Cholesterol intake increases differently plasma aminopeptidase B and aminopeptidase N activities in male and female mice

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Introduction: Aminopeptidase B (APB) and aminopeptidase N (APN) convert angiotensin III (AngIII) to angiotensin IV (AngIV), acting as regulatory enzymes of the renin-angiotensin system (RAS), which controls blood pressure. Due to hypercholesterolemia and hypertension are associated, the regulatory mechanisms mediated by these enzyme activities could be altered by blood cholesterol levels.

Objectives: The purpose of the present study is to evaluate the influence of dietary cholesterol on plasma APB and APN activities in male and female mice.

Material and methods: Specific APB and APN activities are measured in plasma of male and female mice fed a cholesterol enriched diet containing 1% cholesterol and 0.5% cholic acid during 15 days, using alanyl-ß-naphthylamide and arginyl-ß-naphthylamide as enzyme substrates in a fluorogenic assay.

Results: Plasma APB and APN activities are highly increased in both male and female mice after cholesterol treatment, although APB increases in females in a lesser degree than in males.

Discussion: Hypercholesterolemia and hypertension are frequently associated risk factors for vascular diseases; however, the interactions between cholesterol and the regulatory mechanism of blood pressure are poorly understood. Previous reports demonstrated an increase in AngII-degrading activity with cholesterol intake, leading to increased levels of AngIII. However, the increase found here on APB and APN activities suggest a rapid degradation of AngIII to AngIV. It is possible that APB and APN support a mechanism to decrease the pressor effects of AngIII, or to avoid long-lasting effects of this peptide.

Conclusions: RAS regulation through their degrading aminopeptidases is altered with cholesterol intake in both male and female mice, which could be responsible, at least in part, of the pathogenesis of hypertension.

INTRODUCTION
Hypercholesterolemia and hypertension are frequently associated risk factors for vascular diseases; however, the interactions between cholesterol and the regulatory mechanisms of blood pressure are poorly understood [Rubattu et al., 1993]. The renin-angiotensin system (RAS) is an example of a system that may be involved in the pathogenesis of hypertension [Skoog, 1998]. Classically, angiotensin II (Ang II) has been considered as the effector peptide of the RAS, but Ang II is not the only active peptide. Several of its degradation products, including angiotensin III (Ang III) and angiotensin IV (Ang IV) also possess biological functions. These peptides are formed via the activity of several RAS-regulating aminopeptidases [Chansel and Ardaillou, 1998]. Thus, Ang III is converted to Ang IV by aminopeptidase B (APB) (EC 3.4.11.6) or aminopeptidase N (APN) (EC3.4.11.14) [5,6]. Ang III possesses most of the properties of Ang II and shares the same receptors.
The purpose of the present study is to evaluate the influence of dietary cholesterol on plasma APB and APN activities in male and female mice and therefore, its influence on circulating RAS through their regulating peptidases.

MATERIAL AND METHODS

Animals
Twenty male and twenty female BALB/C mice were used in this study. The animals were housed under constant temperature (25 ºC) and day length (12 hours). The experimental procedures for animal use and care were in accordance with the European Community Council Directive (86/609/EEC). All animals were allowed access to water and food ad libitum and were fed during 15 days as follows. Ten male (26.76 ± 1.014 g) and ten female (22.80 ± 1.34 g) mice were fed a standar diet containing 15.6 % of protein, 2.8 % of fat and 55 % of carbohydrate (control groups). The rest of animals, the males (27.096 ± 0.93g) and the females (23.37 ± 0.65 g) were fed the same diet enriched with cholesterol 1 % and cholic acid 0.5 % (cholesterol groups).

Sample preparation
After this time, the animals were anaesthesized under equithesin anaesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged ten minutes at 3000g to obtain the plasma. These samples were frozen and stored at -80ºC, until use.

Aminopeptidase B and Aminopeptidase N activities assay
APB and APN were measured fluorimetrically using arginyl-ß-naphtylamide (ArgNNap) or alanyl-ß-naphthylamide (AlaNNap) as substrates, as previously described by us [García-López et al., 2003]. Thus, ten microliters of sample were incubated in triplicate for 30 min at 37 ºC with 1 ml of the substrate solution containing 100 µM ArgNNap or AlaNNap, 1.5 mM bovine serum albumine (BSA) and 0.65 mM dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4. The amount of ß-naphthylamine released as the result of the enzymatic activity was measured fluorimetrically at 412 nm emission wavelength with an excitation wavelength of 345 nm using a Tecan instrument. Proteins were quantified in triplicate by the method of Bradford using BSA as a standard. Specific APB and APN activities were expressed as pmoles of ArgNNap or AlaNNap hydrolysed per min per mg of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions. The fluorogenic assay was linear with respect to time of hydrolysis and protein content.

Total cholesterol assay.
Blood total cholesterol was determined colourimetrically using a commercial kit (Sigma 352-50).

Analysis of data and statistics.
To analyze the differences between control groups and the animals fed a cholesterol-enriched diet, we used a multiple analysis of variance (MANOVA), followed by Newman-Keuls post-hoc test. All comparisons with p values below 0.05 were considered significant.

RESULTS
Food intake of control and cholesterol groups did not show differences; however, blood total cholesterol levels were significantly increased in male and female (p <0.001) cholesterol fed groups (Table 1). Values (mean ± SEM) of specific APB and APN in plasma of male and female mice fed a cholesterol-enriched diet are presented in Figures 1 and 2. Although control females showed lower levels of APB and APN than control males, both in male and female mice, the cholesterol enriched diet significantly increased APB and APN activities when compared with control groups, although APB increases in females in a lesser degree than in males (p <0.05).
Table 1. Total cholesterol blood levels of control and cholesterol-fed male and female groups (mean ± SEM).
Values are expressed as mg/dL (a p < 0.001).

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Cholesterol</th>
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<tr>
<td>Male</td>
<td>89.99 ± 5.68</td>
<td>129.73 ± 7.29 a</td>
</tr>
<tr>
<td>Female</td>
<td>104.72 ± 5.46</td>
<td>172.93 ± 8.92 a</td>
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Figure 1: Specific plasma aminopeptidase B (APB) activity in control and cholesterol-fed male and female groups. Results are expressed in picomoles of arginyl-ß-naphthylamide hydrolyzed per min and per mg of protein (Mean ± SEM; n=10; * p < 0.05; *** p < 0.001).
DISCUSSION
Hypercholesterolemia and hypertension are frequently associated risk factors for vascular diseases; however, the interactions between cholesterol and the regulatory mechanism of blood pressure are poorly understood. One of this important mechanisms is the circulating RAS. Although in the study of the RAS many attention has been focused on effector peptides, the regulatory mechanism of these peptides has rarely analyzed [Ramirez-Expósito et al., 2001a; Ramirez-Expósito et al., 2001b] . Thus, APB and APN play an important role in their metabolism. In the present work, we have studied the effects of a cholesterol-enriched diet on plasma APB and APN activities in male and female mice. Previous reports demonstrated an increase in AngII-degrading activity with cholesterol intake, leading to increased levels of AngIII. However, the increase found here on APB and APN activities suggest a rapid degradation of AngIII to AngIV. It is possible that APB and APN support a mechanism to decrease the pressor effects of AngIII, or to avoid long-lasting effects of this peptide. Our results also suggest the existence of sex differences in APB and APN. Previous studies performed by our group have demonstrated the influence of cholesterol and steroid hormones on these activities [Martinez et al., 1997; Martinez et al., 1998] . The present results may corroborate the hypothesis that regulatory proteolytic enzyme activities may be modified by the hormonal status; therefore, sex differences in the metabolism and degradation of different modulatory substances and in the general protein metabolism may be possible [Martinez et al., 1998] . In the same way, many evidences suggest that Ang II regulation may be modified by estrogen [Lachowicz et al., 1995; Philips et al., 1995] ; in fact, the most highly recognized factors implicated in the pathogenesis of hypertension, atherosclerosis, congestive failure and associated cardiovascular disease are the RAS and estrogen. A major effects of estrogen results from its influence on the RAS [Krishnamurthi et al. 1999] . Kisley and coworkers [Kisley et al., 1999] have suggested that estrogen may modulate RAS through a

![Specific Plasma APN](image_url)

**Figure 2:** Specific plasma aminopeptidase N (APN) activity in control and cholesterol-fed male and female groups. Results are expressed in picomoles of alanyl-ß-naphthylamide hydrolyzed per min and per mg of protein (Mean ± SEM; n=10; *** p <0.001).
coordinate regulation of the angiotensin receptors and the levels of newly synthesized Ang II. We can conclude that RAS regulation through their degrading aminopeptidases is altered with cholesterol intake in both male and female mice, which could be responsible, at least in part, of the pathogenesis of hypertension.

**BIBLIOGRAPHY**
