Consumption of High Fat Diet may be Associated with the Inhibition of Ntpdase, 5’-Nucleotidase and Acetylcholinesterase activities in the Peripheral Tissues

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Abstract

High fat consumption is a significant risk factor to develop metabolic diseases, leading to chronic inflammatory responses. Atherosclerosis is characterized by the formation of athermannous plaque, where the platelets accumulation can promote the interruption on blood flow. This study evaluates the effect of diets with high fat intake on the purinergic and cholinergic markers in peripheral rats’ tissues. The rats were loaded into 5 groups and they had been receiving the following diets for 3 months: controlled diet, saturated diet, saturated/unsaturated and unsaturated diets. The results show a significant inhibition of overall enzymes activities, mainly after the long-term exposure to saturate and to the saturated/unsaturated diets. The cholesterol, LDL and glucose levels were enhanced in the saturated group. Moreover, the HDL level was significantly lower in rats that took the saturated/unsaturated diet. The inhibition of NTPDase increases the ATP and ADP level that elicits the inflammatory response and the promotion of atherosclerosis. The inhibition of 5’-nucleotidase and AChE leads to an increase of the Adenosine and Acetylcholine (ACh) levels, which may be interpreted as a possible defense mechanism of the organism against the chronic inflammation of the arteries. Thus, we can suggest that the NTPDase, 5’-nucleotidase and AChE may act as an intrinsic control of atherosclerotic lesion and neuro inflammation.

Keywords

• Atherosclerosis
• Cholesterol
• Cholinergic
• Inflammatory response
• Purinergic

HIGHLIGHTS

• High fat consumption contributes to platelet aggregation and to the inflammation process
• ATP and ADP acts as immunomodulators for chronic inflammatory responses
• NTPDase has a putative role in the platelet aggregation control and in the atheroma plaque formation
• Adenosine and Acetylcholine are anti-inflammatory modulators that control the platelet aggregation and the inflammatory process
• NTPDase, 5’-nucleotidase as well as AChE would be used in a therapeutic role

INTRODUCTION

Nowadays, the intake of high fat diets has been a predominant factor to develop the metabolic syndrome. The metabolic syndrome is related to several diseases, such as diabetes type 2 and cardiovascular diseases [1]. It is known that the hypercholesterolemia is responsible for the development of ischemic heart disease, angina and myocardial infarction [2]. In fact, the low-density lipoprotein is a carrier of cholesterol
in the bloodstream and it is associated with the promotion of atherosclerosis [3]. Atherosclerosis is a chronic inflammatory disease of the arteries, related to the endothelial cell activation, promoting the plaque progression and the formation of atheroma, a characteristic artery endothelium lesion [4,5].

The Platelets activation has important roles in the thrombus formation and in the hemostasis, however it is also demonstrated its importance in the formation of atherosclerotic lesions [6]. The models of atherosclerosis present a characteristic relation between the activated platelets and leukocytes in the promotion of the atherosclerotic lesions [7]. In fact, platelets activation elicits the inflammatory response, through the release of inflammatory and immunomodulating factors, and it can be related to the promotion of several diseases [8,9]. Thus, the development of the atheromatous plaque formation and the potential rupture or the interruption of blood flow is considered crucial for the development of acute coronary syndromes and ischemic stroke [5,10].

Extracellular adenine nucleotides as ATP, ADP and adenosine regulate the vascular response to the endothelial injury. ADP induces the platelets aggregation and promotes the coagulation cascade. At the same time, the adenosine, product of AMP hydrolysis, is potent inhibitor acting in the regulation of platelet aggregation [11]. After regulating the platelet activation through purinergic receptors, the receptor-mediated signaling is terminated by the coordinated action in cascade of ectonucleotides, including NTPDase ([E.C. 3.6.1.5] (CD39, ecto-APase, ATP diphosphohydrolase) that hydrolyzes ATP and ADP to AMP, which is subsequently hydrolyzed by the ecto-5’-nucleotidase (E.C.3.1.3.5) in adenosine [12].

It is also known that the progression of the atheroma plaque reduces the vascular lumen, altering the blood flow [5]. Interestingly, cholinergic mechanisms play a role in the modulation of cerebral blood flow [13]. The Acetylcholinesterase enzyme (AChE, 3.1.1.7) is the main mechanism of controlling the cholinergic function, through the degradation of acetylcholine (ACh) in to metabolites, choline and acetate [14,15]. Moreover, AChE is found in several tissues, such as blood cells [16], and the ACh in blood has been related to immune modulation [17].

Thus, since the ectonucleotides, as well as AChE, control the levels of immunomodulatory factors, ATP, adenosine and ACh, and considering that the high fat diets are related of the atherosclerotic inflammation, the aim of this study was to evaluate the effect of fat diets on the purinergic and cholinergic status in peripheral tissues of rats to determine the potential of the neuronal inflammation induction and to contribute to the development of neurological diseases.

**EXPERIMENTAL PROCEDURES**

**Animals**

Twenty-day-old rat pups weighing 60g obtained from our breeding colony were used. The animals were maintained on a 12:12 light/dark cycle, in an air-conditioned (22 ± 1°C) colony room, with free access to water and food, and were weekly weighed. All animal procedures were approved by the Institutional Ethical Committee of the Federal University of Santa Maria (Protocol number 23/2006).

**Materials**

Acetylthiocholine iodide, and 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co (St Louis, MO, USA). All the other reagents used in the experiments were also from analytical grade and made with the highest purity.

**Treatment**

The newly weaned rat pups were submitted to a long-term exposure, for three months, to the different high saturated and unsaturated fat diets. The animals were treated with five different diets: (1) the control group that consumed laboratory chow (Supra-RS, Brazil); (2) standard diet group (Std); (3) diet high in saturated fat group (Sat); (4) diet high in saturated/unsaturated fat group (Sat/Uns); (5) diet high in unsaturated fat group (Uns) (Table 1). The animals were euthanized 24h after the last meal and the blood was collected.

**Platelet-rich plasma preparation**

The platelets were prepared by the method of Pilla et al. [18], modified by Lunkes et al. [19]. The total blood was collected by a cardiac puncture and placed into a flask with 0.129 M sodium citrate as anticoagulant. The total blood-citrate system was centrifuged at 160 x g for 40 min to remove residual blood cells. The platelet-rich plasma (PRP) was centrifuged at 1400 x g for 20 min and washed twice by centrifugation at 1400 x g with 3.5 mmol/L HEPES isomolar buffer containing 142 mmol/L NaCl, 2.5 mmol/L KCl, and 5.5 mmol/L glucose. The washed platelets were resuspended in HEPES isomolar buffer and adjusted to 0.4 – 0.45 mg of protein per milliliter.

**LDH**

The integrity of the platelet preparations was confirmed by determining the lactate dehydrogenase (LDH) activity in which it was obtained platelet lysis with 0.1 % Triton X-100 and it was compared with that of an intact preparation, using the Lab test kit.

**Assay of NTPDase and 5’-nucleotidase activities**

In platelets, the determination of ectonucleotidase activities

<table>
<thead>
<tr>
<th>Table 1: Diet Composition.</th>
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<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Refined-sugar</td>
</tr>
<tr>
<td>Starch</td>
</tr>
<tr>
<td>Lard</td>
</tr>
<tr>
<td>Soybeans oil</td>
</tr>
<tr>
<td>Fiber</td>
</tr>
<tr>
<td>Vitamin/Mineral</td>
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<tr>
<td>Kcal/100g</td>
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</table>
was carried out using the PRP preparation according to Pilla et al. [18]. Briefly, to determine the NTPDase activity, 20 µL of the PRP preparation (8-10 µg of protein) was added to the system mixture, which contained 5 mM CaCl₂, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM tris-HCl buffer, pH 7.4. The reaction started by the addition of 20 µL of ATP or ADP (1 mM final concentration) as substrates. For AMP hydrolysis, the 5'-nucleotidase activity was determined as described above, except that 5 mM CaCl₂ was replaced by 10 mM MgCl₂ and the nucleotide final concentration added was 2 mM AMP. For platelets, 20 µL of the enzyme preparation (8-10 µg of protein) was added to the reaction mixture and pre-incubated for 10 min at 37°C.

Both reactions were stopped by the addition of 200 µL of 10% trichloro acetic acid (TCA) to provide a final concentration of 5%. After chilling on ice for 10 min, 100 µL samples were taken for assay of released inorganic phosphate (Pi) by the method of Chan et al. [20], using malachite green as the colorimetric reagent and KH₂PO₄ as the standard one. Controls were carried out by adding the platelets preparation after TCA addition to correct non-enzymatic nucleotide hydrolysis. All the samples were triplicated. Enzyme activities are reported as nmol Pi released/(min mg) of protein.

**Sample of blood collection**

The blood was collected in vacutainer tubes using EDTA as anticoagulant. The samples were hemolyzed with phosphate buffer, pH 7.4 containing Triton X -100 (0.03%) and an appropriate storage.

**Determination of erythrocyte AChE**

Erythrocyte AChE activity was determined by the modification of Ellmann’s et al. [21], method as described by Worek et al. [22]. Whole blood dilutions were prepared by adding 100 µL blood to 10 mL sodium/potassium phosphate buffer (0.1 mM), pH 7.4 containing Triton X -100 (0.03%). After carefully mixed, the samples were frozen immediately and kept until analysis. The hemolizate (500 µL), phosphate buffer 0.1 mM pH 7.4, DTNB (0.3 mM), and ethopropazine (0.02 mM), a selective butryl cholinesterase (BChE) inhibitor, were pre-incubated during 10 min at 37°C. The reaction was started by the addition of AcSCh (0.45 mM) substrate, and a color development was measured at 436 nm.

The specific activity of erythrocyte AChE was calculated from the quotient between AChE activity and hemoglobin content, and the results were expressed as mU/µmol Hb.

**Protein determination**

Protein was measured by the Coomassie blue method according to Bradford (1976) [23] using bovine serum albumin as standard.

**Statistical analysis**

Data were analyzed by the analysis of variance (One-way ANOVA) followed by the Tukey-Kramer multiple range test, and p<0.05 was considered to represent a significant difference in the analysis. All data was expressed as it means ± S.D.

**RESULTS**

Overall, it was found a significant decrease of the enzymes activities in both peripheral tissue preparations, for platelets and erythrocytes, in young rats exposed to high fat diets (Figures 1 and 2). The AChE activity in erythrocytes was decreased in 39.2% in the saturated group, in comparison to the control group. At the same time, the AChE activity was inhibited in the groups that consumed the saturated/unsaturated 24.5% and unsaturated (22.9%) diets, respectively, when compared to the control group (p<0.05) (Figure 1).

As shown in the Figure (2), the ATP hydrolysis was 58% and 68% inhibited in the rats that consumed the saturated and saturated/unsaturated diets, respectively, in comparison to the control group. For the ADP hydrolysis, it was observed a decrease of 39.1% and 20% after the exposure to saturated and saturated/unsaturated diets, when compared to the control group, respectively. To 5'-nucleotidase activity, the results were consistent with the ATP and ADP hydrolysis, where was found an inhibition of the enzyme activity of the 33.1% and 21.7% in rats exposed to saturated and saturated/unsaturated diets, in comparison to the control group, respectively (Figure 2).

The mean plasma levels parameters, as hematocrit, glucose, cholesterol, cholesterol ester concentrations in lipoprotein fractions, triglycerides, creatine and urea was measured in young rats that consumed different diets (Table 2). The rats that took saturated diet showed higher levels of cholesterol (32%), LDL-cholesterol and glucose (21%), when compared to the control group. At the same time, the rats exposed to saturated diet showed decreased levels of HDL-cholesterol (23%), hematocrit (13%), triglycerides (44%) and urea (36.8%), in comparison with the control group. Although the exposure to the saturated/unsaturated diet showed an enhanced of 320% in the LDL-cholesterol levels, the triglycerides was inhibited in 55.6%, both in comparison to the control group (Table 2).

![Figure 1](https://example.com/figure1.png)

Figure 1 Effect of different diets in AChE activity in erythrocytes of young rats. Each column represents the average of ± S.D. (n=5) as percent of control AChE control value for erythrocytes was 18.68 ± 1.03, respectively, and it was expressed as mL/µmol of Hb. b Different from the control and standard groups. d Different from the unsaturated group. ANOVA – Tukey-Kramer’s Test.
Table 2: Plasma, Glucose, Cholesterol, Cholesterol Ester concentrations in Lipoprotein Fractions, Triglycerides, Creatine and Urea in young rats exposed to High Fat Diets.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Standard</th>
<th>Saturated</th>
<th>Sat/Uns</th>
<th>Unsaturated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>81.8 ± 5.0</td>
<td>84.5 ± 5.4</td>
<td>106.0 ± 13.3</td>
<td>94.3 ± 9.8</td>
<td>61.3 ± 13.0 ***</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>53.7 ± 3.0</td>
<td>60.2 ± 4.9</td>
<td>41.5 ± 1.5 ***</td>
<td>50.5 ± 3.7</td>
<td>53.8 ± 4.9</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>8.8 ± 4.4</td>
<td>10.8 ± 4.9</td>
<td>46.2 ± 1.9 ***</td>
<td>37.0 ± 8.1 ***</td>
<td>4.3 ± 1.1</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>105.8 ± 7.4</td>
<td>118.2 ± 35.4</td>
<td>59.3 ± 24.3 b</td>
<td>47.0 ± 8.3 b</td>
<td>50.2 ± 10.9 b</td>
</tr>
<tr>
<td>Glucose</td>
<td>105.2 ± 7.5</td>
<td>103.3 ± 3.8</td>
<td>127.3 ± 11.2</td>
<td>112.2 ± 18.4</td>
<td>93.7 ± 2.9</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>47.5 ± 1.6</td>
<td>41.5 ± 1.3 a</td>
<td>41.3 ± 1.2 a</td>
<td>47.0 ± 1.3 bc</td>
<td>49.0 ± 2.7 bc</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.62 ± 0.1</td>
<td>0.64 ± 0.2</td>
<td>0.74 ± 0.1</td>
<td>0.60 ± 0.1</td>
<td>0.54 ± 0.1</td>
</tr>
<tr>
<td>Urea</td>
<td>35.3 ± 8.2</td>
<td>22.3 ± 2.2 a,c</td>
<td>32.2 ± 5.7</td>
<td>27.2 ± 1.3</td>
<td>27.6 ± 0.9</td>
</tr>
</tbody>
</table>

Table 2: Values are the average of ± S.D., n=5 observations per group. *** Different from the all groups (p<0.05). *Different from the control group. †Different from the control and standard groups. ‡Different from the saturated group (p<0.05). Data were analyzed by ANOVA followed by Tukey-Kramer’s Test.

**DISCUSSION**

This study shows that the intake of high fat diets promotes alterations in plasma level parameters in young rats. In fact, the cholesterol and low-density lipoproteins (LDLs), as well as, glucose were significant higher in rats that had a saturated diet. At the same time, the intake of conjugated different fats, saturated and unsaturated, shows a significant increase in the LDL-cholesterol levels (320%). In fact, recent studies have demonstrated the relevance of the association between the cholesterol levels and high fat diets on the neuroinflammatory response and the etiology of Alzheimer’s disease [24,25].

Additionally, the results obtained show the inhibition of hydrolysis of the adenine nucleotides and ACh in peripheral tissue markers of young rats exposed to high fat diets [26] demonstrated that the peripheral assays can contribute to determine the status of the central nervous system, in this case the peripheral marker showed a similar behavior when compared to the results obtained in erythrocytes and cerebral preparations from the cholinergic system [26]. Extracellular ATP, ADP and adenosine, products from the hydrolysis, are responsible for the vascular response to endothelial injury [27]. ADP has a putative role in the control of platelets activation, so the presence of enzymes that hydrolyze this diphosphate nucleoside in blood flow is essential to control the platelet aggregation and the thrombus formation [18].

In this case, the enzyme NTPDase inhibits the platelet aggregation by the hydrolysis of ATP and ADP, as also through the inhibitor formation from the metabolite adenosine aggregation, as well as through the bind blocking of the von Willebrand Factor (vWF), inhibiting the activation of platelet receptor complex glycoprotein (GP) Ib-V-IX [28]. In according to this fact, the hydrolysis of tri and diphosphate nucleoside acts as the modifier of the platelet responsiveness to the prothrombotic agonist ADP, thus the NTPase activity can be used as a potential anti-aggregatory agent, preventing the thrombotic lesions [29].

However, in this study we found an inhibition of NTPDase activity in platelets obtained from young rats after the intake of high fat diets, resulting in a decrease in the ATP and ADP hydrolysis. Thus, the enhance in the ADP levels elicit the platelet activation and the formation of thrombus. In addition, it is known that the ATP levels are low in physiological conditions, so it promotes the homeostatic responses, while very high extracellular ATP concentrations elicit the excessive activation...
of purinergic receptors in immune and non-immune cells [5,31,32]. The high ATP levels active the cascade of events to production of proinflammatory mediators that induce the tissue damage and the secretion of immune cells promoting the chronic inflammation [5].

Additionally, the association between the NTPDase inhibition with the enhance in the cholesterol, LDL-cholesterol and glucose levels, after the intake of fats diets can contribute to atheroma development. The increase of plaque lesions reduces the vascular lumen interrupting the blood flow, which along with the artery endothelium calcification associated to plaque progression can result in an eventual rupture of the vascular [4]. Moreover, considering that the cholesterol is a predominant factor to promote the atherosclerosis and as it is regulated by the high-density lipoprotein (HDL), responsible to remove the excess of cholesterol into the bloodstream [5] we can suggest that the decrease of HDL levels after the long-term intake of high fat diets can accelerate the atherosclerosis.

It is known that the ACh has a recognized role as an anti-inflammatory molecule, responsible to inhibit the inflammatory process through the reduction of the lymphocyte proliferation and pro-inflammatory cytokine secretion [33,34] It has become clear that the inhibition of AChE activity supports an anti-inflammatory effect, to increase the ACh levels and to minimize the inflammatory response. In fact, the same mechanism was observed in the increase of adenosine levels obtained through the 5'-nucleotidase activity inhibition.

CONCLUSIONS

There are, therefore, support to the hypothesis that the high fat consumption elicits the aggregation in promoting an inflammatory and prothrombogenic response, through the inhibition of NTPDase resulting to an increase of extracellular ATP and ADP levels and in high cholesterol levels. Additionally, the increase of ACh and adenosine levels can be interpreted as an overall protective effect against the inflammatory response and may contribute to atherosclerosis lesions. Altogether, these results demonstrated the potential of both NTPDase and AChE, as atherosclerosis modulators, showing the neuroprotective role of these enzymes.

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