Apple-procyanidins decrease cholesterol esterification and lipoprotein secretion in Caco-2/TC7 enterocytes

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Abbreviations: LDH, lactate dehydrogenase; XTT, sodium 3'-[(1-phenylaminocarbonil)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate, M

ABSTRACT

Decrease of plasma lipid levels by polyphenols was linked to impairment of hepatic lipoproteins secretion. However, intestine is the first epithelium that faces dietary compounds and contributes to lipid homeostasis by secreting triglyceride–rich lipoproteins during the postprandial state. The purpose of this study was thus to examine the effect of apple and wine polyphenols extracts on lipoprotein synthesis and secretion in human, Caco-2/TC7 enterocytes apically supplied with complex lipid micelles. Our results clearly demonstrate that apple, but not wine polyphenols extract, dose-dependently decreases the esterification of cholesterol and the enterocyte secretion of lipoproteins. Apple polyphenols decrease apoB secretion by inhibition of apoB synthesis without increasing the degradation of the newly synthesized protein. In our conditions, cholesterol uptake, apoB mRNA and MTP activity were not modified by apple polyphenols. The main monomers present in our mixture did not interfere with the intestinal lipid metabolism. By contrast, apple-procyanidins reproduced the inhibition of both cholesterol ester synthesis and lipoprotein secretion. Overall, our results are compatible with a mechanism of action of polyphenols resulting into an impaired lipid availability that could induce inhibition of intestinal lipoprotein secretion and contribute to the hypolipidemic effect of these compounds *in vivo*.

INTRODUCTION

Fruits and vegetables consumption is consistently associated with a decrease in lipemia along with a decrease in the risk for cardiovascular diseases (CVD) and stroke, diabetes, and obesity (1-3). Such effects were attributed to dietary fibers and phenolic compounds. Polyphenols, which are abundant in fruits, vegetables and beverages, e.g. tea and red wine, are very heterogenous and their main classes are defined from the nature of their carbon skeleton (4). Flavonoids, the most abundant polyphenols in our diet, can be divided into several classes : flavones, flavonols, isoflavones, anthocyanins, flavanols, procyanidins and flavanones. The protective effect of flavonoids against CVD has been attributed to several mechanisms, including inhibition of LDL oxidation (5) and of platelet aggregation (6), and improved endothelial function (7). More recently, it has been shown that flavonoids could interfere with the synthesis and secretion of triglyceride-rich lipoproteins (TRL) in hepatocytes (8-10).

In addition to hepatocytes, enterocytes are able to synthesize intestine-specific TRL, namely chylomicrons, the secretion of which ensure the delivery of dietary lipids to the body. TRL are composed of a core of neutral lipids, mostly triglyceride (TG) but also cholesterol esters (CE), surrounded by a monolayer of amphipathic lipids such as phospholipids and cholesterol. Apolipoprotein B (apoB) plays a major structural role in the assembly of TRL and is required for their secretion. In humans, hepatic TRL, i.e. VLDL, comprise an apoB100 isoform, whereas intestinal TRL, i.e. chylomicrons, comprise an apoB-48 isoform, resulting from the edition of apoB mRNA. TRL assembly has been mostly characterized in hepatocytes as a two-steps process : the formation of a lipid-poor apoB-particle , followed by its fusion with a TG-rich apoB-free lipid droplet under the action of the microsomal triglyceride protein

(MTP) (for review see (11, 12)). However, major differences in TRL formation exist between hepatocytes and enterocytes, including the mode of fatty acid supply. In hepatocytes, fatty acids are brought by plasma at the basolateral pole of the cell. In enterocytes, dietary lipids, after their emulsion by bile and their subsequent digestion by pancreatic enzymes, are provided at the apical pole of enterocytes as complex lipid micelles made of fatty acids (FA), monoacylglycerol (MG), lysophospatidylcholine, cholesterol and biliary salts (13). After absorption, FA and MG are used for intracellular *de novo* synthesis of TG. Concomitantly, absorbed cholesterol is esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT), but its requirement for chylomicron assembly and secretion is still debated (14).

Though postprandial lipemia is a physiological phenomenon occurring several times a day after each ingestion of dietary fat, clinical data show a correlation between postprandial lipemia and the progression of coronary artery disease (15). Indeed, exaggerated postprandial hyperlipidemia, resulting from an excess of dietary sources or an ineffective clearance of TRL, leads to an accumulation of TRL remnants in plasma that may have adverse effects on vascular endothelium (16). In this context, it is obvious that modulation of intestinal lipoproteins secretion should play an important role in the control of CVD risk factors.

Apples and wine being demonstrated to reduce plasma lipids levels and atherosclerosis progression (17-19), the objective of our study was to examine the potential role of polyphenols on the synthesis and secretion of intestinal lipoproteins using the human enterocyte Caco-2/TC7 cells (20). To get closer to physiology in analysing intestinal lipid metabolism, Caco-2/TC7 cells were cultured on microporous filters which delineate apical and basal compartments. Dietary lipids were supplied in

the apical compartment as complex micelles (21) mimicking the postprandial duodenal content after a fat-rich meal (13).

Our results clearly demonstrate that a procyanidin-enriched fraction isolated from apple polyphenols extract decreases the esterification of cholesterol and impairs the intestinal lipoprotein secretion. Such results may account for the protective effect of polyphenols against postprandial hypertriglyceridemia observed *in vivo*.

MATERIALS AND METHODS

Materials

Unless indicated, all chemicals were purchased from Sigma (Saint-Quentin-Fallavier, France). Applephenon C100, an apple (*Pyrus Malus*) polyphenols extract, was from Jan Dekker France (St Germain en Laye, France). Wine polyphenols were supplied by Vin et Santé (ONIVINS, Paris). Chlorogenic acid, phloridzin, (-)-epicatechin, (+)-catechin were from Extrasynthese, Genay, France. [1-^{14}C]-labeled oleic acid and [1, 2-^{3}H(N)] cholesterol were purchased from ICN (Orsay, France) and [^{35}S]-methionine/cysteine mix from NEN (Perkin-Elmer, Courtaboeuf, France). The rabbit anti-human ApoB antibodies were generously provided by Dr A. Mazur (INRA, Theix, France) and the peroxidase-conjugated goat anti-rabbit antibody was purchased from Vector Laboratories (Burlingame, CA). CP-113818 was generously provided by Dr O. Francone and N. Fournier.

Cell culture

Caco-2/TC7 cells (20) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, glucose 25mM and glutamax) from GibcoBRL (Life Technologies, Cergy Pontoise, France) supplemented with 20% heat inactivated fetal calf serum (Abcys, France), 1% non essential aminoacids (GibcoBRL), penicillin (100 IU/ml) and streptomycin (10µg/ml) (GibcoBRL) and maintained under 10% CO_2 atmosphere at 37°C. Cells were used between passages 13 and 34. For experiments, cells were seeded at a density of 50x10^3 cells/cm^2 on microporous (1µm pore size) PET membranes (Falcon, Becton Dickinson, Meylan, France). The medium was changed in both compartments two days after seeding and daily thereafter. Transepithelial electrical resistance (TEER) was regularly measured using a Millicell-ERS apparatus

(Millipore, St Quentin en Yvelines, France) to assess confluence, which is usually reached one week after seeding. After confluence, cells were switched to asymmetric conditions with a serum-free medium in the upper compartment for one week more. Polyphenols were dissolved in ethanol and supplied on the apical side of cell culture such as the maximum final concentration of ethanol was 1%. The number of surviving cells was measured by the XTT-{sodium 3'-[(1-phenylaminocarbonil)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate} assay (Cell Proliferation Kit II; Roche Biochemicals, Meylan, France) following a 24 hours incubation period.

Micelles preparation and incubations

Lipids were brought to the cells as a complex lipid emulsion containing 0,6 mM oleic acid, 0.2 mM L-α-lysophosphatidylcholine, 0.05 mM cholesterol, 0.2 mM 2-monooleoylglycerol and 2 mM taurocholic acid. Preparation of micelles was done as previously described (21). Where appropriate, micelles were supplemented with 2 µCi [1-^{14}C]-oleic acid (56 mCi/mmol, ICN) per ml final volume of medium and, according to experiment, 2µCi of [1, 2-^{3}H(N)]-cholesterol (35-50 mCi/mmol) per ml final volume of medium. Freshly prepared micelles-containing medium (1.5 ml) was placed in the upper compartment.

Analysis of lipids

Lipids extraction from media (1 ml) or sonicated cell lysates (0.1 ml adjusted to 1 ml with serum-free medium) were carried out as previously described (21) except that counting was performed in a Tri-Carb scintillation counter (Packard, Rungis, France).

Western blotting

Immediately after collection, cell culture media (200 µl) were mixed with 20 µl of PBS 10X containing 10% triton X100, 50 mM EDTA and 2 µl of a protease inhibitor cocktail (P8340, Sigma), then stored at –20°C until analysis. Culture media (20 µl) were boiled for 4 min in Laemmli buffer and fractionated under reducing conditions in a 6% polyacrylamide gel. Proteins were transferred on Bio-Rad nitrocellulose membranes for two hours at 50V using a mini-Trans-Blot Cell (Bio-Rad) in 25 mM Tris, 192 mM glycine. The membrane was soaked overnight at 4°C in TBS-T (20 mM Tris-HCl, PH 7.6, 137 mM NaCl, 0.1% Tween-20) containing 10% non fat dry milk. Blots were probed for one hour at room temperature using rabbit polyclonal anti-apo B antibodies (1/10000), then peroxidase conjugated goat anti-rabbit immunoglobulins and developed using ECL western blotting reagents according to the manufacturer's instructions (Amersham-Pharmacia, Orsay, France). Films were scanned and quantified using Scion Image software.

Separation of lipoproteins

After incubation for 30 min in a cys/met-free medium (GibcoBRL), cells were incubated during 24h in a cys/met-free medium supplemented with 150 µCi/ml [^{35}S]-cys/met in the apical compartment in the presence or not of lipid micelles. After collection, basolateral media were immediately adjusted to 0.005 % gentamicin, 1 mM EDTA, 0.04% sodium azide and 0.02% sodium(ethylmercurithio)-2 benzoate. Media (4 ml) were layered under 4 ml of 0.15 M NaCl and centrifuged at 10 000 x g for 30 min at 20°C. The 1.5 ml top fraction, containing chylomicrons, was recovered and washed once by ultracentrifugation at 100 000 x g for 18h in a new tube containing 4 ml of culture medium containing 20 % FCS and adjusted to 8 ml with 0.15 M NaCl. The chylomicron-depleted basolateral media were adjusted to 8 ml with 0.15M NaCl and sequential ultracentrifugation was performed, as described

previously (22), to isolate lipoproteins that float at the density range of the major human lipoprotein classes (VLDL, LDL and HDL) i.e. 100 000 x g and 10 °C for 18 h at density 1.006, for 20 h at density 1.063 and for 40 h at density 1.21, respectively. [^{35}S]-Meth/Cys labeled fractions of d>1.006 were dialyzed against PBS then all fractions were concentrated using YM10 centricons (Millipore) and adjusted to 120 µl. Samples (20 µl) were separated onto 6 % SDS-PAGE and gels were processed for fluorography and/or placed against Phosphorimager screens for the quantification of individual bands corresponding to apoB100 and apoB48.

Pulse-chase analysis

After incubation for 1h in a cys/met-free medium (GibcoBRL), cells were pulsed for 30 min in cys/met-free medium supplemented with 200 µCi/ml [^{35}S]-cys/met and lipid micelles in the apical compartment and in the presence or not of polyphenols. After the pulse, cells were rinsed and then chased, for 3 hrs, in the presence of an excess of unlabeled methionine (10 mM) and cysteine (2mM) in both compartments, and with lipid micelles in the presence or not of polyphenols in the apical one. Cell lysates were prepared as described above and centrifuged to remove cell debris. Basolateral media were collected and adjusted to 2% of protease inhibitors cocktail. For each sample to immunoprecipitate, 40 µl of a 30% suspension of protein A-Sepharose beads (Pharmacia) in buffer 1 (1% BSA, 0.1% sodium azide in 0.1 M sodium phosphate buffer pH8) were incubated for 2h at room temperature with 200 µl of rabbit polyclonal anti-apoB antibodies (1/100 in the same buffer). The beads were washed twice in buffer 1, once in buffer 2 (0.2% BSA, 1% triton X100, 0.1% sodium azide in 0.1 M sodium phosphate buffer pH8) and then 200 µl of cell lysate containing 200 µg of proteins, or 200 µl of basolateral media, were added. After 90 min incubation at 4°C, the beads were washed four times with buffer 2, twice with 0.1M

phosphate buffer pH 8, and once with 10 mM phosphate buffer pH 8. The beads were boiled for 4 min in 50 µl of Laemmli buffer. Aliquots were counted in Optiphase Highsafe 2 scintillation fluid in a Beckman scintillation counter.

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was prepared according to the manufacturer's instruction (Tri Reagent, Euromedex, France). cDNA was synthesized from 1 µg of total RNA in 20 µl using random hexamers and murine Moloney leukemia virus reverse transcriptase (Invitrogen, Cergy Pontoise, France) as recommended by the manufacturer. Synthesis of primers for ACAT1, ACAT2, MTP and APOB gene (table 1) was done by Proligo (France). Quantitative RT-PCR analyses were performed with 50 ng of reverse transcribed total RNA in 1X LC FastStar DNA Master SYBR Green I buffer (Roche Diagnostics, Meylan, France), 3 mM $MgCl_2$, 200 nM of each sense and antisense primers in a final volume of 20 µl using the LightCycler apparatus (Roche Diagnostics, Meylan, France). Quantification of HMGCoA reductase, and 18S rRNA gene expression were done using Assays-on-demand and Pre-developed Taqman Assay Reagent respectively from Applied Biosystems (Les Ulis, France) and FastStart DNA Master Hybridization probes (Roche Diagnostics, Meylan, France) as recommended by manufacturer. Control Caco-2/TC7 lipid-induced total RNA was used to make standard curves. In summary, cDNA of control cells was diluted into tenfold serial dilutions (10^{-1} to 10^{-6}), amplified along with samples and plotted in arbitrary units to acquire quantitative data with the LightCycler software. After expression of data as percentage relative to control (micelles), mRNA levels of target genes were normalized to 18S rRNA and expressed in arbitrary units. All samples were assayed in duplicate, and the average value of the duplicates was used for quantification.

Preparation of Procyanidin-enriched fraction

Procyanidin-enriched fraction was prepared from Applephenon C100. Procyanidins-enriched fraction was purified on a Sephadex LH-20 column (55x3 cm i.d.; 100 µm; Pharmacia) from 1.7 g of Applephenon C100. The following eluents were successively applied: water (1000 mL), methanol/water 1:1 (3200 mL), and acetone/water 7:3 (220 mL). Procyanidin-enriched fraction (in the acetone/water eluent) and procyanidin-depleted fractions (in the methanol/water eluent) were dried by removing the solvents under reduced pressure and freeze-drying. 169 mg were recovered from the procyanidin-enriched fraction. Bidimensional chromatography was carried out with high-performance cellulose plates on aluminum foils (10x10 cm, 0.1 mm, Merck). Eluent for procyanidins were t-buthanol/acetic acid/water 3:1:1 (by vol., eluent A), acetic acid/water 6:94 (eluent B). Plates were sprayed with a mixture of equal volumes of aqueous $K_3Fe(CN)_6$ (2% m/v) and $FeCl_3$ (2% m/v) to visualize phenolic compounds.

Microsomal triglyceride transfer protein (MTP) activity assay

MTP activity was measured by an MTP assay Kit according to the manufacturer's instructions with some modifications (Roar Biomedical, New York, USA). The assay is based on a transfer of fluorescence, due to MTP activity, between donor and acceptor vesicles. Caco-2/TC7 cells were scraped and sonicated in homogenization buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1/50 protease inhibitor cocktail. The MTP assay was performed by incubating 200 µg protein with 10 µl of donor and 10 µl of acceptor solutions in a total volume of 250 µl cell homogenates and incubated for different times at 37°C. MTP activity was calculated by measuring fluorescence at 465 nm excitation wavelength and 538 nm

emission wavelength every 20 min using the Fluostar Ascent FL (Labsystems S.A., Paris, France). Results are expressed in arbitrary units calculated as fluorescence intensity transferred per min.

Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed by the Student's *t* test for unpaired data.

RESULTS

Polyphenols were supplied at the apical side of Caco-2/TC7 cells, using concentrations similar to those reached in physiological conditions. Indeed, the maximal dose of apple or wine polyphenols extracts used in this study corresponded to the consumption of three apples or half a litter of wine per day, respectively (4). These concentrations did not induce any toxic effect, as assessed by XTT assay and lactate dehydrogenase (LDH) activity measurement (data not shown).

Apple polyphenols decrease the intracellular incorporation of [1-^{14}C] oleic acid into cholesterol ester and inhibit lipids secretion by Caco-2/TC7 cells

In order to evaluate the effect of apple or wine polyphenols on the intestinal lipid metabolism and secretion, cells were supplied during 24h, in the apical medium, with complex micelles containing [1-^{14}C] oleic acid, and increasing concentrations of polyphenols. As shown in **Fig. 1**, 200 and 500 μg/ml of apple polyphenols reduced the intracellular accumulation of [1-^{14}C]-oleate incorporated into cholesterol ester by 45% (0.38 ± 0.04 vs 0.69 ± 0.11 nmol / mg. prot; $p<0,01$) and 64 % (0.25 ± 0.03 vs 0.69 ± 0.11 nmol / mg prot.; $p<0,001$), respectively. No difference was observed for the intracellular accumulation of newly synthesized phospholipids or triglycerides. By contrast, wine polyphenols exerted no effect on [1-^{14}C]-oleate incorporation into cholesterol ester, phospholipids and triglycerides.

In the same time, apple polyphenols dose-dependently lowered the secretion of all classes of newly synthesized lipids, resulting at 500 μg/ml in a 77% decrease in cholesterol esters (0.032 ± 0.008 vs 0.14 ± 0.02 pmol / dish; $p< 0.001$), a 61% decrease in phospholipids (0.24 ± 0.07 vs 0.61 ± 0.12 nmol / dish; $p<0.001$) and a 92% decrease in triglycerides (0.16 ± 0.1 vs 1.9 ± 0.4 nmol /dish; $p <0.001$) in the

basal media. Again, no effect on the secretion of lipids was observed with wine polyphenols, whatever their concentration.

Apple polyphenols decrease apoB and lipoproteins secretion

Lipids being secreted associated with apoB, we quantified by western blotting the apoB secreted in the basal medium after 24h of contact with micelles supplemented or not with apple polyphenols. Micelles supply induced a 4-fold increase in apoB48 secretion in the basal medium (**Fig 2A**). The addition of apple polyphenols dose-dependently decreased the secretion of apoB48, as compared to micelles added alone (65% and 81% at 200 and 500 µg/ml, respectively). To further characterize the effect of polyphenols on the pattern of secreted lipoproteins, cells were incubated for 24 hours in the presence of [^{35}S] met/cys, with or without micelles that were supplemented or not with 200 µg/ml apple polyphenols. Then, basolateral media were subjected to sequential ultracentrifugation, and the recovered fractions were analysed by SDS-PAGE and fluorography. Results are shown in **Fig. 2B**. As reported elsewhere (23), micelles supply dramatically increased the secretion of triglyceride-rich lipoproteins (d<1.006 g/ml) (compare lane 1 and 3). For cells that were supplied with micelles (lanes 3 and 4), polyphenols addition resulted in the inhibition of both apoB100 and apoB48 secretion. Moreover, this effect was observed in all the lipoprotein fractions. Interestingly, the inhibitory effect of polyphenols on apoB100 and apoB48 secretion was found very efficient on cells that were not incubated with micelles. Indeed, the secretion of triglyceride-rich lipoproteins (d<1.006 g/ml) was not detectable and that of lipoproteins floating at d>1.006 g/ml was strongly impaired (lane 2).

Apple polyphenols inhibit [^{35}S] met/cys incorporation into apoB

Previous studies have demonstrated that decrease of apoB secretion could be due to a decrease of its synthesis or to an increase of its degradation (24, 25). In order to identified the points of control of apoB decrease under supplementation with apple polyphenols, pulse-chase experiments were performed. After 30 min pulse, incorporation of [^{35}S] met/cys into apoB decreased by 60% when 500 µg/ml apple polyphenols were added on the apical medium (figure 3A). Over the 3h chase, the newly synthesized apoB remained at the same level with or without apple polyphenols (figure 3B), thus indicating that apple polyphenols inhibit early step of apoB synthesis and do not increase apoB degradation. As shown in figure 3C, after 3h chase, labeled secreted apoB secretion was decreased by 66% in the presence of apple polyphenols, i.e. in the same range that inhibition of apoB synthesis (figure 3A) and inhibition of apoB48 secretion over 24h period of treatment with apple polyphenols (figure 2A).

MTP activity is not affected by apple polyphenols

It is well known that lipid transfer by MTP enzyme is necessary for the lipidation of nascent apoB and this enzyme is a major actor in the assembly and subsequent secretion of intestinal lipoproteins (26). As it was previously shown that MTP activity was inhibited by flavonoids such as naringenin, hesperitin and quercetin (9, 27), we tested the possibility that apple polyphenols impair MTP activity in our model. MTP activity was measured after 24h incubation with or without 200 µg/ml apple polyphenols and micelles in the apical compartment. As shown in **Fig. 4**, no obvious change was induced by micelles and/or apple polyphenols supply. Similar results were obtained after 2, 4 or 8 hours of incubation (data not shown) indicating that changes in MTP activity did not account for the decrease in TRL secretion that was induced by apple polyphenols.

Apple polyphenols inhibit cholesterol esterification but not cholesterol uptake

Since reports have suggested that the level of cholesterol esters might regulate apoB secretion in Caco-2 cells (28, 29), we investigated whether apple polyphenols could interfere with cholesterol uptake and/or cholesterol esterification, and in turn hinder lipoprotein secretion. As quantified by intracellular [1,2-^{3}H(N)]-cholesterol (**Fig 5A**), apple polyphenols did not impair the intracellular accumulation of free cholesterol from micelles, suggesting that apple polyphenols did not interfere with cholesterol uptake or catabolism. In contrast, the incorporation of [1-^{14}C]-oleic acid into cellular cholesterol esters was decreased by 38% (2375±643 vs 3779±248 cpm/mg protein, $p<0.05$) after 4h and by 47% (5863±541 vs 10791±1409 cpm/mg protein, $p<0.01$) after 8h incubation (**Fig. 5B**). Moreover, measurement of [1,2-^{3}H(N)] cholesterol esters showed a 68% inhibition (11528±1933 vs 34692±5798 cpm/mg protein, $p<0.01$) at 8h (**Fig. 5C**). Altogether these data suggest that ACAT activity was inhibited by polyphenols. Wilcox and colleagues recently demonstrated that flavonoids decreased ACAT activity as well as ACAT2 gene expression in HepG2 cells (9). We therefore quantified ACAT mRNA levels by relative-quantitative real-time RT-PCR. As shown in **Table 2**, the addition of apple polyphenols in the apical compartment did not induce any modification of ACAT2, ACAT1, MTP, apoB and HMGCo-A reductase mRNAs, underlying differences between hepatocytes and enterocytes. Thus, in the enterocytes, our results imply that ACAT is inhibited by polyphenols at the activity level.

Inhibition of ACAT activity is not sufficient to decrease intestinal lipoproteins secretion

In order to evaluate the effect of cholesterol esterification on intestinal lipoproteins secretion, Caco-2/TC7 cells were incubated with micelles in the presence or not of

CP-113818, an ACAT inhibitor that was shown to inhibit ACAT intestinal activity (30). CP-113818, used at 40 nM in the range of IC50 of ACAT activity in human intestine (30), inhibited the incorporation of [1-^{14}C]-oleic acid in cholesterol esters by 71% ($p<0.001$) (**Fig. 6A**). The non significant decrease in intracellular newly synthesized triglycerides and phospholipids, demonstrated the specificity of the CP-113818 inhibitor when used at this concentration. Moreover, CP-113818 decreased the secretion of cholesterol esters by 61 % while that of triglycerides, phospholipids and apoB remained unchanged (**Fig. 6 B and C**), suggesting that inhibition of cholesterol esters synthesis was not sufficient to inhibit the secretion of lipoproteins in Caco-2/TC7 cells.

Proacyanidin-enriched fraction reproduces the effects of apple polyphenols extract.

The apple polyphenols extract that we used contained procyanidins, chlorogenic acid, caffeic acid, (-)-epicatechin, (+)-catechin and phloretin-2'-glucoside (40-45%, 15-20%, 7-8%, 3-5%, 4-6% and 3-4% respectively). In order to identify the molecule(s) that could be involved in the control of intestinal lipoproteins metabolism, we carried out experiments with the different components of the extract, i.e. monomers or procyanidins. As procyanidins molecules differ in fruits and vegetables species, we performed procyanidins enrichment from total apple polyphenols extract using a Sephadex LH-20 column. All molecules were used at a concentration equivalent to that present in 200 μg/ml of apple polyphenols extract. As shown in **Table 3**, chlorogenic acid, phloridzin, (-)-epicatechin, (+)-catechin, supplied alone or mixed together, did not modify the synthesis and secretion of cholesterol esters, triglycerides and phospholipids. By contrast, the procyanidin-enriched fraction purified from apple polyphenols, but not the procyanidin-depleted fraction, induced an

inhibition of both cholesterol esters synthesis and lipids secretion, similarly to the pattern obtained with the total apple polyphenols extract (**Fig. 7**).

DISCUSSION

Phenolic compounds which are present in fruits and vegetables have hypolipidemic properties in animal models fed a high-fat and/or a high-cholesterol diet (31-33). Studies performed in the liver attributed these beneficial effects to a decrease in the assembly and secretion of hepatic apoB-containing lipoproteins (8, 34, 35). However, it must be emphasized that intestinal epithelium, the first tissue to face dietary compounds, plays a pivotal role in lipid homeostasis through TRL synthesis and secretion during the postprandial state. In the present study we demonstrate that, while polyphenols from wine had no effect, a procyanidin-enriched fraction isolated from apple polyphenols decreases the esterification of cholesterol and the secretion of apoB-containing lipoproteins by the human Caco-2/TC7 enterocytes apically supplied with lipid micelles.

We have shown that in intestinal cells, apple polyphenols lowered intracellular cholesterol ester, measured as accumulation of [1,2-^{3}H(N)]-cholesterol ester or incorporation of [1-^{14}C]-oleic acid, without changing cholesterol uptake, most likely through an inhibition of cholesterol esterification, as reported for flavonoids such as taxifolin, naringenin or hesperitin in HepG2 cells (8, 9). Two ACAT genes, ACAT1 and ACAT2, have been identified, which differ by their tissue expression and presumably their cellular function (36). Wilcox *et al* have shown that naringenin and hesperitin inhibit ACAT activity in AC29 cells stably transfected with either ACAT1 or ACAT2 cDNA (9). ACAT2 mRNA was significantly reduced when HepG2 cells were incubated with naringenin and hesperitin, whereas ACAT1 mRNA was unaffected (9). We did not observe any change in ACAT1 and ACAT2 mRNA levels in our conditions. This suggests that, in enterocytes, the polyphenols-induced decrease in

cholesterol esterification, does not occur through the control of ACAT gene expression. Altogether these results demonstrate that polyphenols interfere with esterification of both endogenous and newly absorbed cholesterol.

The second effect of apple polyphenols was to decrease the secretion of apoB100- and apoB48-containing lipoproteins. This inhibition concerned all the lipoprotein fractions and was dramatic in the absence of micelles supply. The requirement of apoB100 for triglycerides in order to escape intracellular degradation (37) explains that apoB100-containing lipoproteins mainly float at $1.006<d<1.063$ mg/ml in the absence of exogenous lipid supply. By contrast, apoB48 is almost exclusively secreted as lipid-poor lipoproteins, i.e. “HDL-like” lipoproteins as reported by Liao et al (38), corresponding to its lesser dependence towards triglycerides. In the presence of polyphenols, both apoB100- and apoB48-containing lipoproteins were barely detectable, which could indicate a blockade of apoB synthesis However, in the presence of micelles, polyphenols addition resulted in the secretion in apoB100- and apoB48-containing lipoproteins, which were recovered essentially in TG-enriched fractions. We demonstrated that apple polyphenols inhibited synthesis of apoB but did not change the degradation of the newly synthesized protein. This inhibition could occur during the initial steps of synthesis, as it was described for the action of insulin on hepatic apoB (39, 40). Furthermore, it has been recently reported in HepG2 cells that lipid availability or transfer to apoB is required for the completion of its translation and translocation across the endoplasmic reticulum membrane (41, 42).

In this regard, the amount of intracellular cholesterol esters has been proposed to control apoB secretion in HepG2 and Caco-2 cells (29, 43). In contrast, we show that the amount of secreted apoB48 in the basal medium was not decreased by the ACAT inhibitor, CP113818, despite its efficient inhibition of cholesterol esterification. This

suggests that inhibition of TRL secretion could be a specific effect of some molecules identified as inhibitors of ACAT activity. Wilcox *et al*. have shown that DuP 128, an ACAT inhibitor, failed to significantly alter apoB secretion in HepG2 cells in the presence of oleic acid but that CI-1011, another ACAT inhibitor, did (43). Altogether, these results demonstrate that the decrease of intracellular cholesterol esterification is not sufficient to explain by itself the inhibition of apoB-containing lipoprotein secretion.

Impairment of MTP activity has already been involved in the decrease of apoB secretion (12). Casaschi *et al*, using Caco-2 cells grown on filters under supply of oleic acid complexed to BSA, have shown that quercetin, a flavonoid, inhibited apoB secretion along with MTP activity and triglycerides synthesis (27). But, we did not observe any decrease in MTP activity in Caco-2/TC7 cells incubated between 2 to 24 hours with apple polyphenols indicating that inhibition of MTP could not explain the decrease of TRL secretion. An alternative explanation could be a blockade upstream the transfer of triglycerides to apoB. Indeed, secreted triglycerides derive from lipolysis of cytosolic stores followed by their re-esterification in the endoplasmic reticulum membrane and MTP-dependent transfer to the endoplasmic reticulum lumen (44). In hepatocytes, inhibition of the cytosolic triglycerides lipolysis step results in a dramatic decrease in secretion of triglycerides, cholesterol esters and phospholipids (45). Furthermore, it has been shown recently that VLDL secretion depends on a verapamil-sensitive mechanism for triglycerides accumulation in endoplasmic reticulum (46). Accordingly, our results could be explained by a blockade of triglycerides availability in the endoplasmic reticulum lumen, upstream of MTP-mediated transfer, resulting in an impairment of apoB synthesis. Another hypothesis could be that polyphenols could impair the secretory process itself.

Another important finding in our study was that the procyanidin-enriched fraction from apple polyphenols was able to reproduce the effects of the total extract. Procyanidins are oligomeric structures made of flavanols, catechin and epicatechin. They are present in plants and have attracted increasing attention in the fields of nutrition and medicine, due to their potential health benefits (47). To explain the biological effects of procyanidins, it was assumed that these molecules are bioavailable in their target tissues. However, it must be emphasized that, among all the oligomeric procyanidins structures, only monomers and dimers can be transferred through the intestinal barrier, the first epithelium that is met by these compounds. In our study, monomers were found unable to reduce cholesterol ester synthesis and lipoprotein secretion, thus suggesting that dimers or larger oligomers interfered with intestinal lipoprotein metabolism. Dimeric procyanidins were found to inhibit NF-kB activation and IL-2 release when Jurkat cells were incubated with PMA (49). Recently Eng *et al*, demonstrated that procyanidin dimers specifically inhibit estrogen biosynthesis through their binding to the active site of aromatase. In addition, they showed that mixture of procyanidin dimers was more active than individual molecules and that mice fed daily with this mixture had significant reduction in androgen-dependent-tumor growth (50). Moreover, other reports have shown that the relaxing activity of procyanidins, in rat and human artery endothelium tended to increase as a function of polymerisation degree (51, 52). Further studies are needed to analyse whether procyanidin polymers exert their inhibiting effects in our system through a signalling pathway, which remains to be determined. Finally, the differences observed between the effects of apple and wine polyphenols could depend of procyanidins content. Indeed, apple polyphenols extract contains 10% of B1 and B2 procyanidin dimers, as

compared to 1.5% in wine polyphenols extract. These differences could explain why wine polyphenols do not display the effects of apple polyphenols.

Exaggerated postprandial hyperlipemia has been found associated with cardiovascular disease, probably by accumulation of atherogenic remnants of triglyceride-rich lipoproteins in the postprandial state. Therefore, the prevention of postprandial hyperlipemia could be of importance to decrease the risk factor of cardiovascular disease. Recently, atorvastastine, a potent HMG-CoA reductase inhibitor, has been shown to improve postprandial lipoprotein metabolism by decreasing the incremental area under the curve for cholesterol and triglyceride associated with TRL in hypertriglyceridemic patients (53). A number of new agents are in development, which target the inhibition of lipids absorption (54), transport (55) or esterification (56). In this respect, procyanidins are interesting candidates, because we demonstrate in this study that these natural molecules are able to inhibit cholesterol esterification and intestinal lipoprotein secretion at nutritional amounts.

Acknowledgments

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Figure legends

Figure 1 : Synthesis and secretion of triglycerides, phospholipids and cholesterol esters in Caco-2/TC7 cells incubated with apple or wine polyphenols.

Caco-2/TC7 cells were cultured for 15 days on microporous membranes and in asymmetric conditions during the last seven days. Complex micelles containing 2µCi [1-^{14}C]-oleic acid per ml of medium and increasing concentration of apple or wine polyphenols were added on the apical side of the cells for 24 hours. Lipids were extracted from cells (cellular) and basal medium (secreted), separated by thin-layer chromatography and radioactive spots corresponding to phospholipids, triglycerides, and cholesterol esters were counted. Results are from 3 different experiments in duplicate and are expressed as nmol of [1-^{14}C]-oleic acid incorporated into lipids per mg of protein for cellular lipids and per dish for secreted lipids. $*$ $p<0.05$, $**$ $p<0.01$, $***$ $p<0.001$

Figure 2 : Effect of apple polyphenols on apoB48 and lipoproteins secretion by Caco-2/TC7 cells.

A, Caco-2/TC7 cells were cultured as described in figure 1 and incubated for 24 h with or without micelles and increasing concentrations of apple polyphenols extract. Basal media (20 µl) were subjected to 6 % SDS-PAGE and apoB was revealed by western blotting. Quantification of apoB48 was done by film scanning. Intensity of the bands was expressed as arbitrary units (AU).

B, Caco-2/TC7 cells were incubated for 24 h in medium supplemented with [^{35}S]-met/cys (100 µCi/ml), with or without micelles and 200 µg/ml of apple polyphenols.

Basal media were collected and lipoproteins were isolated by sequential ultracentrifugation. Each fraction was analysed by 6% SDS-PAGE and fluorography to visualize [^{35}S]-labeled apoB100 and apoB48. Different exposures were used to reveal chylomicrons and other lipoprotein fractions.

Figure 3 : Effect of apple polyphenols on synthesis and degradation of apoB

Pulse-chase experiments of apoB in Caco-2/TC7 cells cultured with micelles and with (closed bars) or without 500 µg/ml of apple polyphenols (open bars). After 30 min labeling with [^{35}S] met/cys and 3h chase, apoB was immunoprecipitated from 200 µg of cell extracts (A) and (B) and 200 µl of basal media (C) and counted. Results correspond at one representative experiment of 3 independent determinations.

Figure 4 : Effect of micelles and apple polyphenols on MTP activity.

Caco-2/TC7 cells were incubated with or without micelles, apple polyphenols (200 µg/ml) or both. MTP activity was measured using an MTP assay kit (Roar Biochemical) as described in the material and methods section. The reaction was started with 200 µg of proteins and transfer of fluorescence from donor to acceptor particles was measured at 37°C every 20 min for 6h. Results are expressed in arbitrary units corresponding to the fluorescence intensity transfer per min. The values represent the mean ± SD from 3 experiments performed in triplicate.

Figure 5 : Kinetics of cholesterol uptake and esterification.

Cells were incubated for various times with micelles containing both [1, 2-^{3}H(N)]-cholesterol and [1-^{14}C]-oleic acid and with (□) or without (■) 200 µg/ml apple polyphenols. Lipids were extracted from cells. Free cholesterol and cholesterol esters

were separated by TLC. (A) Cholesterol uptake was evaluated by measuring the accumulation of [1,2-^{3}H(N)]-cholesterol in cell lysates. (B) Cholesterol ester synthesis was quantified by incorporation of [^{14}C] oleic acid or (C) accumulation of [^{3}H] cholesterol ester. Values are the mean ± SD of 3 experiments performed in triplicate. ∗ $p<0.05$, ∗∗ $p<0.01$

Figure 6 : Effect of CP-113818, an ACAT inhibitor, on cholesterol esters synthesis and apoB secretion

Caco-2/TC7 cells were cultured as described in figure 1 and incubated with micelles containing [^{14}C]-oleic acid (control), supplemented or not with 200 µg/ml apple polyphenols or 40 nM CP-113818. Lipids were extracted from cells (A) and basal media (B), separated by TLC and [1-^{14}C] oleic acid incorporated into phospholipids (open bars), triglycerides (grey bars) and cholesterol ester (hatched bars) were counted. Data are expressed as a percentage of control (set at 100%). Values represent the mean ± SD of two different experiments performed in triplicate, ∗ $p<0.05$, ∗∗ $p<0.01$, ∗∗∗ $p<0.001$. A representative SDS-PAGE of apoB revealed by western blotting from 20 µl of basal media is shown in (C).

Figure 7 : Effect of apple procyanidin-enriched fraction on cholesterol ester synthesis and lipids secretion

Caco-2/TC7 cells were incubated for 24h with micelles containing [1-^{14}C]-oleic acid supplemented or not with 200 µg/ml apple polyphenols, or 90 µg/ml apple procyanidin-enriched fraction, or 110 µg/ml apple procyanidin-depleted fraction. Lipids were extracted from cells (A) or basal media (B), separated by TLC and [^{14}C] oleic acid incorporated in phospholipids (open bars), triglycerides (grey bars) and cholesterol esters (hatched bars) was counted. Data are expressed as a percentage

of control (set as 100%). Values represent the mean ± SD of two different experiments performed in triplicate, ∗ $p<0.05$, ∗∗ $p<0.01$, ∗∗∗ $p<0.001$.

Table 1 : Sequences of primers for quantitative RT-PCR

Gene		Primer sequence (5'→3')
ACAT1	sense	CCTGAGGAAGATGAAGACC
	antisense	CTCTGCCTCTGCTGTCAAC
ACAT2	sense	CGTCTGCAGAGGACAGAAG
	antisense	GTGTCGGGTCCATTGTACC
MTP	sense	TTCAGCACCTCAGGACTGC
	antisense	GTCTGAGGTCTGAGCAGAG
APOB	sense	CCCACAGCAAGCTAATG
	antisense	GTCTGCAGTTGAGATAG

Table 2 : Relative gene expression in Caco-2/TC7 cells quantified by real-time PCR

Gene	Micelles Arbitrary units (AU)	Micelles and apple polyphenols Arbitrary units (AU)
ACAT1	1 ± 0.22	1.23 ± 0.18
ACAT2	1 ± 0.003	0.84 ± 0.15
MTP	1 ± 0.04	1.02 ± 0.25
ApoB	1 ± 0.04	0.92 ± 0.28
HMGCR	1 ± 0.07	1.06 ± 0.18

Caco-2/TC7 cells were grown on filters for 15 days and then incubated with micelles or micelles and apple polyphenols for 24 hours. Quantification of gene expression was done by real-time RT-PCR. The mRNA abundance was expressed as the ratio between the target gene mRNA and 18S rRNA. The values represent the mean ± SD from 3 experiments in duplicate.

Table 3 : Effects of flavonoids monomers on lipids synthesis and basal secretion in caco-2/TC7 cells

	Intracellular			*Secreted*		
Polyphenols	Phospholipids (%)	Triglycerides (%)	Cholesterol ester (%)	Phospholipids (%)	Triglycerides (%)	Cholesterol ester (%)
Control	100±10	100±5	100±10	100±3	100±3	100±3
Apple extract 200µg/ml	95±18	77±20	49±5[a]	66±15[a]	47±21[b]	41±13[c]
Chlorogenic acid	102±5	101±16	85±15	85±17	96±6	72±6
Phloridzin	90±15	78±16	72±17	97±5	90±4	80±4
(-)-Epicatechin	88±24	84±22	69±26	96±8	94±9	77±9
(+)-Catechin	106±3	96±3	79±19	94±9	92±6	76±6
Mix monomers	74±7	99±1	74±7	103±5	99±8	80±8

Caco-2/cells were grown on filters during 15 days and then incubated with micelles or micelles and apple polyphenols during 24 hours. [1-^{14}C] oleic acid was used as tracer and was incorporated in micelles. Monomers of flavonoids were supplied at

concentrations equivalent to those present in 200 μg/ml apple polyphenols (chlorogenic acid, 40 μg/ml; phloridzin, 12 μg/ml; (-)-epichatechin, 16 μg/ml; (+)-catechin, 10 μg/ml). Mix monomers contained chlorogenic acid, phloridzin, (-)-epichatechin, (+)-catechin. Lipids were extracted from basal medium and cells. Lipids were separated on thin layer chromatography and [^{14}C] oleic acid incorporated into triglycerides, cholesterol esters and phospholipids were counted. Results were expressed as percentage of the intracellular lipid content or of secreted lipids as compared with cells incubated with micelles. The values represent the mean ± SD from 2 experiments in triplicate. a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

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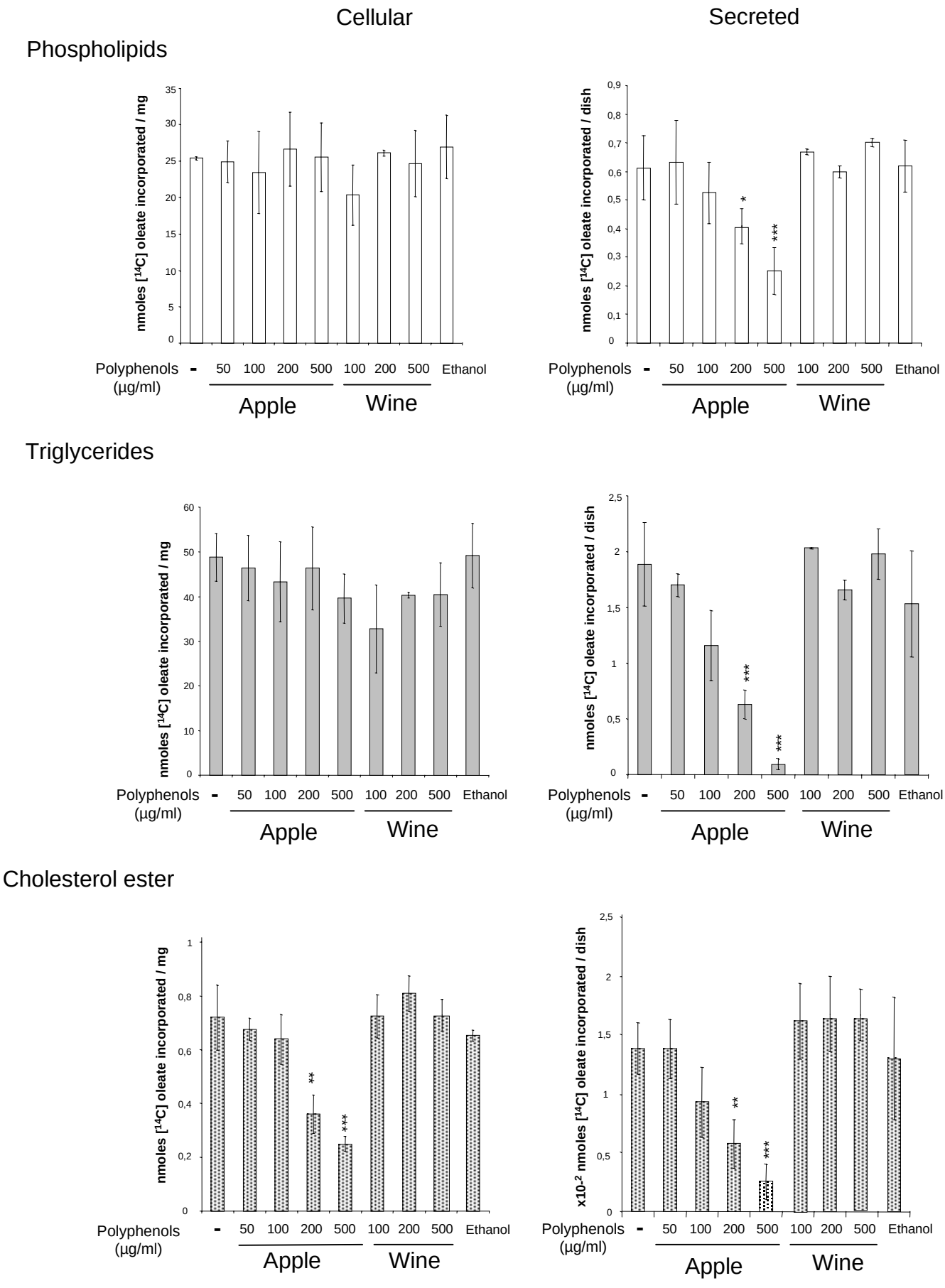
Figure 1


Cellular
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nmoles [14C] oleate incorporated / dish
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Ethanol
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Wine
Triglycerides
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Figure 2

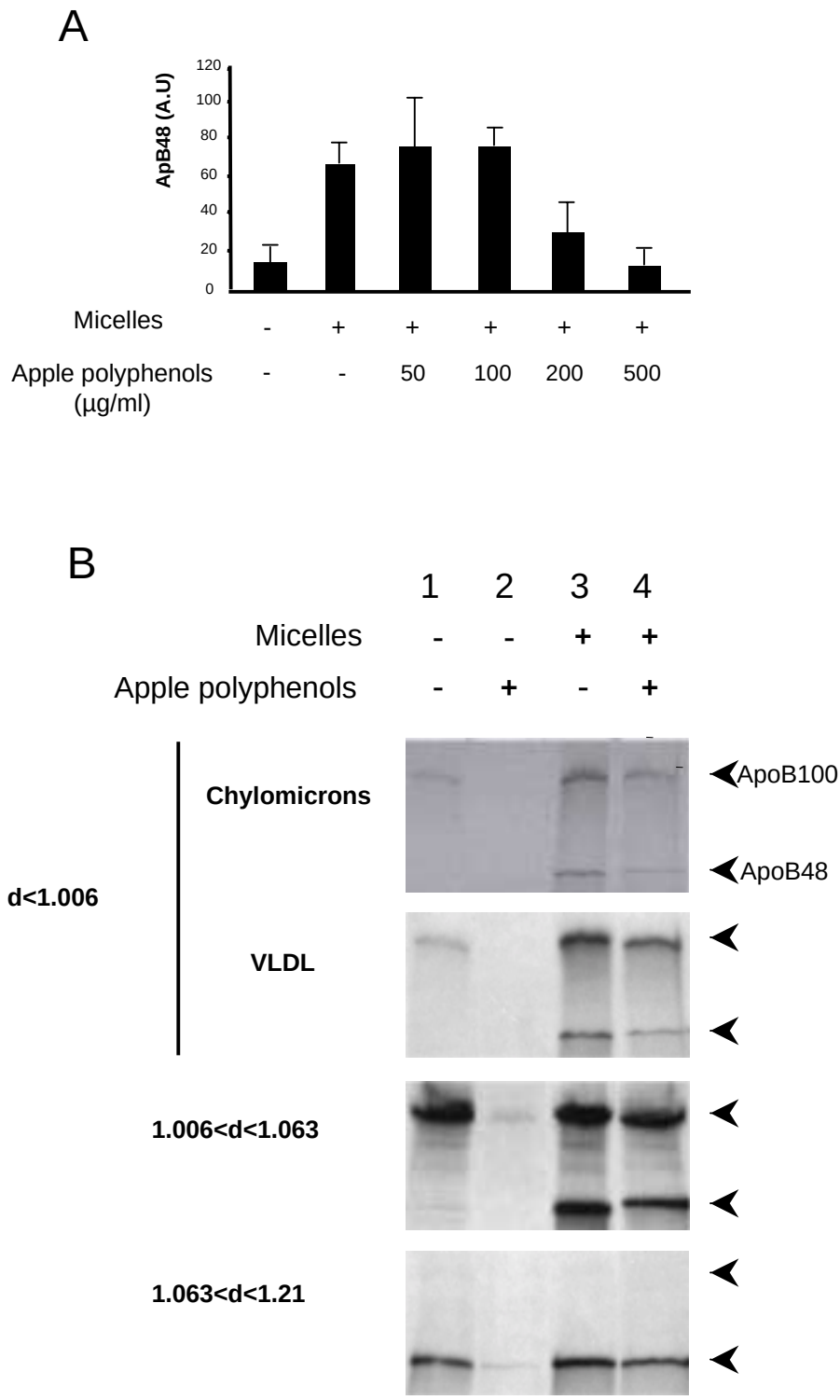
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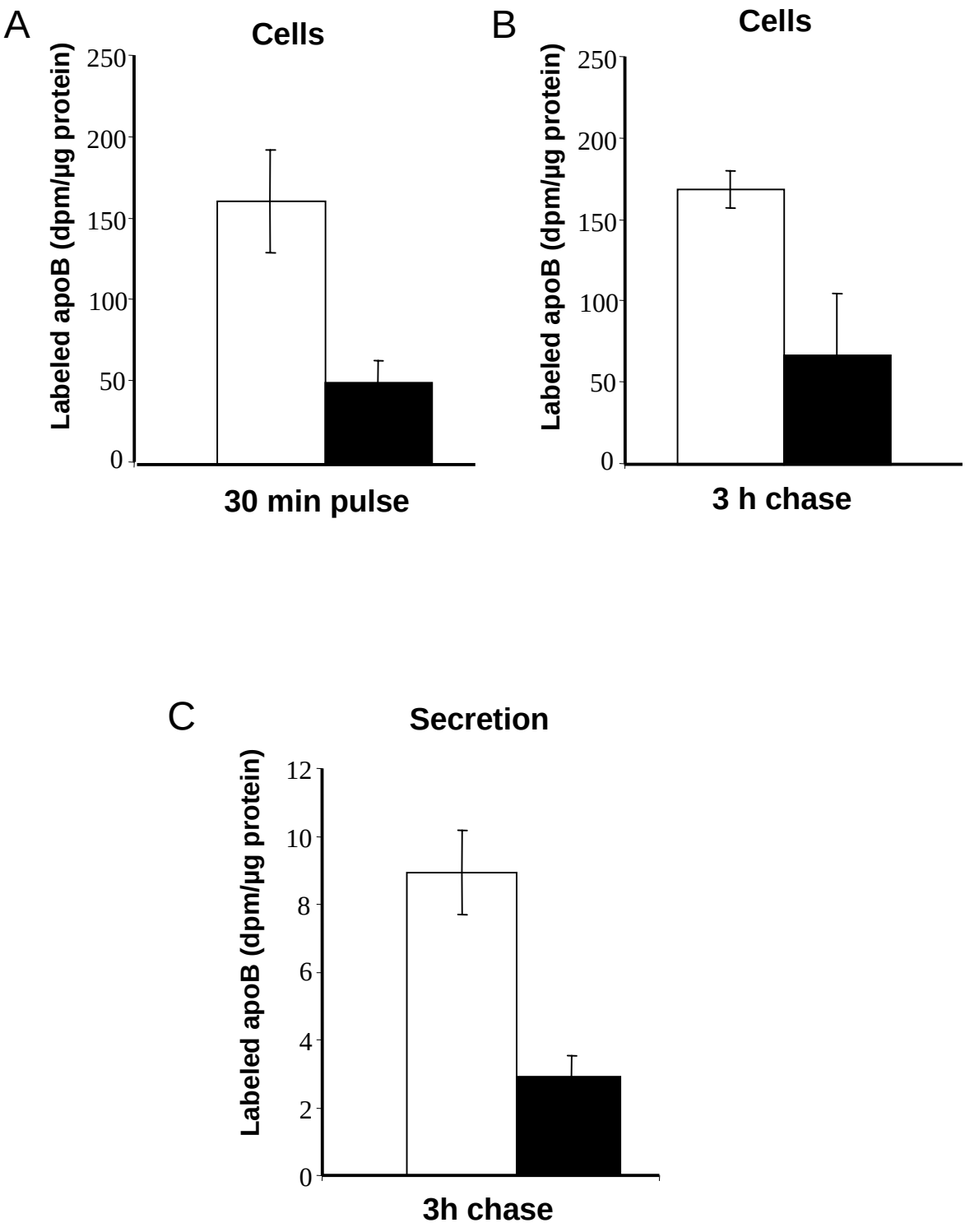
Figure 3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Secretion
Labeled apoB (dpm/µg protein)
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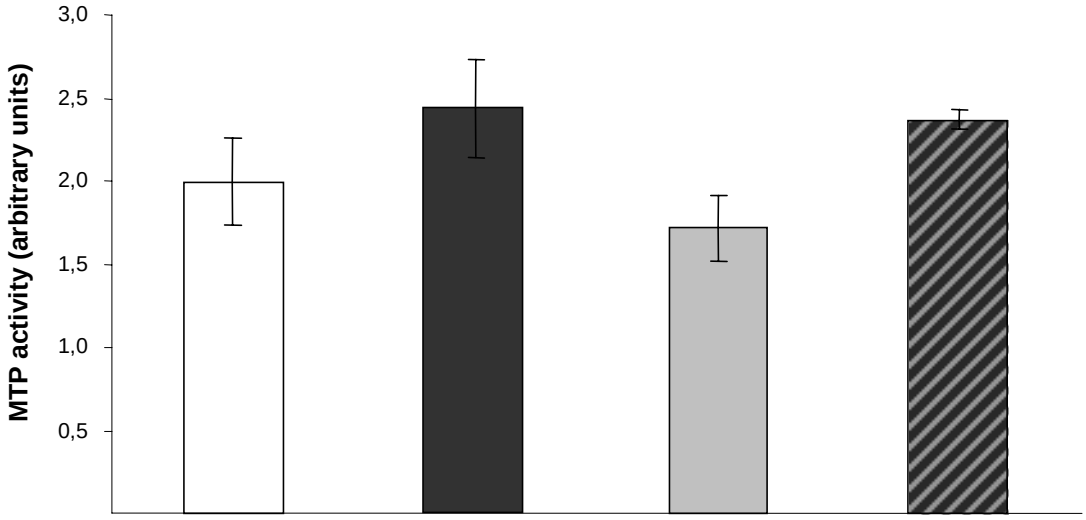
3h chase

- Figure 4



Micelles	-	+	-	+
Apple polyphenols	-	-	+	+

- Figure 5

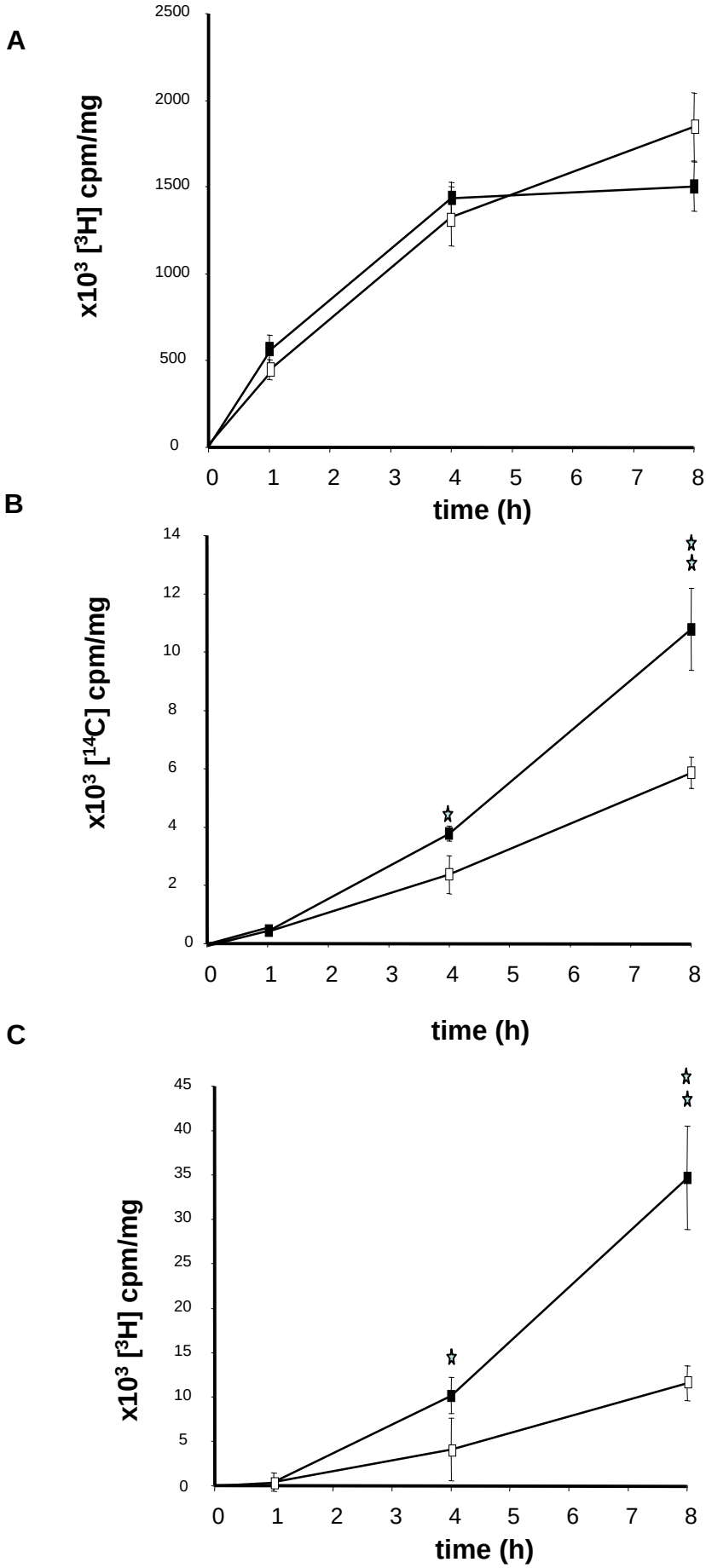

A
2500
2000
1500
1000
500
0
x10³ [³H] cpm/mg
0 1 2 3 4 5 6 7 8
time (h)
B
14
12
10
8
6
4
2
0
x10³ [¹⁴C] cpm/mg
0 1 2 3 4 5 6 7 8
time (h)
C
45
40
35
30
25
20
15
10
5
0
x10³ [³H] cpm/mg
0 1 2 3 4 5 6 7 8
time (h)

Figure 6

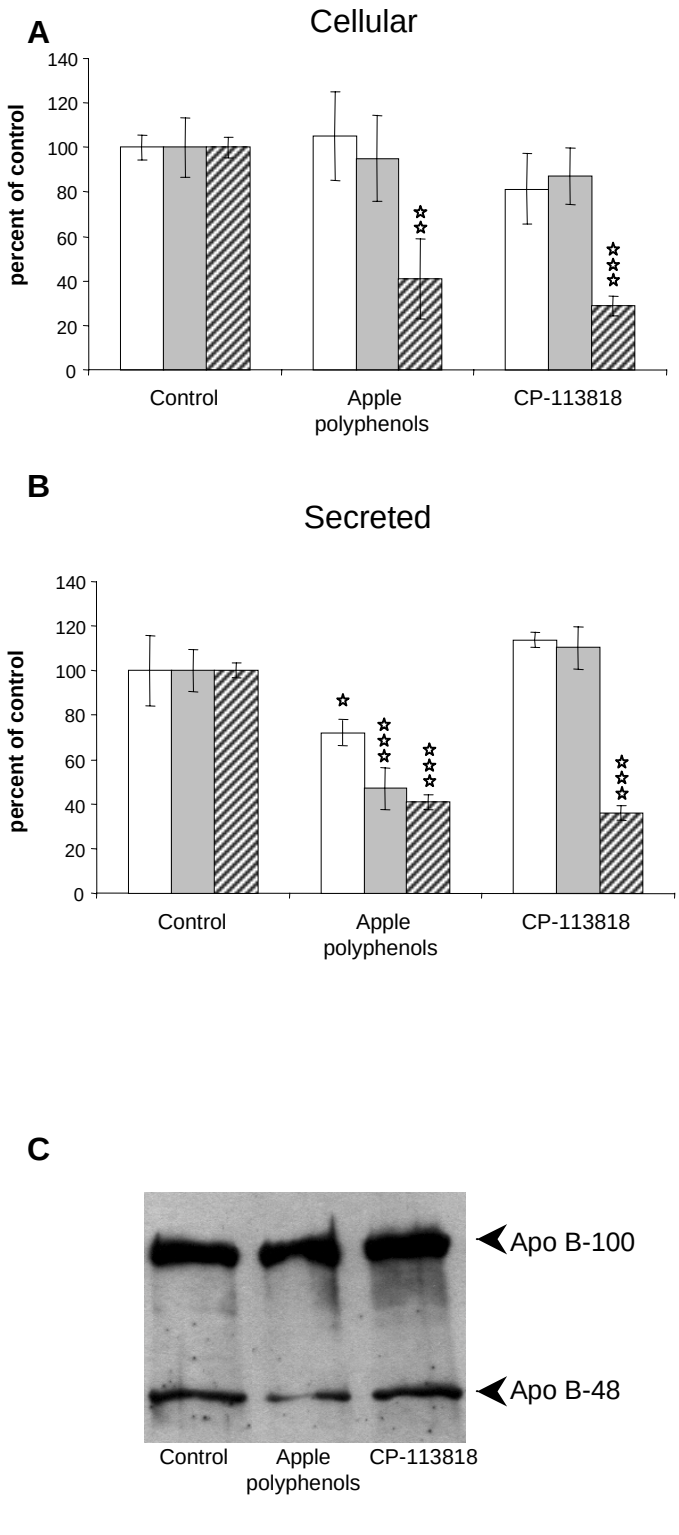
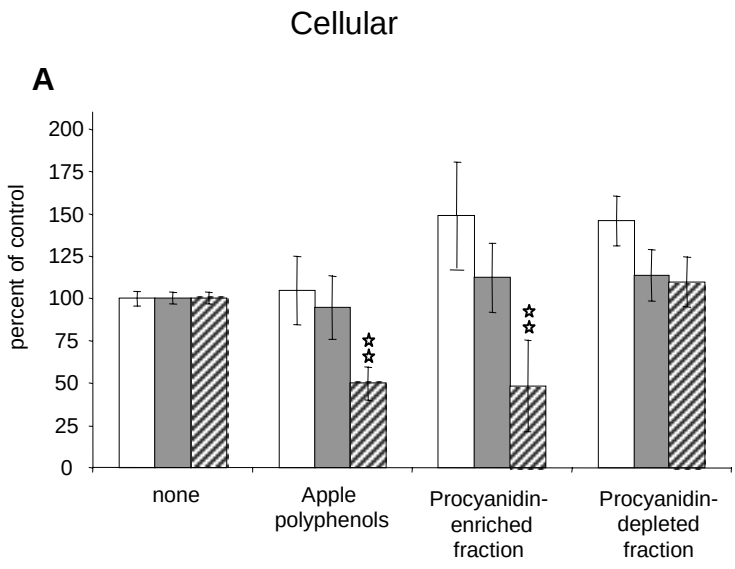
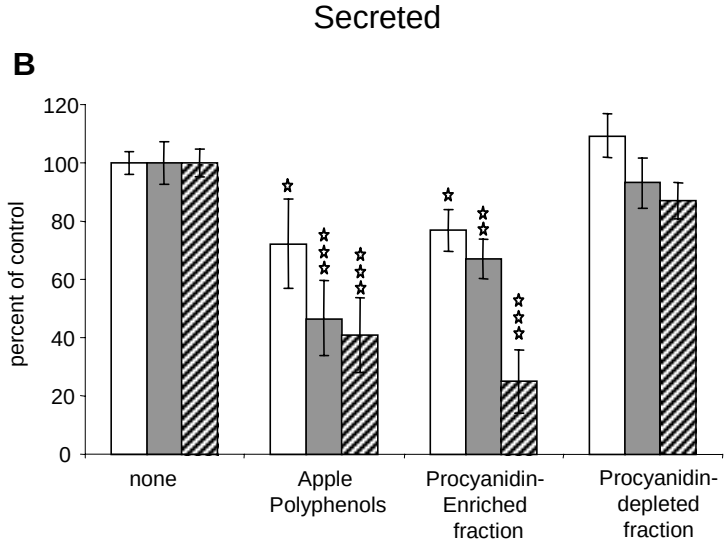

A
Cellular
percent of control
140
120
100
80
60
40
20
0
Control
Apple polyphenols
CP-113818
B
Secreted
percent of control
140
120
100
80
60
40
20
0
Control
Apple polyphenols
CP-113818
C
Apo B-100
Apo B-48
Control
Apple polyphenols
CP-113818

Figure 7


Cellular
A
percent of control
200
175
150
125
100
75
50
25
0
none
Apple polyphenols
Procyanidin-enriched fraction
Procyanidin-depleted fraction


Secreted
B
percent of control
120
100
80
60
40
20
0
none
Apple Polyphenols
Procyanidin-Enriched fraction
Procyanidin-depleted fraction