorvastatin significantly increased the level of HMG-CoA reductase mRNA compared with control cells (Fig. 4). Cells incubated with dealcoholized red wine and alcoholized wine had similarly enhanced levels of HMG-CoA reductase mRNA, whereas ethanol alone had no effect.

**ApoB100 secretion.** Incubation of cells with resveratrol and quercetin significantly decreased apoB100 secretion compared with control cells (Fig. 5). Gallic acid also significantly decreased apoB100 secretion but not as much as the phytochemicals.
DISCUSSION

The aim of the present studies was to determine whether polyphenolics affect apoB100 production and secretion in HepG2 cells. Red wine polyphenolics were chosen as the medium for administration of polyphenolics into cells. When cells were incubated with dealcoholized red wine, apoB100 secretion was significantly suppressed by 50% compared with control cells. This effect was similar to the effect of the lipid-lowering drug, atorvastatin. Both atorvastatin and dealcoholized wine decreased cholesterol availability in the cells; this lipid regulates VLDL synthesis and secretion (31,32). Generally, a decrease in intracellular cholesterol also triggers the cell to upregulate the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, and also LDL receptor gene expression, to increase cholesterol concentrations in the cell (33,34). Dealcoholized wine, alcoholized wine and atorvastatin upregulated HMG-CoA reductase mRNA and dealcoholized red wine increased LDL receptor mRNA levels compared with control cells. Consequently, LDL receptor binding activity was also increased in cells incubated with these agents. Collectively, our data 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. Thus, polyphenolics appear to regulate lipoprotein metabolism in liver cells via a mechanism similar to that of atorvastatin.

Our results suggest that decreased cholesterol availability in the presence of dealcoholized red wine and atorvastatin may decrease the secretion of apoB100 from hepatocytes. ApoB100 is synthesized and secreted by hepatocytes mainly as VLDL before it is converted to LDL in circulation. The formation of hepatic apoB100 containing lipoprotein particles is a complex process that requires the coordinate synthesis and assembly of apoB100, triglyceride, cholesterol esters, phospholipids and other components (32). Studies suggest that apoB100 secretion is primarily regulated post-translationally because...
apoB100 production rates are directly correlated to the fraction of newly synthesized apoB100 that escapes intracellular degradation during translocation across the endoplasmic reticulum (35). It is the availability of lipid that seems to influence the proportion of newly synthesized apoB100 that is degraded (36). However it is unclear whether the regulation exclusively involves triglycerides, cholesterol, cholesterol esters, phospholipids or a combination of these. Many in vitro (37,38) and in vivo (39,40) studies have reported the importance of cholesterol and cholesterol esters in the control of apoB100 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 apoB100 secretion will also be attenuated (31). Previous studies have suggested that flavonoids decrease apoB100 secretion due to increased degradation of apoB100 in the cell. These studies suggest that apoB100 in liver cells is degraded due to the limited availability of cholesterol (19,41,42). Borradaile et al. (40,41) showed that citrus and soy flavonoids decrease apoB secretion from liver cells due to increased degradation of apoB100. Increased degradation of apoB100 secretion was also associated with a reduced cholesterol mass in the cells. Similarly, we found that dealcoholized red wine and atorvastatin both significantly suppressed intracellular total, esterified and free cholesterol levels (Fig. 1). We suggest that polyphenolics decrease apoB100 levels in the medium due to increased degradation, which in turn is due to the limited availability of lipids. Therefore, dealcoholized red wine possibly attenuates the secretion of apoB100 from liver cells by limiting the availability of intracellular cholesterol.

Alcoholized wine did not decrease apoB100 secretion to the same extent as dealcoholized wine, suggesting that the presence of ethanol masked the effects of the polyphenolics. Consistent with these findings, alcohol-containing wine did not decrease intracellular cholesterol levels as much as alcohol-stripped wine, suggesting that increased lipid availability due to ethanol stimulated apoB100 secretion. Collectively, our data indicate that the presence of alcohol in red wine masks some of the beneficial effects of the polyphenolics.

Incubation of cells with the polyphenols, resveratrol and quercetin, significantly decreased apoB100 secretion compared with control cells (Fig. 5), suggesting that these compounds may be responsible for the attenuation in apoB100 secretion in cells incubated with alcohol-stripped red wine. The concentrations of these compounds used were equivalent to those found in plasma after the consumption of red wine.

The hepatic LDL receptor is critical for removing cholesterol-carrying lipoproteins from the circulation, thereby directing these particles away from atherogenic sites (16). Our results showed that red wine polyphenolics upregulated the LDL receptor gene expression and binding activity (Fig. 3), similar to atorvastatin. The observed increase in LDL receptor expression may be related to a decrease in intracellular cholesterol levels. Similar to our findings, Wilcox et al. (19), demonstrated that incubation of liver cells with citrus flavonoids caused a fivefold increase in LDL receptor mRNA levels and a twofold increase in LDL binding activity. The expression of the LDL receptor in the liver is tightly regulated and geared to maintain an optimum cellular content of cholesterol (17). When cellular cholesterol synthesis is suppressed; conversely, when cellular cholesterol declines, synthesis increases. Cholesterol balance is also maintained in the cell by sterol-mediated feedback repression of several other genes including HMG-CoA reductase and HMG-CoA synthase, both of which are involved in the synthetic pathway of cholesterol. These genes are regulated coordinately by the sterol status of the cell in parallel with the LDL receptor (43). However, the expression of the LDL receptor is regulated mainly at the transcriptional level, whereas HMG-CoA reductase is regulated at the transcriptional, translational and protein levels (43).

We demonstrated previously that green tea polyphenolics decrease intracellular cholesterol levels in cells, resulting in upregulation of the LDL receptor and HMG-CoA reductase (44). Green tea polyphenolics also stimulate the production of active sterol regulatory element-binding protein (SREBP) in the nucleus as a result of decreased cholesterol levels in cells. The work described here showed that red wine polyphenolics, similar to green tea polyphenolics, might be involved in regulating LDL receptor and HMG-CoA reductase transcription through a common transcriptional factor, such as SREBP-1.

Red wine polyphenolics may act like a HMG-CoA reductase inhibitor agent, similar to atorvastatin. HMG-CoA reductase inhibitors are usually competitive inhibitors (45). However, treatment with these inhibitors usually results in large increases in the mRNA and protein for HMG-CoA reductase, the LDL receptor and other genes. This may be a consequence of a decreased supply of cholesterol in the liver in the presence of these agents. A study by Vinson et al. (12) demonstrated that hyperlipidemic hamsters fed dealcoholized red wine had a 45% reduction in plasma LDL concentrations relative to the control hamsters drinking water. Plasma total triglycerides and cholesterol levels were decreased by 13 and 25%, respectively. The hamsters fed dealcoholized wine had significantly less atherosclerosis (−22%) than controls. Collectively, our cell data suggest that the decrease observed in circulating lipoproteins in hamsters fed dealcoholized red wine may be a result of increased LDL receptor binding activity and a suppression of hepatic lipoprotein production.

Red wine consumption has modest effects on LDL cholesterol levels in humans; however, particle number has never been measured. LDL particle size (small, dense vs. large, buoyant) and LDL concentration (i.e., apoB100 number) contribute to an atherogenic phenotype. Small dense LDL penetrate arterial tissue more easily, are preferentially retained within the subendothelial space and are more readily oxidized (14). Red wine polyphenols may attenuate cardiovascular risk by...
modulating LDL phenotype without influencing cholesterol levels. The effect of polyphenols on LDL size and number has not been reported previously. In vitro experiments in our laboratory showed that exposure of human liver cells to physiologic levels of wine polyphenolics (range of 0–50 μmol/L) generated fewer particles and was comparable to the effects of atorvastatin (Fig. 2). Similar changes in the number of lipoprotein particles secreted were observed when individual polyphenolics, gallic acid, quercetin and resveratrol were examined (Fig. 5). Another supposition is that polyphenolic cardioprotective effects may be due to a reduction in LDL oxidation. In vitro, red wine catechins have been shown to inhibit LDL oxidation better than ascorbic acid and vitamin E (46). Interestingly, in another study, gallic and caffeic acids did not affect LDL oxidation ex vivo, indicating that their beneficial effect in preventing coronary artery disease may be not related to antioxidant effects (47).

Our data suggest that the effect of alcoholized red wine on lipid and lipoprotein metabolism was mediated by the polyphenolics and not the alcohol component. Ethanol alone had no effect on apoB100, LDL receptor activity or HMG-CoA reductase mRNA level compared with the control. This would suggest that the effect of both alcoholized and dealcoholized wine on these pathways could be attributed to the polyphenolics present in the red wine. Collectively, our data suggest that it was the polyphenolics in the red wine, and not the alcohol component, that was affecting these major pathways in lipoprotein metabolism.

Our present cell culture findings suggest that red wine polyphenolics decrease the intracellular levels of cholesterol, which consequently appears to trigger a feedback mechanism involving the upregulation of LDL receptor and HMG-CoA reductase expression. Limited cholesterol availability also appears to increase the degradation of apoB100 in cells, resulting in an attenuation of apoB100 secretion from cells. Collectively, these results may explain why circulating VLDL and LDL levels are attenuated in hamsters supplemented with red wine polyphenolics. Because decreasing the production and secretion of atherogenic lipoproteins decreases the risk of development of CVD, diets supplemented with red wine polyphenolics may provide beneficial effects in humans.

LITERATURE CITED


