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Acute Effect of Tea, Wine, Beer, and Polyphenols on ecto-Alkaline Phosphatase Activity in Human Vascular Smooth Muscle Cells

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Alkaline phosphatase (ALP) is an ecto-enzyme widely distributed across species. It modulates a series of transmembranar transport systems, has an important role in bone mineralization, and can also be involved in vascular calcification. Polyphenol-rich diets seem to have protective effects on human health, namely, in the prevention of cardiovascular diseases. We aimed to investigate the effects of polyphenols and polyphenol-rich beverages upon membranar alkaline phosphatase (ecto-ALP) activity in intact human vascular smooth muscle cells (AALTR). The ecto-ALP activity was determined at pH 7.8, with *p*-nitrophenyl phosphate as the substrate, by absorbance spectrophotometry at 410 nm. Cell viability was assessed by the lactate dehydrogenase (LDH) method, and the polyphenol content of beverages was assessed using the Folin−Ciocalteu reagent. All polyphenols tested inhibited ecto-ALP activity, in a concentration-dependent way. Teas, wines, and beers also inhibited ecto-ALP activity, largely according to their polyphenol content. All tested compounds and beverages improved or did not change AALTR cell viability. Stout beer was an exception to the described behavior. Although more studies must be done, the inhibition of AALTR ecto-ALP activity by polyphenolic compounds and polyphenol-containing beverages may contribute to their cardiovascular protective effects.

KEYWORDS: Alkaline phosphatase; smooth muscle cells; polyphenols; wine; tea; beer

INTRODUCTION

Atherosclerosis is widely prevalent in western populations. It is a complex process, associated with high low-density lipoprotein (LDL) levels in serum and inflammation of injured vascular tissue, frequently resulting in dystrophic calcification. It begins much earlier in life than initially thought, a reason why its prevention should begin in adolescence (*1*). The migration of vascular smooth muscle cells toward the intima and the calcification of the extracellular matrix initiated, at least in part, by these cells, constitute also important mechanisms in the establishment of atherosclerosis. Atherosclerotic calcification as well as medial arterial calcification, processes highly characteristic of diabetes and end-stage renal disease, resemble bone formation (*2*).

Alkaline phosphatase (ALP) (EC 3.1.3.1.) has been investigated for more than 70 years (*3*). Its position as a front-line chemical test in hepatobiliary and bone diseases justifies that interest (*4*). In eukaryotes, it is a membrane-bound metalloenzyme consisting of a group of isoenzymes, all glycoproteins. Human ALPs are encoded by four different gene loci, which express the tissue nonspecific (TNALP), intestinal (IALP), placental (PALP), and germ-cell (GALP) isoenzymes. Because kidney, bone, and liver express relatively high amounts of the same ALP protein (isoform), it is usually denominated TNALP. Liver, bone, and kidney ALP isoforms are formed through posttranslational modifications of the tissue nonspecific gene product. IALP, PALP, and GALP are expressed in high amounts in intestine, placenta, and germ cells, respectively, and are denominated tissue-specific ALPs (*5*). Despite its wide distribution in nature, ALP-specific functions remain unclear. ALP cellular location mainly in the plasma membranes of exchange surfaces, where extensive transport takes place, is suggestive of a biological role related to transport processes (*5*, *6*). It has been suggested that ALP is involved in the renal transport of phosphate (*5*), in the modulation of hepatocyte P-glycoprotein transport activity (*7*, *8*), in the transport of organic cations in the blood−brain barrier (*9*), in taurocholate uptake by rat hepatocytes (*10*), and in the intestinal transport of lipids (*5*). IALP is present in surfactant-like particles (SLPs) that surround fat droplets (*11*). SLPs are formed inside enterocytes and then

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secreted into either the intestinal lumen or the blood stream (*12*). IALP gene expression is regulated by ZBP-89 and seems to have an important role in retarding the rate of fat absorption (*13*, *14*). The most well-established function of TNALP is its role in bone mineralization, as clearly shown by the clinical situation of hypophosphatasia (*15*) and confirmed by studies with knockout mice models (*16*, *17*). ALP may also contribute to pathological mineralization in other tissues, including the vascular endothelium (*18*, *19*), a biological process sharing similarities with bone formation, for example, in the presence of bone regulatory factors (*2*, *20*, *21*).

Polyphenols occurring in fruits, vegetables, grains, and beverages, such as tea, beer, or red wine, have been extensively studied in the last few years due to the emerging possibility of their disease-preventive properties on cancer, coronary heart disease, and osteoporosis suggested by several epidemiological studies (*22*−*28*).

Tea and red wine intakes have been epidemiologically shown to be inversely related to coronary heart disease. Tea and red wine are very rich in polyphenols. Their properties as powerful antioxidant beverages and as protectors of the cardiovascular system have been extensively studied (*22*, *25*−*28*). Recent studies have identified compounds with antioxidant and estrogenic characteristics (*29*−*33*) with protective effects against cardiovascular diseases in hops and beer (*29*, *32*, *33*).

Our aim was to investigate the putative direct modulation of ALP activity in vascular smooth muscle cells by polyphenols and some beverages rich in these compounds (such as wine, tea, and beer). We hypothesize that the modulation of this enzyme activity may be another process that explains polyphenol protection against cardiovascular diseases.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl phosphate (pNPP), *p*-nitrophenol (pNP), levamisole, sodium orthovanadate, (+)-catechin hydrate, (−)-epicatechin, (−)-epigallocatechin-3-gallate (EGCG), *trans*-resveratrol, quercetin dihydrate, myricetin, rutin hydrate, chrysin, coomassie brilliant blue G, albumin, tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride], Hepes, Dulbecco's modified Eagle's medium (DMEM) cell culture medium, fetal bovine serum, penicillin, streptomycin, amphotericin B, and tricine were purchased from Sigma (Sigma Alcobendas, Madrid, Spain). Dimethyl sulfoxide (DMSO), ethanol, Folin−Ciocalteu reagent, D-glucose, Triton X-100, sodium hydrogencarbonate, sodium chloride, potassium chloride, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All other reagents were of the highest quality and purity available. Xanthohumol was kindly supplied by Hopsteiner (Mainburg, Germany) through iBeSa, Beverage and Health Institute (Porto, Portugal). Lager type beer (SuperBock), stout type beer (SuperBock Stout), and alcohol-free beer (Cheers) were purchased from the local market (Portuguese beers produced by UNICER, Portugal). Green and black teas (both from Tetley) were purchased from the local market. Red wine (Terraços do Douro, 1999, Sandeman) and white wine (Terraços do Douro, 2000, Sandeman) were produced in the Douro region (North of Portugal) and were also purchased from the local market. Alcohol-free red and white wines were prepared by extracting ethanol from the corresponding intact wines (kindly prepared and supplied by Prof. Paula Guedes de Pinho from ESBUC, College of Biotechnology, Portuguese Catholic University, Porto, Portugal).

Beverages Preparation. All beverages were used at pH 7.8. The gas was removed from the beers before use. Green and black teas were prepared, according to the recommendations of the supplier, by infusing one tea bag (green tea, 1.75 g for 5 min; black tea, 1.5 g for 2 min) in 250 mL of boiling water.

Cells and Culture Conditions. The adult aortic smooth muscle cell line (AALTR) was generously provided by Dr. McDougall (Fred Hutchinson Cancer Research Center, United States) (*34*). AALTR cells were maintained in a humidified atmosphere of 5% CO_2/95% air and grown in DMEM supplemented with 4.5 g L^{-1} glucose, 3.7 g L^{-1} sodium hydrogencarbonate, 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B. The culture medium was changed every 2−3 days, and the cell culture was split every 7 days. For subculturing, cells were washed with phosphate-buffered saline solution, enzymatically removed with 0.25% trypsin−0.02% EDTA (3 min, 37 °C), split 1:4, and subcultured in plastic culture dishes (21 cm^2; Ø 60 mm; Corning Coastar, Corning, NY). For enzymatic assays, the cells were seeded, after splitting, on 24 well plastic cell culture clusters (2 cm^2; Ø 16 mm; Corning Coastar). Membranar alkaline phosphatase (ecto-ALP) activity studies were generally performed 5 days after seeding when cells formed a monolayer, between passage numbers 18−24.

Assay of ecto-ALP in Intact Cells. ecto-ALP assays were performed in intact cells. On the experiment day, the culture medium was removed and cell monolayers were washed once with buffer A [pH 7.8, with the following composition (mM): 5.5 glucose, 136 NaCl, 2.6 KCl, 20 Hepes, and 10 tricine] at 37 °C. Then, cell monolayers were preincubated at 37 °C for 30 min in buffer A. The ecto-ALP reaction was carried out in 0.3 mL of buffer A containing 5 mM pNPP and 1 mM $MgCl_2$. After 60 min of incubation, 0.2 mL of the supernatant solution was transferred into hemolyse tubes containing 0.02 M NaOH at 4 °C; the amount of pNP released from pNPP, reflecting ecto-ALP activity, was measured at 410 nm [adapted from Anagnostou (*35*)]. In experiments performed to evaluate the effect of polyphenols or polyphenol-rich beverages on ecto-ALP activity, polyphenols (10−200 μM) and polyphenol-rich beverages (200 μL/mL buffer A) were present during both the preincubation and the incubation periods. EGCG was dissolved in water. Catechin, epicatechin, and xanthohumol were dissolved in 100% ethanol. Resveratrol, quercetin, myricetin, rutin, and chrysin were dissolved in DMSO. The final concentration of the solvents used, in the incubation medium, was 0.1%. Controls of these drugs and beverages (12.0% ethanol for both red and white wines, 5.6% ethanol for alcohol-containing beers, and water for alcohol-free beverages and teas) were run in the presence of the corresponding solvents. The results were expressed as ecto-ALP specific activity (nmol pNP released/min/mg cell protein) or as percentage of activity recovered in the presence of a given compound or beverage, when compared with the corresponding control.

Determination of Cell Viability. Cell viability was assessed by measuring the lactate dehydrogenase (LDH) activity, as described by Bergmeyer and Bernt (*36*). The release of the intracellular LDH into the media was used to calculate cell viability, since released LDH is a stable enzymatic marker correlating linearly with cell death. The LDH activity was derived by measuring NADH oxidation at 340 nm during the reduction of pyruvate to lactate. Optical density values were recorded for 2 min, and the rate of reduction was calculated. Cells from control cultures were solubilized with 0.3 mL of 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed for 30 min at 37 °C. This cell-associated LDH activity was added to the LDH activity released from control cultures, and the total activity was considered to represent 100% cell death. Then, for each treatment [polyphenols (200 μM) or polyphenol-rich beverages (200 μL/mL buffer A)], the amount of LDH present in the medium was calculated as a percentage of total LDH, which reflects the percent cell death in the sample.

Total Polyphenol Content. The total polyphenol content of the beverages was determined following the Folin−Ciocalteu method adjusted to a microscale (*37*). In an Eppendorf tube, 790 μL of distilled water, 10 μL of sample, and 50 μL of Folin−Ciocalteu reagent were mixed. After 1 min, 150 μL of aqueous 20% (w/v) Na_2CO_3 was added, and the mixture was mixed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve, using catechin as the standard. The results were expressed as mg/L catechin equivalents.

Protein Determination. The protein content of cell monolayers was determined as described by Bradford (*38*), with human serum albumin as the standard.

Calculations and Statistics. K_m and V_{max} values for ALP activity were calculated from nonlinear regression analysis of the saturation

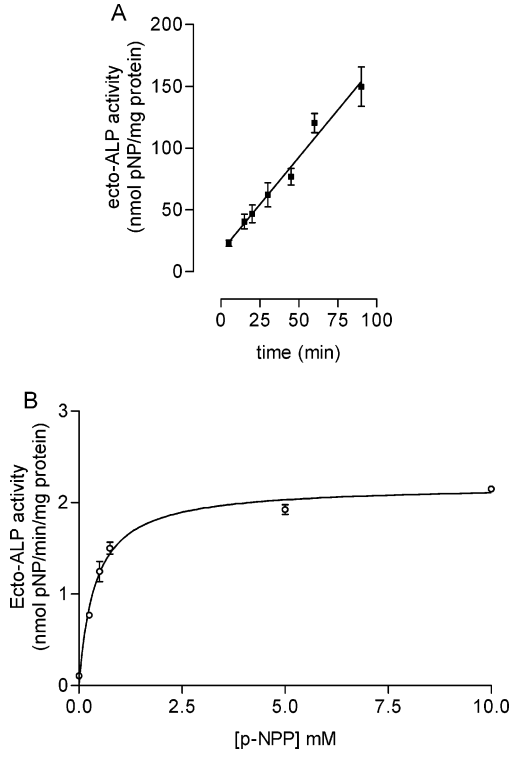

Figure 1. pNP formation dependence on time (**A**) and substrate (pNPP) concentration (**B**) in AALTR cells. Cells were incubated at 37 °C with 5 mM (**A**) or increasing (0.125–10.0 mM) concentrations of pNPP (**B**) at pH 7.8. Data represent arithmetic means ± SEM of 3–6 experiments, each performed in triplicate.

curves using the Graphpad Prism statistics software package (*39*). The activity of an enzyme is defined by the rate constant K_{enzyme} ($K_{enzyme} = V_{max}/K_m$), indicating that the higher the V_{max} and the lower the K_m are the higher is the activity of the enzyme (*40*).

For IC_{50} calculation, Hill equation parameters for multisite inhibition (*41*) were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (*39*). The IC_{50} values were given as geometric means (*42*) with 95% confidence limits.

The ecto-ALP activity, cell viability, and polyphenol content results were expressed as arithmetic means ± standard error of the mean (SEM). Statistical significance of difference between various groups was evaluated by one-way analysis of variance followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $p < 0.05$.

RESULTS

ecto-ALP Activity in AALTR Cells. The time course of pNP formation was determined using pNPP as the substrate in a final concentration of 5.0 mM. pNP formation increased in a time-dependent way up to 90 min of incubation time (**Figure 1A**). So, in subsequent determinations of ecto-ALP activity, an incubation period of 60 min was used.

To determine the kinetic parameters of ecto-ALP activity in AALTR cells, intact cells were incubated with increasing concentrations of pNPP (0.125−10.0 mM). As can be seen in **Figure 1B**, the ecto-ALP activity appeared to be saturable and dependent on the concentration of pNPP used. The kinetic parameters obtained by nonlinear analysis of the saturation curves were as follows: $K_m = 389.3 \pm 46.3$ μM pNPP, $V_{max} = 2.18 \pm 0.05$ nmol pNP/min/mg protein, and $K_{enzyme} = 0.005$ nmol pNP/min/mg protein/μM pNPP.

In subsequent experiments, performed to evaluate the modulation of ecto-ALP activity in AALTR cells by levamisole, orthovanadate, polyphenolic compounds, and polyphenol-rich beverages, intact cells were incubated with 10.0 mM pNPP to ensure that the enzyme was working under saturated conditions. Enzymatic assays were performed at pH 7.8 since viability of AALTR cells was not affected by this pH value (results not shown) and ecto-enzyme activity was higher.

Ecto-ALP activity modulation by levamisole and orthovanadate was investigated in order to characterize the ALP isoenzyme present in the AALTR cell line. A 500 μM concentration of levamisole and 100 μM orthovanadate significantly inhibited ecto-ALP activity from AALTR cells (57.94 ± 6.02 and 51.04 ± 3.95% of recovered activity, respectively).

Effect of Polyphenols on ecto-ALP Activity and AALTR Cells Viability. The effect of various polyphenols on ecto-ALP activity in AALTR cell line was studied. All of the polyphenols tested (10−200 μM) (the stilbene resveratrol; the prenylated chalcone xanthohumol; the flavanols EGCG, catechin, and epicatechin; the flavonols quercetin, myricetin, and rutin; and the flavone chrysin) produced a significant concentration-dependent decrease of ecto-ALP activity in AALTR cells. The maximal reductions found and the IC_{50} values that could be determined were summarized in **Figure 2A** and **Table 1**, respectively. As can be seen (**Table 1**), EGCG, xanthohumol, and myricetin were the most potent inhibitory polyphenols with very similar IC_{50} values: 51.91, 56.22, and 54.40 μM, respectively, followed by quercetin and chrysin. None of the compounds studied diminished cell viability in the conditions tested, when compared with the respective controls; on the contrary, EGCG and myricetin appeared to have a beneficial effect on AALTR viability (**Figure 2B**).

Effect of Polyphenol-Rich Beverages on ecto-ALP Activity and AALTR Cells Viability. Tea, wine, and beer are currently described as polyphenol-rich beverages. We quantified the total polyphenol content of all tested beverages, summarized in **Table 2**. The effect of these beverages on ecto-ALP activity was also assessed.

All tested beverages inhibited ecto-ALP activity in intact AALTR cells: Teas and red wine were more potent than white wines and beers. Green and black teas inhibited enzyme activity almost completely. There was no difference between the two teas (green tea, 7.29 ± 3.07%; black tea, 13.89 ± 2.96%) although green tea tended to have a stronger effect, in agreement with its higher polyphenol content (**Figure 3A** and **Table 2**). Green tea significantly increased the viability of AALTR cells, and black tea showed the same tendency (**Figure 3B**).

The effect of red wine was similar to that obtained for teas (14.01 ± 5.16%; **Figure 4A**), in accordance with the fact that these beverages showed the highest values for total polyphenol content (**Table 2**). White wine also reduced the ecto-ALP activity (44.67 ± 3.02%), albeit with less potency than red wine. These effects are again correlated with red and white wine polyphenol contents (**Table 2**). There were no differences between the effects obtained for wines and the corresponding alcohol-free beverages (**Figure 4A**) (alcohol-free red wine, 1.44 ± 1.02%; alcohol-free white wine, 42.18 ± 3.81%). Nevertheless, alcohol-free red wine seems to have a stronger effect on ecto-ALP activity (**Figure 4A**), in agreement with its polyphenol content (**Table 2**). Cell viability was improved by all tested wines. Red wine seems to be more protective in what concerns AALTR cell viability than white wine (**Figure 4B**).

Beers also reduced the ecto-ALP activity as shown in **Figure 5A**. Lager type beer was more potent than stout type beer in decreasing ecto-ALP activity (26.99 ± 4.09 vs 47.23 ± 5.10%). Once again, there were no differences between lager type beer and alcohol-free beer (39.51 ± 3.88%; **Figure 5A**). None of

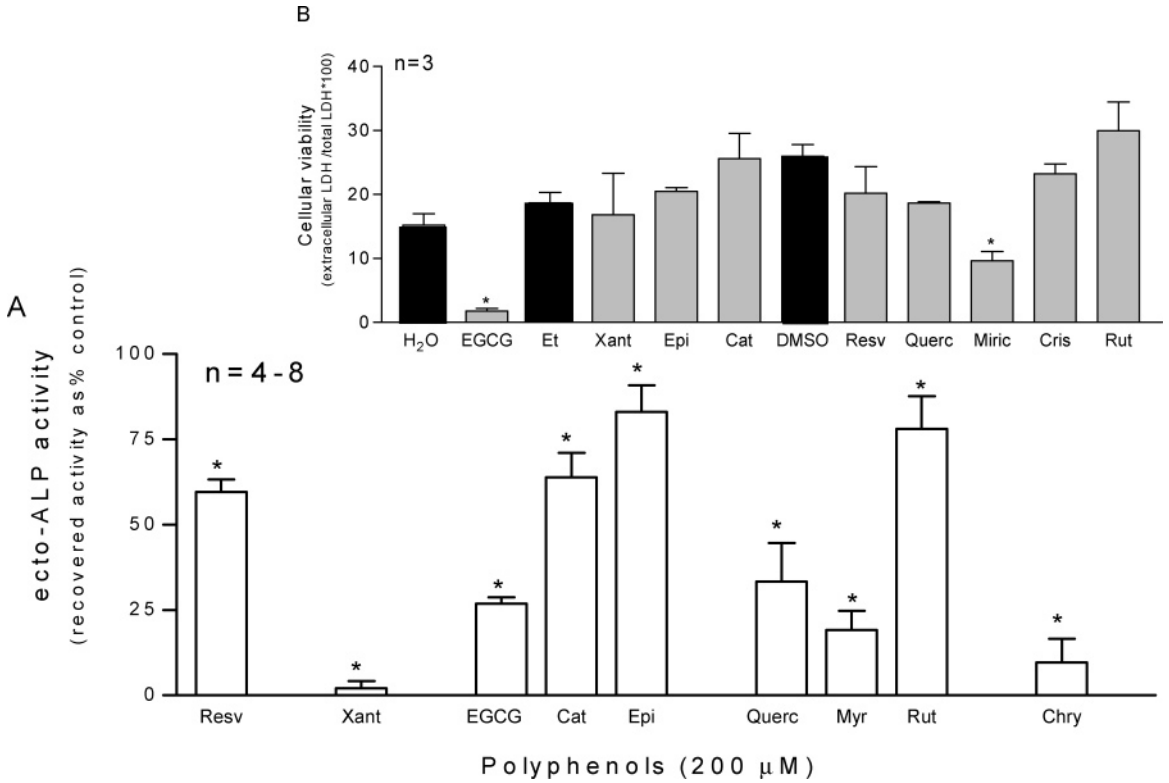

Figure 2. Effect of various polyphenols on ecto-ALP activity (**A**) and in cell viability (**B**) in AALTR cell line. Cells were preincubated for 30 min and incubated for 60 min at 37 °C with 10.0 mM pNPP, in the absence (water, H_2O; ethanol, Et; and DMSO controls) or in the presence of different polyphenols (200 μM): resveratrol (Resv), xantohumol (Xant), EGCG, catechin (Cat), epicatechin (Epi), quercetin (Querc), myricetin (Myr), rutin (Rut), and chrysin (Chry). AALTR cells viability was then assessed by the LDH method. Each value represents the arithmetic mean ± SEM. *Significantly different from the control; $p < 0.05$.

Table 1. Effect of Polyphenols on ecto-ALP Activity in AALTR Cells[a]

	AALTR ecto-ALP inhibition		
polyphenol	IC_{50} (μM)	95%	*n*
xantohumol	56.22	16.65–189.8	5–8
EGCG	51.91	39.24–68.67	4–8
quercetin	65.43	38.36–111.6	6–8
myricetin	54.40	26.33–112.4	5–6
chrysin	93.18	23.13–375.5	5–6

[a] IC_{50} values are geometric means with 95% confidence intervals.

Table 2. Total Polyphenol Content of Studied Beverages[a]

beverage	total polyphenol content (mg/L catechin equivalents)
green tea	937.4 ± 41.9 a
black tea	653.5 ± 5.3 b
red wine	1672.6 ± 186.8 c
alcohol-free red wine	2102.9 ± 124.1 d
white wine	221.7 ± 0.5 e
alcohol-free white wine	225.6 ± 10.8 e
lager type beer	445.6 ± 10.7 f
alcohol-free beer	249.2 ± 18.2 f
stout beer	987.4 ± 15.3 g

[a] Values represent means ± SEM of triplicate determinations ($n = 3$). The concentration is expressed as mg/L catechin equivalents. The mean values followed by different letters, in each group of beverages, are significantly different at $p < 0.05$.

the beers diminished cell viability with the exception of stout beer (**Figure 5B**).

Finally, to ensure that ethanol, by itself, did not alter the enzyme activity, AALTR cells were incubated with solutions

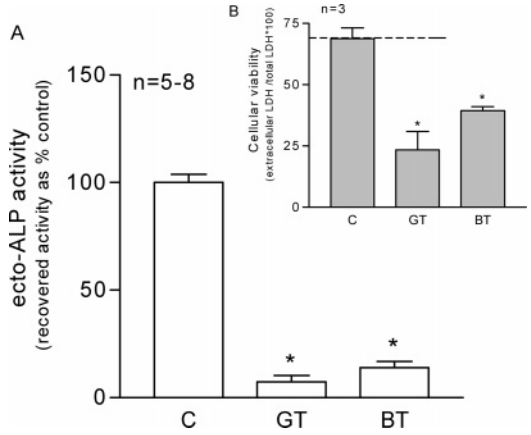

Figure 3. Effect of teas on ecto-ALP activity (**A**) and in cell viability (**B**) in AALTR cells: green tea (GT) and black tea (BT). Cells were preincubated for 30 min and incubated for 60 min at 37 °C with 10.0 mM pNPP in the absence (water control, **C**) or in the presence of teas (200 μL/mL buffer). AALTR cells viability was then assessed by the LDH method. Each value represents the arithmetic mean ± SEM. *Significantly different from the control; $p < 0.05$.

with different ethanol concentrations. There was no alteration in ecto-ALP activity (results not shown).

DISCUSSION

ALP has been characterized as an ecto-phosphatase enzyme in eukaryotes, being anchored to the plasma membrane via a covalent linkage to glycosylphosphatidylinositol. This ecto-phosphatase activity is maintained during vesicles formation in

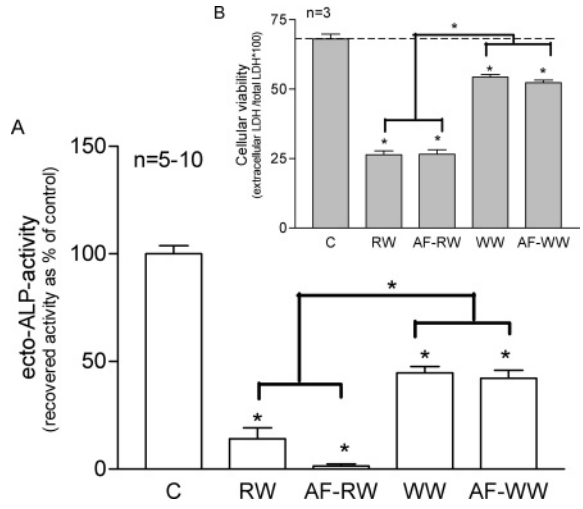

Figure 4. Effect of wines on ecto-ALP activity (**A**) and in cell viability (**B**) in AALTR cells: red (RW) and white wine (WW), alcohol-free red (AF-RW), and alcohol-free white wine (AF-WW). Cells were preincubated for 30 min and incubated for 60 min at 37 °C with 10.0 mM pNPP in the absence (control-ethanol 12%, **C**) or in the presence of wines (200 μL/mL buffer). AALTR cells viability was then assessed by the LDH method. Each value represents the arithmetic mean ± SEM. *Significantly different from the control; $p < 0.05$.

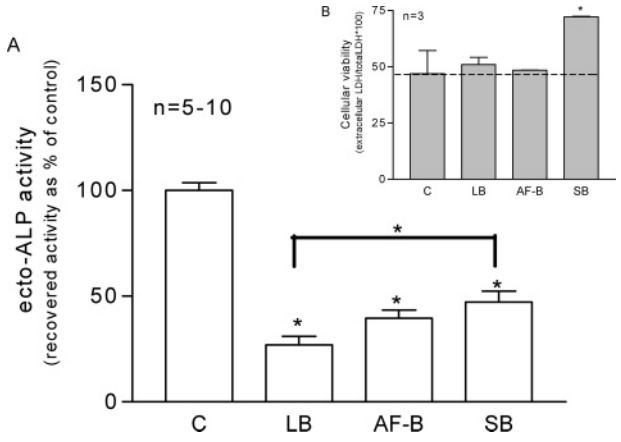

Figure 5. Effect of beers on ecto-ALP activity (**A**) and in cell viability (**B**) in AALTR cells: lager type beer (LB), alcohol-free beer (AF-B), and stout type beer (SB). Cells were preincubated for 30 min and incubated for 60 min at 37 °C with 10.0 mM pNPP in the absence (control-ethanol 5.6%, **C**) or in the presence of beers (200 μL/mL buffer). AALTR cells viability was then assessed by the LDH method. Each value represents the arithmetic mean ± SEM. *Significantly different from the control; $p < 0.05$.

bone and is important for mineralization (*5*, *15*−*17*, *35*, *43*−*47*) as well as for the regulation of lipid absorption (*11*, *12*).

The phosphatase activity studied in this work can be characterized as an ecto-phosphatase activity: The substrate used was only present in the external medium since it does not penetrate plasma membranes (*45*); cell viability did not diminish, under the described conditions; substrate hydrolysis was linear with time, confirming that cells were intact during the incubation period; pNPPase activity was not detected in the external medium (data not shown), which excludes enzymatic action of contaminants or leakage of intracellular enzymes.

As previously mentioned, we chose pH 7.8 to study ALP in AALTR intact cells. Other authors have already described the physiological pH value as optimal for the ALP enzyme activity in several cell lines (*35*, *43*, *44*). McComb et al. (*6*) defined an alkaline pH optimal for ALP in their studies with the purified enzyme.

The kinetic parameters obtained in this work show that k_m and V_{max} values for ALP in AALTR cell lines were in agreement with those obtained by other authors also for TNALP (*48*).

In AALTR cells, ecto-ALP inhibition by levamisole was far from complete (57.94 ± 6.02%). Taking into consideration the cell line origin, we expected to obtain a higher degree of inhibition since levamisole is known to be a specific inhibitor of TNALP (*35*). In agreement with our results, there are some reports describing differences in the behavior of TNALP isoforms activity when levamisole and other compounds are tested, probably due to different glycosylation patterns associated with the isoform source (*49*). A 100 μM concentration of orthovanadate also inhibited the enzyme activity in AALTR cells (51.04 ± 3.95%). Vanadate is a phosphotyrosyl protein phosphatase inhibitor (*50*), and so, the inhibition obtained with vanadate may indicate that ALP works, at least in part, as an ecto-tyrosine phosphatase in AALTR cells.

Teas, wines, and beers were used in this experimental work because of their extensive worldwide consumption and described health benefits in preventing cardiovascular diseases (*22*−*28*, *32*, *33*). The evolution of these pathologies seems to be exacerbated in estrogen-deficient women. Health benefits of these beverages have been attributed mainly to their polyphenol contents, and the classification of many polyphenols as phytoestrogens (ligands for estrogen receptors) led the scientific community to consider this estrogenic effect as a possible mode of action (*51*, *52*). Nevertheless, it has been demonstrated that polyphenols also inhibited the oxidation of LDL, increased high-density lipoprotein cholesterol, induced nitric oxide-dependent vasorelaxation, and inhibited platelet aggregation, inflammation, and proliferation of vascular smooth muscle cells (*23*, *27*) mechanisms that contribute to their cardiovascular protection.

Studying ALP activity using direct modulation of polyphenols and polyphenolic-rich beverages, we are trying to understand the relation between the above-mentioned compounds and their cardiovascular protection as well as the involvement of ALP in the process. All polyphenols tested inhibited ecto-ALP activity in a concentration-dependent way. Chrysin is not known to exist in these beverages, but it is present in many plants and is part of human nutrition (*23*).

Published results of long-term effect of polyphenols on ALP activity are inconclusive and almost all refer to osteoblasts or osteoblast-like cells. Yamaguchi and Jie (*53*) referred to an increase in ALP activity and in calcium tissue content with 100 μM genistein, a reduction in ALP activity with 100 μM EGCG, and no alterations in ALP activity with 100 μM catechin. Choi et al. (*54*) described an increase in ALP activity with 10−100 μM catechin. The precise mechanisms by which polyphenols interact with ALP in long-term treatments are not yet known.

None of the tested polyphenols diminished cell viability; on the contrary, myricetin and EGCG improved the viability of AALTR cells. Similar results in osteoblasts have been described for 10−100 μM catechin (*54*).

As far as we know, this is the first study of the direct effect of these beverages on ecto-ALP activity in this cell line. The results of experiments with polyphenol-rich beverages are in accordance with the results for individual polyphenols. All tested beverages significantly inhibited ecto-ALP activity in AALTR cells. This inhibition is tightly related to the polyphenol content for each type of beverage.

There were no differences in ecto-ALP activity inhibition between green and black teas, although green tea seems to have a stronger effect, in agreement with its polyphenol contents. Other authors have found a different variety of polyphenols but the same total polyphenol contents for green and black teas (*55*, *56*). The effects of green tea ingestion have been intensively studied, and some authors defend that only green tea has health benefits. Others attribute to black tea the same potentialities. Red wine reduction of ecto-ALP activity was similar to that of teas, despite its higher polyphenol contents. These results as well as the results obtained for alcohol-free red wine can be explained by the very high inhibition of ecto-ALP activity, already observed for teas. On the other hand, red wine ALP inhibition was higher than that obtained with white wine, in accordance with the respective polyphenol contents. Concerning beers, lager type beer, unexpectedly, had a stronger inhibitory effect on ALP activity than stout type beer. Theoretically, stout type beer has a higher antioxidant content than lager type beer (*57*). We confirmed that the total polyphenol content of the stout beer used in the study was higher than that of lager type beer. Therefore, the beer polyphenol content does not seem to be determinant of these beverages effects on ecto-ALP activity. In this context, it must be stressed that polyphenolic compounds present in beers are generally very different from polyphenols of other beverages. None of the beverages tested diminished cell viability, with the exception of stout beer.

Ethanol does not seem to interfere with the effect of alcoholic beverages on ecto-ALP. As a matter of fact, no differences between alcohol-free, intact beverages and ethanol (the most important alcohol present in these beverages) effects on ALP activity have been observed. Maner et al. described reduced ALP activity with chronic treatments with ethanol (*58*).

Our results suggest that polyphenols may, at least in part, be one of the agents contributing to the effect of wine, tea, and beer on ecto-ALP activity: (i) Individual polyphenols and beverages have a coincident pattern of effects on this enzyme activity, and (ii) the polyphenol contents of the beverages tightly and positively correlated with the beverage inhibitory effects on the enzyme (*22*, *26*). Even knowing that many other compounds present in the three tested beverages (like carbohydrates, proteins, organic acids, mineral compounds, phenolic acids, and xanthines) (*27*, *59*) can also function as ALP modulators, the observation of the inhibitory effect of those intact beverages on ecto-ALP activity in AALTR cells remains an interesting result.

More studies, namely, involving chronic treatments with these compounds and mineralization assays, are now needed and are strongly encouraged by our present data. Results obtained for ecto-ALP modulation in AALTR cells open the possibility for ALP pharmacological manipulation in vivo in specific tissues.

ABBREVIATIONS USED

ALP, alkaline phosphatase; DMSO, dimethyl sulfoxide; ecto-ALP, membranar alkaline phosphatase; EGCG, epigallocatechin-3-gallate; IALP, intestinal alkaline phosphatase; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenyl phosphate; TNALP, tissue-nonspecific alkaline phosphatase; PALP, placental alkaline phosphatase; GALP; germ-cell alkaline phosphatase; SLPs, surfactant-like particles; LDH, lactate dehydrogenase; AALTR, human adult aortic smooth muscle cell line.

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