

Characterization of rat heart alkaline phosphatase isoenzymes and modulation of activity

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Alkaline phosphatase (ALP) is important in calcification and its expression seems to be associated with the inflammatory process. We investigated the *in vitro* acute effects of compounds used for the prevention or treatment of cardiovascular diseases on total ALP activity from male Wistar rat heart homogenate. ALP activity was determined by quantifying, at 410 nm, the *p*-nitrophenol released from *p*-nitrophenylphosphate (substrate in Tris buffer, pH 10.4). Using specific inhibitors of ALP activity and the reverse transcription-polymerase chain reaction, we showed that the rat heart had high ALP activity (31.73 ± 3.43 nmol *p*-nitrophenol-mg protein⁻¹·min⁻¹): mainly tissue-nonspecific ALP but also tissue-specific intestinal ALP type II. Both ALP isoenzymes presented myocardial localization (striated pattern) by immunofluorescence. ALP was inhibited a) strongly by 0.5 mM levamisole, 2 mM theophylline and 2 mM aspirin (91, 77 and 84%, respectively) and b) less strongly by 2 mM L-phenylalanine, 100 μ L polyphenol-rich beverages and 0.5 mM progesterone (24, 21 to 29 and 11%, respectively). β -estradiol and caffeine (0.5 and 2 mM) had no effect; 0.5 mM simvastatin and 2 mM atenolol activated ALP (32 and 36%, respectively). Propranolol (2 mM) tended to activate ALP activity and corticosterone activated (18%) and inhibited (13%) (0.5 and 2 mM, respectively). We report, for the first time, that the rat heart expresses intestinal ALP type II and has high total ALP activity. ALP activity was inhibited by compounds used in the prevention of cardiovascular pathology. ALP manipulation *in vivo* may constitute an additional target for intervention in cardiovascular diseases.

Key words: Heart; Alkaline phosphatase; Polyphenol-rich beverages; Steroid hormones; Methylxanthines

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Introduction

Alkaline phosphatase (ALP; EC 3.1.3.1.; orthophosphoric-monoester phosphohydrolase, alkaline optimum) is ubiquitous in nature (from bacteria to humans); however,

its physiological role and natural substrates remain largely unknown (1,2). The first established function of ALP is its role in bone mineralization (1-3). More recently, it has been proposed that ALP regulates lipid transport (4). ALP expression in vascular endothelia of small arterioles in the

brain and heart, which normally do not mineralize, could contribute to vascular hardening and calcification that could, in turn, be related to vascular aging and vascular disease (1,5).

Vascular calcification, including coronary artery calcification and aortic valve calcification, is a common and clinically significant component of atherosclerosis and cardiac valvular disease. The amount of coronary calcification correlates with the overall coronary plaque burden and an increased risk of myocardial infarction. The degree of aortic valve calcification is a strong predictor of both the progression and the outcome of aortic stenosis. These calcified lesions contain not only various components associated with bone mineralization, such as ALP, but also inflammatory cells such as macrophages and lymphocytes. Although the mechanisms underlying valve calcification have not been established, recent data suggest that valve calcification is an active process, much like atherosclerosis, that is preceded by inflammation, lipid deposition and the accumulation of extracellular bone matrix proteins (1,6-11).

ALP represents a family of phosphomonoesterases, which, in humans, are expressed by four different gene *loci*: tissue-nonspecific (Tn-ALP), intestinal (Int-ALP), placental (P-ALP) and germ-cell isoenzymes (Gc-ALP) (2). Tn-ALP is present in a large number of tissues (mainly located in the external side of plasma membranes via a glycosyl-phosphatidylinositol anchor linkage) and is most abundant in liver, kidney and bone. Int-ALP, P-ALP and Gc-ALP are tissue-specific ALPs (Ts-ALP). Ts-ALPs are named for the tissue with highest expression and activity (none are expressed in just one tissue). Only humans and great apes have P-ALP; all other mammals have instead Int-ALP (2,12). Tissue differential processing of each of the ALP gene products, for example through differential glycosylation, gives rise to tissue-specific isoforms (2,13). Depending on the physiological/pathological conditions ALP can be released into intestinal lumen, plasma or bile in various isoforms: with or without its anchor and in the former case associated or not with membrane fragments (2). ALP isoenzymes (and isoforms) can be distinguished by their heat stability, optimum pH, substrate affinity or effect of several specific inhibitors or activators (2,13-19).

The variety of ALP forms led our group to characterize the effect (*in vitro* and/or *in vivo*) of several endogenous metabolites, drugs, hormones, beverages and food components on ALP isoenzyme and/or isoform activity and/or expression. *In vitro* data from our group regarding ALP activity modulation will be presented shortly in the discussion of this article, since some of our previous results will be useful for comparison with the present data (13,14,17-19).

In order to further clarify the physiological role of ALP, we have determined *in vitro* the acute effect of compounds, drugs, hormones and polyphenol-rich beverages, with reported cardiovascular protective effects (for the prevention or treatment of cardiovascular diseases) and/or known effects on ALP activity from other sources (cell lines, tissues and species) on total ALP activity from rat heart. We hypothesize that the modulation of ALP activity may be another process contributing to explain the protection given by aspirin, steroid hormones, statins, β -blockers and polyphenol-rich beverages against cardiovascular diseases.

Material and Methods

Beverages and their preparation

Lager-type beer (SuperBock®), stout-type beer (SuperBock Stout®) and alcohol-free beer (Cheers®) were purchased at the local market (Portuguese beers produced by UNICER, Portugal). Green and black teas (both from Tetley®) were also from the local market. Red wine (Vinha das Garças®) was produced in Palmela region (Terras do Sado, South of Portugal) by UNICER and provided to us by this company. Green and black teas were prepared according to the recommendations of 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 boiling water. Gas was removed from beers before use. All beverages were used at pH 10.4.

Animals

Sixteen male adult Wistar rats (free from liver and bone diseases, purchased from Harlan Interfauna Ibérica, Barcelona, Spain), weighing 300-340 g, were used. The animals were maintained under controlled environmental conditions (12-h light/dark cycle, at 24°C room temperature), in groups of 3, fed a suitable commercial diet (Harlan) with water *ad libitum*. Animal procedures were according to the European Community guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals [the corresponding author holds a Portuguese authorization for working with laboratory animals (according to category C of the recommendations of the Federation of European Laboratory Animal Science Associations)]. Animals were anesthetized with pentobarbital (60 mg/kg) and the heart was perfused *in situ* with ice-cold isotonic NaCl to wash out blood. The organ was then removed and rinsed in ice-cold isotonic NaCl. For reverse transcription-polymerase chain reaction (rt-PCR) analysis, a segment from the left ventricle was rinsed with diethylpyrocarbonate-water and stored at -80°C until the moment of the experiment. Rat heart was frozen at -80°C

until the immunofluorescence experiment. Rat heart was placed in two times (mL/g) homogenization solution [phosphate-buffered saline (PBS, pH 7.4) + 0.5% Triton X-100; 4:1], with no homogenization or other form of cell disruption, and stored at -80°C until the moment of ALP activity assay performance.

Reverse transcription-polymerase chain reaction

All molecular biology reagents used were purchased from Sigma (Sigma Alcobendas, Spain) unless otherwise stated. After total RNA extraction with Tripure® (Roche, Germany), 20 µg total RNA was incubated at 37°C for 30 min with 23 units of RNase-free DNase I in 100 µL 5 mM MgCl₂, 50 mM triethanolamine-HCl, pH 7.5, to degrade any residual DNA. The RNA was extracted with phenol-chloroform, precipitated with ethanol and dissolved in water. For cDNA synthesis, 5 µg of the RNA thus prepared was incubated at 45°C for 1 h in a total volume of 20 µL with 200 units of Superscript™ II Reverse Transcriptase (RT; Gibco BRL, Life Technologies, Gaithersburg, MD, USA), in 10 µM random hexamers, 0.375 mM per dNTP, 3 mM MgCl₂, 75 mM KCl, 50 mM Tris-HCl, pH 8.3 (25°C), 10 mM dithiothreitol and 40 units RNase inhibitor (RNaseOUT™; Gibco BRL). For paired negative controls RT was omitted. Following heat-inactivation of the proteins (10 min at 95°C) and addition of 5 µL 0.5 mg/mL DNase-free RNase A in 10 mM Tris-HCl, pH 8.0, and 50% (v/v) glycerol, the cDNA was incubated at 37°C for 30 min to degrade unreacted mRNA. PCR was performed on 4 µL of this preparation. The PCR product mixture (50 µL) contained 0.5 µM per primer, 0.2 mM per dNTP, 2.3 mM MgCl₂ and 2 units of Taq DNA polymerase in the buffer provided (Gibco BRL). The following primers for rat Int-ALP type I, Int-ALP type II, Tn-ALP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used: 5'-CCA GCC AGC TTA CCA ATG AGA-3' (forward primer Int-ALP type I), 5'-CTG CCT GCT GCT TGT AGT TGA-3' (reverse primer Int-ALP type I), 5'-CCT GGA GCC CTA CAC CGA CTG-3' (forward primer Int-ALP type II), 5'-GCC CCC AGT AGT AGC ATC AGC-3' (reverse primer Int-ALP type II), 5'-CAG TGT CAG CCG TTA ATT GAC-3' (forward primer Tn-ALP), 5'-ATC GTG CTG ACC TTG CCA CA-3' (reverse primer Tn-ALP), 5'-ACT GGC GTC TTC ACC ACC AT-3' (forward primer GAPDH), 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse primer GAPDH). Thermocycling consisted of 1 cycle of 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 26°C and 2 min at 72°C for Int-ALP type I, Int-ALP type II and GAPDH primers or 1 cycle of 2 min at 94°C, followed by 40 cycles of 0.5 min at 94°C, 0.5 min at 26°C and 1.5 min at 72°C for Tn-ALP and GAPDH. The predicted sizes (in bp) of the PCR products were: 249 (Int-ALP type I), 218

(Int-ALP type II), 246 (Tn-ALP) and 682 (GAPDH). Ten microliters of each individual PCR product was then run on 1.6% agarose gel and visualized with an ultraviolet transilluminator (Vilber Lourmat, France) using ethidium bromide staining, a COHU CCD camera and the appropriate filters for UV light (Cohu, USA).

Immunofluorescence

The frozen rat heart was included in ornithine carbamyl transferase solution (Bright, England) and 5-µm thick sections were cut with a Leica cryostat (Leica Microsystems GmbH, Germany) and placed onto poly-L-lysine coated microscopy slides (Polysine™, Menzel-Glaser, Germany). Immunofluorescence was performed using primary antibodies (Santa Cruz Biotechnology, USA) against Int-ALP [goat polyclonal antibody (PLAP L-19: sc-15065), diluted 1/25] and Tn-ALP [goat polyclonal antibody (TNAP N-18: sc-23430), diluted 1/25], for 60 min at room temperature, after a 10-min blocking period at room temperature [10% bovine serum albumin (Sigma) w/v in PBS]. PBS was used instead of the primary antibodies for the negative controls. Although we have used a polyclonal antibody against P-ALP and Int-ALP, only Int-ALP was detected since rats do not express P-ALP (2,5). A 60-min incubation was performed with the secondary antibody (donkey anti-goat IgG-FITC, sc-2024, from Santa Cruz Biotechnology, diluted 1/200) at room temperature. Washings between incubations were performed twice for 5 min each with PBS + 0.1% Tween 20 (Sigma). Antibody dilutions were made using UltraAb diluent (LabVision Corporation, USA). Nuclei counterstaining was achieved with DAPI (4'-6-diamidino-2-phenylindole, diluted 1/100, Sigma) for 15 min at room temperature. Slides were mounted with Vectashield (mounting medium for fluorescence; Vector Laboratories, Inc., USA) and observed with an ApoTome Microscope (AxioImager.Z1, Zeiss System, Germany), with a light source of fixed wavelength of 488 and 350 nm, for IgG-FITC (green) and DAPI (blue), respectively. The AxionVision 3.0 program (Zeiss) was used to acquire images and photographs.

Alkaline phosphatase activity

p-Nitrophenylphosphate (*p*-NPP), *p*-nitrophenol (*p*-NPL), levamisole, L-phenylalanine, theophylline, caffeine, acetylsalicylic acid, propranolol, atenolol, progesterone, corticosterone, β-estradiol and simvastatin were purchased from Sigma. On the day of ALP quantification, tissue samples diluted further in homogenization solution (to 1 g tissue + 12 mL homogenization solution) were homogenized and kept on ice. ALP activity assays were carried out in triplicate, as previously described (13,15). The reac-

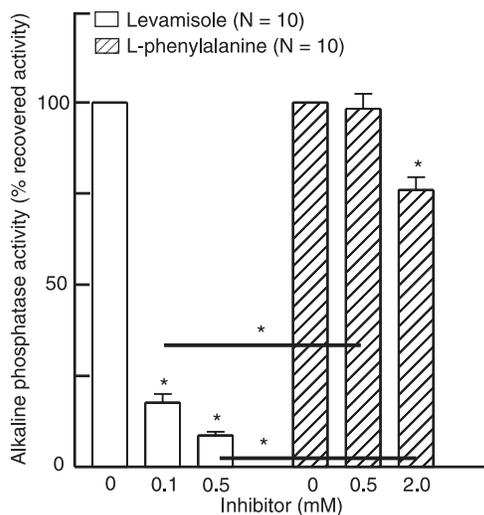


Figure 1. Effect of alkaline phosphatase (ALP) classic inhibitors, levamisole and L-phenylalanine, on ALP activity from rat heart homogenate. Results are reported as mean \pm SEM and represent a percent of the corresponding control activity (first column of each set of results, 100%). The ALP-specific activity in the control was 31.73 ± 3.43 nmol *p*-nitrophenol·mg protein⁻¹·min⁻¹. The horizontal lines indicate significant differences between effects of inhibitors. * $P \leq 0.005$ (paired or unpaired Student *t*-test).

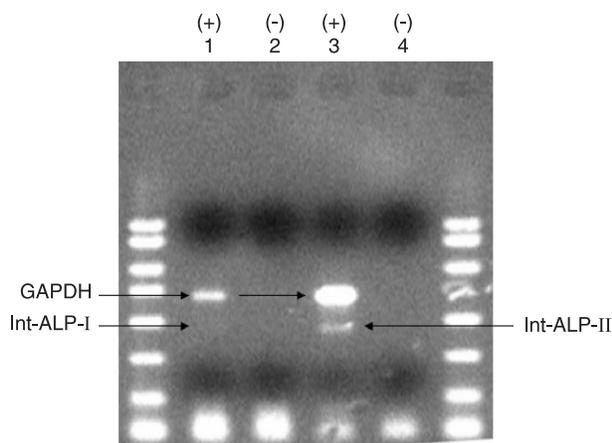


Figure 2. Detection of Int-ALP type I (Int-ALP-I) and Int-ALP type II (Int-ALP-II) mRNA in total RNA from rat heart homogenate using rt-PCR. rt-PCR analysis with specific primers for Int-ALP-I (lane 1), Int-ALP-II (lane 3) and GAPDH (lanes 1 and 3) was performed with total RNA prepared from rat heart homogenate (left ventricle). GAPDH mRNA detection was used as control for intactness of mRNA. Lanes (+) and (-) correspond to samples generated in the presence and absence of RT enzyme during reverse transcriptase reaction, respectively; lanes 2 and 4 correspond to negative controls for Int-ALP-I and Int-ALP-II, respectively. PCR products were separated by agarose gel electrophoresis, followed by staining with ethidium bromide. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Int-ALP = intestinal alkaline phosphatase.

tion mixtures, with a final volume of 500 μ L, contained: 80 mM Tris buffer, pH 10.4, 0.4 mM MgCl₂, 0.376 mg *p*-NPP, the compound (0.1 to 2 mM) or the beverage (50 or 100 μ L) to be tested and heart homogenate. The reactions were started by the addition of the tissue enzyme sample: 20 μ L heart homogenate in a final dilution of 1/13 in homogenization solution. Phosphate concentration in the incubation medium was 0.353 mM, similarly to other studies by our group on ALP activity from tissue homogenates (13,15). Levamisole and L-phenylalanine were dissolved in water. Theophylline, caffeine and simvastatin were dissolved and diluted in dimethylsulfoxide. Acetylsalicylic acid (aspirin), propranolol, atenolol, progesterone, corticosterone and β -estradiol were dissolved and diluted in ethanol. Controls for drugs were run in the presence of a corresponding volume of solvent. Ethanol 5, 5.6 and 13% solutions were used as solvent controls for stout-type beer, lager-type beer and red wine, respectively. Water was used as solvent control for green and black teas. Incubations were carried out at 37°C for 30 min and the enzymatic reaction was stopped by adding 2 mL 20 mM NaOH (4°C). The absorbance of the *p*-NPL produced by ALP activity was measured at 410 nm (Spectronic Genesys 5, Milton Roy, USA). A *p*-NPL calibration curve was constructed in order to calculate ALP activity. The effect of each compound or beverage on ALP activity was calculated as the percent of the activity of the appropriate ALP control.

Protein determination

Protein concentration was determined as described by Bradford (20), with bovine serum albumin as standard.

All data are reported as mean \pm SEM and represent the percent of the corresponding control activity (N = number of cases). The significance of differences between means was assessed by paired or unpaired Student *t*-test.

Results

The effect of eleven different compounds (belonging to different chemical and/or pharmacological classes) and six different beverages (with high and distinct polyphenol content) was evaluated on total ALP activity from rat heart homogenate. Assay conditions allowed linearity of the enzymatic reaction (data not shown).

Levamisole and L-phenylalanine significantly inhibited ALP activity from rat heart homogenate, although levamisole had a significantly stronger effect (Figure 1). While 0.1 and 0.5 mM levamisole inhibited more than 80% of ALP activity (in a concentration-dependent way) 2 mM L-phen-

ylalanine inhibited just 24% of the enzyme activity. Total ALP-specific activity present in rat heart homogenate was 31.73 ± 3.43 nmol *p*-NPL·mg protein⁻¹·min⁻¹. As can be seen in Figure 2, rat heart expresses Int-ALP type II, but not Int-ALP type I. Both Tn-ALP (Figure 3A) and Int-ALP (Figure 3B) were expressed in the myocardium; Tn-ALP clearly showed a striated pattern of distribution.

Theophylline and caffeine had different effects on ALP from rat heart homogenate: theophylline significantly inhibited the enzyme (in a concentration-dependent manner) but caffeine had no effect (Figure 4).

All polyphenol-rich beverages (red wine; lager-type, stout-type and alcohol-free beers; green and black teas) tested on ALP activity from rat heart homogenate significantly inhibited the enzyme (red wine having the least volume-dependent pattern of inhibition; Figure 5). Polyphenol-rich beverages had different inhibitory effects on ALP: 50 μ L red wine had a significantly stronger effect than the same volume of stout-type beer or green tea; 50 μ L lager-type beer and 50 μ L alcohol-free beer had a significantly stronger effect than the same volume of green tea (Figure 5).

Aspirin (acetylsalicylic acid, 2 mM) strongly inhibited ALP activity (84%) from rat heart homogenate (Figure 6); aspirin effect was concentration-dependent.

Steroid hormones behaved differently towards ALP activity. β -estradiol (0.5 and 2 mM) had no effect on enzymatic activity. Corticosterone (0.5 mM) activated (18%) but 2 mM corticosterone inhibited (13%) ALP activity. Progesterone (0.5 mM) inhibited (11%) ALP activity, with no effect at 2 mM. At both concentrations, corticosterone effect was significantly different from that of the other two steroid hormones (Figure 6).

Simvastatin (0.5 mM) and atenolol (2 mM) significantly activated (32 and 36%, respectively) ALP activity from rat

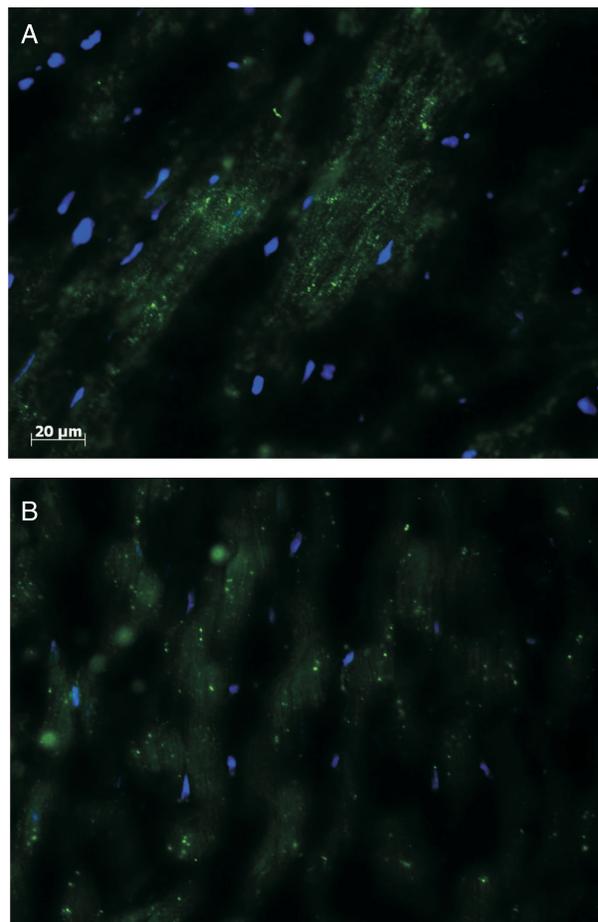


Figure 3. Distribution of alkaline phosphatases (ALP) isoenzymes in the rat myocardium by immunofluorescence. The presence of ALP was identified in green, showing a striated pattern for both isoenzymes, but stronger for tissue-nonspecific ALP. Cell nuclei were colored in blue with DAPI (4'-6-diamidino-2-phenylindole). Scale is identical in both figures. A, Tissue-nonspecific ALP. B, Intestinal ALP.

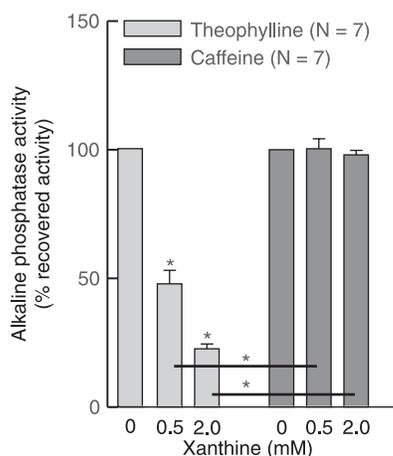


Figure 4. Effect of xanthines (theophylline and caffeine) on alkaline phosphatase activity from rat heart homogenate. Results are reported as mean \pm SEM and represent a percent of the corresponding control activity (first column of each set of results, 100%). The horizontal lines indicate significant differences between effects of xanthines. * $P \leq 0.005$ (paired or unpaired Student *t*-test).

heart homogenate (Figure 6). Atenolol (2 mM) induced a significantly stronger activation (36%) than 2 mM propranolol; propranolol presented a tendency to activate ALP (effects of both β -blockers were concentration-dependent; Figure 6).

Discussion

To our knowledge, this is the first report concerning rat heart ALP isoenzyme identification (in terms of its mRNA expression as well as protein localization and percentage

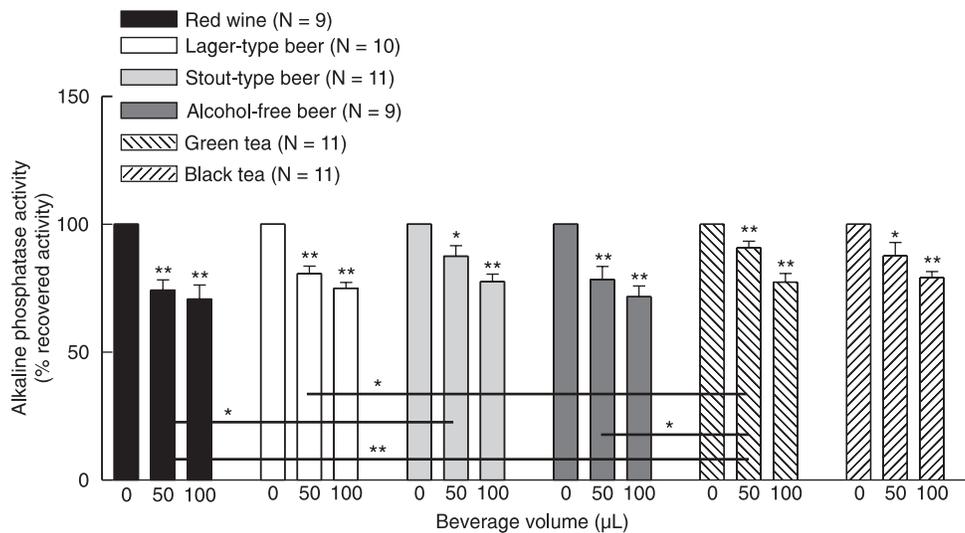


Figure 5. Effect of polyphenol-rich beverages, red wine (13% ethanol), beer [lager-type (5.6% ethanol), stout-type (5.0% ethanol) and alcohol-free] and tea (green and black) on alkaline phosphatase activity from rat heart homogenate. Results are reported as mean \pm SEM and represent a percent of the corresponding control activity (first column of each set of results, 100%). The horizontal lines indicate significant differences between effects of polyphenol-rich beverages. * $P \leq 0.05$, ** $P \leq 0.005$ (paired or unpaired Student *t*-test).

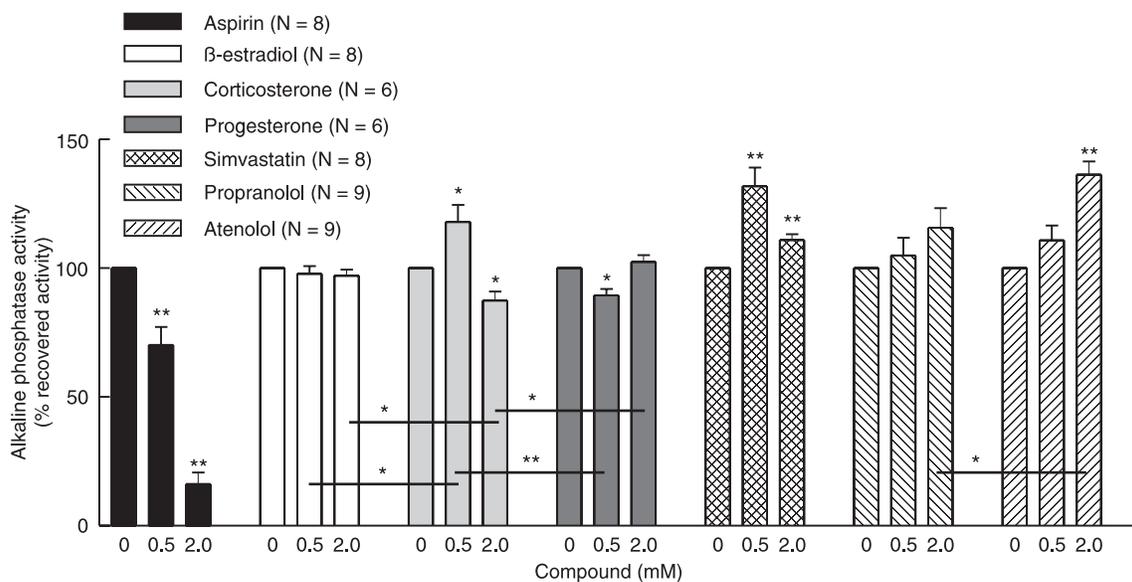


Figure 6. Effect of acetylsalicylic acid (aspirin), steroid hormones (β -estradiol, corticosterone and progesterone), simvastatin and β -blockers (propranolol and atenolol) on alkaline phosphatase activity from rat heart homogenate. Results are reported as mean \pm SEM and represent a percent of the corresponding control activity (first column of each set of results, 100%). The horizontal lines indicate significant differences between effects of compounds. * $P \leq 0.05$, ** $P \leq 0.005$ (paired or unpaired Student *t*-test).

of activity), total ALP activity quantification in the rat heart and its *in vitro* acute modulation by compounds and polyphenol-rich beverages reported in the literature to have cardiovascular beneficial effects.

The inhibitory effects of levamisole and L-phenylalanine showed that both Tn- and Ts-ALPs were present in rat heart (with Tn-ALP presenting a greater activity than Int-ALP), since these compounds are, respectively, specific inhibitors of these two ALP groups (2,13,19). Our results are in agreement with those reported by Van Belle (16) regarding the effects of levamisole and L-phenylalanine on ALP extracted from normal rat heart (without any treatment). Additionally, we showed, by rt-PCR analysis, that Int-ALP, namely Int-ALP type II, was indeed expressed in this tissue.

The rat has two Int-ALP genes (Int-ALP type I and Int-ALP type II) coding for two Int-ALPs with different primary structures (79% amino acid homology), temporal postnatal expression, substrate specificity, tissue localization and response to fat feeding and to cortisone. After an acute fat feeding, Int-ALP type II expression is increased in liver (where Int-ALP type I is not expressed) and Int-ALP type II expression increases five times more than Int-ALP type I in the duodenum (4,21,22). It is interesting to note that rat liver and heart, tissues that can use fat as an energy source in several physiological states, express the same Int-ALP gene.

We hypothesized that the high total ALP activity observed in the normal rat heart (without any treatment) homogenate could not be accounted for only by the presence of ALP in the vascular compartment and, by immunofluorescence, we showed that both Int- and Tn-ALPs are present in rat cardiomyocytes, which is in agreement with our results of total ALP activity in the rat heart and of rat heart ALP activity inhibition by levamisole and L-phenylalanine. Autofluorescence of vascular tissue did not allow us to visualize ALP in blood vessels. Nevertheless, the presence of Int-ALP in the human vascular compartment of different organs has already been reported by Domar et al. (12) and the presence of Tn-ALP in both the heart vascular compartment and muscle has already been reported by Schultz-Hector et al. (5). Müller et al. (23) reported an increase of ALP activity, after a single injection of isoprenaline into the right atrium, particularly on the Schwann cell and its membranes, membranes enveloping nerve axons, the sarcolemma of the muscle cell and the fibrocyte cell surface. These investigators (23) suggested that the Schwann cell is responsible for the production of ALP. Here we report the presence of Int-ALP in cardiomyocytes and the presence of both ALP isoenzymes in normal rat heart (without any treatment). Although speculative, we

suggest that the distribution of specific fluorescence along the myofibrils of myocardial cells is compatible with the presence of ALP in the sarcoplasmic reticulum.

Several lines of evidence suggest ALP as a putative target for prevention and treatment of cardiovascular diseases. ALP is important for the initiation of calcification and its inhibition prevents calcification, either normal or ectopic (1-3,6-8,10,11). ALP activity has been associated with human valve calcification *in vitro* and *in vivo* (7,8,11), with calcification of bioprosthetic heart valves from bovine pericardium *in vitro* and *in vivo* models (10) and with *in vitro* sheep aortic valve interstitial cell calcification induced by transforming growth factor- β 1 (11). These *in vitro* results are supported by the rt-PCR studies of gene expression pattern differences between human calcific aortic stenosis and human normal aortic valve cusps demonstrating that calcific aortic cusps have increased ALP and transforming growth factor- β 1 expression (11). An association between cardiac valve calcification and inflammation has been reported (9,11,24). ALP seems to be associated with inflammatory processes and their modulation/regulation (25,26). *In vitro* and *in vivo*, ALP activity is modulated by oxidative stress (25,27,28). The ingestion of nutrients/beverages with anti-oxidant properties has positive effects on cardiovascular health (29-31).

In the present study, we sought to understand the reported "usefulness" of various compounds, hormones and beverages for prevention and/or treatment of cardiovascular diseases. A marked ALP inhibition was observed with theophylline and aspirin, and some inhibition with wine, beers and teas. β -estradiol and caffeine had no effect; simvastatin and atenolol activated ALP (propranolol also showing an activation tendency). Corticosterone presented a mixed behavior on ALP and progesterone slightly inhibited it.

An inhibitory effect on rat heart ALP activity would be acceptable as a similar background for the cardioprotective effects of the compounds, hormones and beverages tested. By reducing rat heart ALP activity they would contribute to reduce or stop progression of cardiomyocyte, vascular, valvular and/or aorta calcification improving or stabilizing the cardiovascular system; however, that did not occur for all of them. We concluded that only the agents we have tested with reported preventive effects against cardiovascular pathology have ALP inhibitory activity. The meaning of this association is presently unknown but the importance of cardiovascular diseases strongly recommends further exploration of this link.

Methylxanthines, caffeine and theophylline, have been included in this study because a) caffeine is probably the most frequently ingested pharmacologically active sub-

stance in the world and recent research indicates that moderate coffee intake may be associated with beneficial effects on cardiovascular health (32); b) theophylline is described as an inhibitor of ALP: Tn-ALP isoforms and Ts-ALP isoenzymes are inhibited/modulated at different degrees (13). The theophylline inhibitory effect on rat heart ALP was similar to its effect on rat kidney cortex ALP but stronger than that on rat liver ALP (13).

Tea, wine and beer were used in this research because of their extensive worldwide consumption, anti-oxidant capacity (related to their polyphenol content) and described health benefits in preventing cardiovascular diseases (29-31). The evolution of these pathologies seems to exacerbate in estrogen-deficient women, thus, our interest in β -estradiol (33). Wine, beer and tea were tested directly *in vitro* (in an acute treatment) upon ecto-ALP activity from a human vascular smooth muscle (AALTR) cell line for the first time by our group (19). A much stronger inhibitory effect of these beverages had been observed on AALTR ecto-ALP activity (19) than on rat heart ALP. These AALTR results are largely and positively correlated with the polyphenol content of the beverages tested (19,29), what did not happen in the case of rat heart ALP.

Progesterone, β -estradiol and/or corticosterone effects on rat liver and kidney cortex ALP activity as well as on rat brain microvessel endothelial (RBE4) cell line (13,14) are different from those presented here. However, similarly to our present results, in AALTR cell line β -estradiol had no effect while corticosterone (and progesterone) activated the enzyme (18).

Aspirin has been reported to have a protective role in the cardiovascular system, exhibiting anti-platelet, anti-thrombotic, anti-inflammatory and anti-oxidant properties. It also inhibits calcification of bovine pericardium used for bioprosthetic heart valves (10,34). Our results with aspirin are in accordance with published data concerning the *in vivo* and *in vitro* effects of aspirin upon ALP activity. Aspirin inhibits, *in vitro* and *in vivo*, the calcium deposition and/or ALP activity in bioprosthetic heart valves from bovine pericardium (10). Aspirin, in a therapeutic concentration range-dependent manner, inhibits ALP secretion and the stimulatory insulin effect upon ALP production by human osteoblasts *in vitro* (35).

It is interesting to note that two drugs sharing anti-oxidant and anti-inflammatory properties have opposite effects on rat heart ALP: aspirin and simvastatin (34,36).

Statins have well-known protective effects on cardiovascular health, mainly ascribed to their hypolipidemic activity. Beyond this, statins exert pleiotropic effects on vascular wall cells, including improvement of endothelial dysfunction, stabilization of atherosclerotic plaque, de-

crease of oxidative stress and vascular inflammation (17,36,37). On the other hand, they are reported to increase bone density (17,36). We have recently reported an increase on ALP activity in rat heart homogenate for lovastatin (17), but not as intense as with simvastatin. These ALP activating effects may explain the lack of statin protection against aortic valve calcification reported by Cowell et al. (for a discussion of this subject, see Ref. 17). Simvastatin inhibits rat liver and kidney cortex ALP activity (17).

β -blockers are currently used to treat cardiovascular problems (38) and have been reported to have positive effects on bone mineralization and/or osteoporosis (39). The ALP activating effects of propranolol and atenolol observed here fit well with their putative beneficial effects on bone.

Epidemiological studies show a positive correlation between osteoporosis and vascular calcification suggesting that when bone does not mineralize properly the vessels do and vice versa (6). For most of the compounds and beverages tested there are data about beneficial effects on bone mineralization and/or osteoporosis (17,30,31,36,39,40).

Glycosylation can influence/interfere with ALP catalytic activity and ALP activity modulation (2,13,19). Differential glycosylation of the Tn- and Int-ALPs and/or distinct level of expression of Tn- and Int-ALPs (assuming their distinct modulation by the compounds and beverages tested) can explain the distinct ALP modulation results obtained with xanthines, polyphenol-rich beverages, steroid hormones and simvastatin on the various cell lines and/or tissues used so far as ALP sources. Thus, we concluded that it is fundamental that ALP activity studies take the enzyme source into account.

To our knowledge this is the first time that ALP was *in vitro* acutely treated with aspirin, simvastatin, atenolol and propranolol (simvastatin results are discussed in Ref. 17), and there are no data in the literature relating caffeine, theophylline, β -blockers (atenolol and propranolol) and polyphenol-rich beverages (wine, beer and tea) and cardiomyocyte/vascular/valvular/aorta valve calcification. More studies, namely involving chronic treatments (measuring ALP expression and performing mineralization assays) with these compounds and beverages on cardiovascular (and bone) tissues or cell lines are needed and strongly encouraged by our present results.

Both Tn- and Int-ALPs are associated with membrane surfaces characterized by significant transport activity (1,2,4,12,14,17,22,23,25). The apparent Tn-ALP localization in the sarcoplasmic reticulum leads us to raise the hypothesis of ALP being involved in the modulation of the

crucial transport of Ca^{2+} across the sarcoplasmic reticulum membrane, what should be investigated in the future. Also, evaluation of a putative participation of Int-ALP type II in lipid transport and deposition in rat heart muscle and vascular compartment would be interesting, since lipid deposition contributes to atherosclerosis and Int-ALP is involved in regulating intestinal lipid transport (4). Furthermore, exploration of this hypothesis (Int-ALP and lipid transport at the heart) may help to clarify the pathogenesis

of lipid overstorage in cardiac myocytes in type 2 diabetes mellitus and the development of heart failure and/or the reduction in triacylglycerol storage and mobilization in situations of early heart failure.

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