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Diet-induced hypercholesterolemia impaired testicular steroidogenesis in mice through the renin–angiotensin system

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ABSTRACT

Hypercholesterolemia and low testosterone concentrations in men are associated with a high risk factor for atherosclerosis. It is known that cholesterol serves as the major precursor for the synthesis of the sex hormones. The bioactive peptides of the renin–angiotensin-system localized in the gonads play a key role in the relation between cholesterol and testosterone by modulating steroidogenesis and inhibiting testosterone production. In the present work, we evaluated the effects of diet-induced hypercholesterolemia on circulating testosterone levels and its relationship with the testicular RAS-regulating specific aminopeptidase activities in male mouse. A significant decrease in serum circulating levels of testosterone was observed after induced hypercholesterolemia. The changes found in aminopeptidase activities suggest a role of Ang III and Ang IV in the regulation of steroidogenesis.

1. Introduction

Hypercholesterolemia is a complex disorder associated with several genetic and environmental factors, and a major risk factor for atherosclerosis. Also, epidemiological data has widely demonstrated that low testosterone concentrations in men are associated with a higher risk of atherosclerosis [4]. However, the detailed relationship between low testosterone and hypercholesterolemia waits to be clarified, although it is well known that cholesterol serves as the major precursor for the synthesis of the sex hormones, including testosterone.

Several studies have provided evidence for the presence of the renin–angiotensin system (RAS) in reproductive tissues. Thus, immunoreactive renin has been detected in the Leydig cells of rat and human testes [14,18]. Also, renin, angiotensin I (AngI), angiotensin II (AngII) and angiotensin receptors have been detected in normal rat Leydig cells and a murine Leydig cell line [16,17]. Finally, angiotensin-converting enzyme (ACE) activity has been assayed in rat testis, localized predominantly in the germinal cells. Thus, a putative role has been proposed for AngII in modulating the action of gonadotropin in Leydig cells, thus modulating steroidogenesis and inhibiting testosterone production [13]. Classically, AngII has been considered the main bioactive peptide of the RAS; however, some studies suggest that in certain tissue RAS, such as the brain and the gonads, the major player is angiotensin III (AngIII) [6,7,19]. In fact, AngIII has most of the properties of AngII and shares the same receptors. AngIII arises through deletion of the N-terminal aspartic residue by aminopeptidase A (APA; EC 3.4.11.7) and aspartyl-aminopeptidase (ASAP; EC 3.4.11.21). Furthermore, AngIII is metabolized to angiotensin IV (AngIV) by aminopeptidase B (APB; EC 3.4.11.6) and aminopeptidase N (APN; EC 3.4.11.14) [3,23].

In this study, the effects of diet-induced hypercholesterolemia on circulating testosterone levels and its relationship with the testicular RAS-regulating specific aminopeptidase activities were evaluated.

2. Materials and methods

2.1. Animals and treatment

2.1.1. Animals

Thirty male Balb/C mice were used in this study and randomly divided into two groups. The animals were housed under constant temperature (25 °C) and day length (12 h). The experimental procedures for animal use and care were in accordance with the European Community Council Directive (86/609/EEC) and approved by the bioethical committee of the University of Jaen. All animals

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were allowed access to water and food *ad libitum* and were fed during 15 days as follows. Fifteen mice (26.76 ± 1.014 g body weight) were fed a standard diet containing 15.6% of protein, 2.8% of fat and 55% of carbohydrate (control group). The other fifteen animals (27.096 ± 0.93 g body weight) were fed the same diet enriched with cholesterol 1% and cholic acid 0.5% (cholesterol group).

2.1.2. Sample preparation

After the treatment period, the animals were killed under equithesin anesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged 10 min at 3000*g* to obtain the serum. Samples were frozen and stored at −80 °C until use. The right testis was quickly removed and also frozen until use. To obtain the soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl–Tris buffer (pH 7.4) and ultracentrifuged at 100,000*g* for 30 min (4 °C). The resulting supernatants were used to measure soluble enzymatic activity and protein content in triplicate. To solubilize membrane proteins, the pellets were rehomogenized in HCl–Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100,000*g*, 30 min, 4 °C), the supernatants were used to measure solubilized membrane-bound activity and proteins in triplicate.

2.1.3. RAS-regulating aminopeptidase assays

ASAP was determined fluorometrically in triplicate, using aspartyl-β-naphthylamide (AspNNap) as the substrate, as previously described [11]. Briefly, 10 μL of each supernatant was incubated with 100 μL of the substrate solution (100 μM AspNNap, 1.5 mM BSA, and 2 mM $MnCl_2$ in 50 mM HCl–Tris buffer pH 7.4.) for 30 min at 37 °C.

APA was measured in the same way [11] using as substrate glutamyl-β-naphthylamide (GluNNap): 10 μL of supernatant was incubated for 30 min at 37 °C with 100 μL of the substrate solution (100 μM GluNNap, 1.5 mM BSA, 0.65 mM DTT, and 50 mM $CaCl_2$ in 50 mM HCl–Tris buffer pH 7.4).

APN N and APB were also measured fluorometrically using alanyl-β-naphtylamide (AlaNNap) or arginyl-β-naphthylamide (ArgNNap) as the substrate, as previously described [11]. Ten microliters of each supernatant were incubated for 30 min at 37 °C with 100 μL of the substrate solution: 100 μM AlaNNap or 100 μM ArgNNap, 1.5 mM bovine serum albumin (BSA), and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4.

All reactions were stopped with the addition of 100 μL of 0.1 M acetate buffer, pH 4.2. The amount of β-naphthylamine released as a result of the enzymatic activity was measured fluorometrically at a 412-nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified in triplicate using the Bradford methods [5], with BSA as a standard. Specific soluble and membrane-bound ASAP, APA, APN, and APB activities were expressed as picomoles of Asp- or Glu- or nanomoles of Ala- and Arg- β-naphthylamide, hydrolyzed per min per mg of protein, 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.

2.1.4. Total cholesterol assay

Blood total cholesterol was determined colorimetrically using a commercial kit (Cayman Chemical) and following the instructions of the manufacturer.

2.1.5. Circulating testosterone assay

Serum samples were measured by dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) for testosterone (Perkin–Elmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), according to manufacturer instructions. The lower limit of assay detection was 0.3 nmol/L (0.09 ng/mL); the intra-assay coefficient of variation was between 2.6% and 3.3%; and the inter-assay coefficient of variation was between 6.1% and 7.3%.

2.2. Statistics

To analyze the differences between control and hypercholesterolemic groups, we used unpaired Student-t-test. Comparisons with p values below 0.05 were considered significant.

3. Results

Compared with control diet, hypercholesterolemic diet produced a significant increase in serum total cholesterol levels (129.73 ± 7.29 mg/dL in cholesterol group vs. 89.99 ± 5.68 mg/dL in control group (Fig. 1). Also, there was a significant decrease in serum circulating levels of testosterone in control group (3.98 ± 1.04 ng/mL) vs. cholesterol group (2.45 ± 0.9 ng/mL) (Fig. 2).

Figs. 3–6 show testicular soluble and membrane-bound specific activities of ASAP (Fig. 3), APA (Fig. 4), APN (Fig. 5), and APB (Fig. 6) in control and cholesterol groups. Dietary cholesterol significantly increased ($p < 0.05$) soluble but not membrane-bound ASAP activities in rat testis. However, no significant changes were found in APA activities either in soluble or membrane-bound fraction. In the same way, dietary cholesterol significantly increased ($p < 0.01$) both soluble but not membrane-bound APN and APB activities in rat testis.

4. Discussion

Here we present a line of evidence that documents a testosterone deficiency in hypercholesterolemic male mice. Thus, compared with control mice, hypercholesterolemic male mice had lower serum testosterone levels than control group. Several authors have also described an impaired testicular function with hypercholesterolemia. Thus, Tanaka et al. [22] have investigated the effects of hypercholesterolemia on different parameters of testicular function, including serum circulating testosterone in Sprague–Dawley rats, using a standard chow containing 2% cholesterol. They found, after 4 weeks of treatment, that serum cholesterol was significantly higher (206% that of controls), and serum testosterone was significantly lower (49% that of controls), and suggested that hypercholesterolemia is an independent risk factor for testicular dysfunction. In the same way, Feng et al. [9] have investigated the effects of diet-induced hypercholesterolemia on testosterone

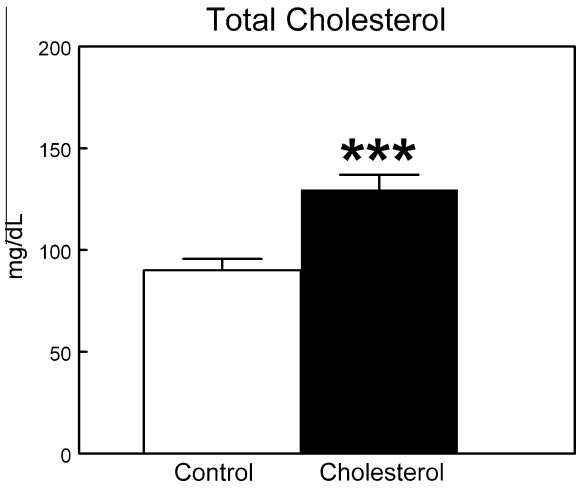

Fig. 1. Total cholesterol levels in control and hypercholesterolemic Balb/C mice. Results are expressed in milligrams deciliter (mean ± SEM; $n = 15$; $^*p < 0.05$).