Hydrochlorothiazide and high-fat diets reduce plasma magnesium levels and increase hepatic oxidative stress in rats

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Abstract. This study was designed to develop a rodent model of hydrochlorothiazide (HCTZ) toxicity by associating its intake with a high-fat (HF) diet. Rats were fed for 16 weeks with a control diet or with an HF diet supplemented or not with different doses of HCTZ. HCTZ, in a similar way to the HF diet, caused a significant increase in fructosamine levels. HCTZ and HF diet intake caused a significant reduction in magnesium and potassium levels, as well as an increase in lipid peroxidation and vitamin C in liver. Importantly, negative correlations were found between magnesium and glucose levels as well as between magnesium and fructosamine levels. The association between HCTZ and the HF diet caused additional worsening of biochemical parameters related to glucose homeostasis, and further increased hepatic oxidative stress. Our results suggest that chronic intake of HCTZ or an HF diet causes metabolic changes that are consistent with the development of insulin resistance. In addition, the association of an HF diet and HCTZ treatment can exacerbate some of these biochemical alterations, suggesting that this model might be useful for studying HCTZ metabolic toxicity.

Key words: high-fat diet, hydrochlorothiazide, hyperglycemia, oxidative stress, magnesium

Hydrochlorothiazide (HCTZ) is a diuretic that belongs to the thiazide class of compounds and is widely used for the treatment of hypertension [1]. The safety and efficacy of HCTZ in reducing morbidity and mortality in hypertensive patients have been demonstrated [2]. However, HCTZ can have low efficacy, and it can increase cardiovascular death and coronary artery disease [3, 4], if taken alone or in combination with other anti-hypertensive agents (for instance, benazepril [3]), and as such, its use has recently been questioned. Furthermore, HCTZ might be associated with the development of metabolic abnormalities such as hyperglycemia and type 2 diabetes mellitus [5, 6]. Therefore, the use of this class of diuretic alone is not recommended [3, 4].

Similarly, long-term, HF diets fat can promote the development of insulin resistance, which ultimately leads to an increased risk of developing type 2 diabetes mellitus [7, 8]. Experimentally,
an HF diet can be used in rodents to mimic human insulin resistance [9, 10]. Several studies have reported reduced insulin-mediated glucose metabolism in animals fed HF diets [8, 11]. More recently, peripheral magnesium levels have gained attention as an important factor associated with insulin resistance and metabolic syndrome development [12-14].

Hyperglycemia, the primary clinical manifestation of diabetes mellitus, is associated with non-enzymatic glycation of proteins and free radical generation [15-17]. These processes can cause permanent chemical alterations in proteins, and increase oxidative stress in a variety of experimental models of hyperglycemia [8, 10, 15-17]. In this context, excessive production of reactive oxygen species (ROS) or inadequate antioxidant protection can facilitate the development and progression of diabetes and its complications [18].

The liver is one of the primary, insulin-responsive organs and has a central role in modulating normal glucose homeostasis. Literature reports have demonstrated that hepatic damage induced by ROS can disrupt cellular homeostasis and aggravate metabolic syndrome features [19, 20]. Thus, in an attempt to develop a rodent model for studying the metabolic side effects of HCTZ, the main aim of this study was to investigate whether an association between an HF diet and HCTZ could have a negative, synergistic influence on fructosamine concentration and on other biochemical parameters associated with type 2 diabetes mellitus, including a depletion of plasma magnesium levels. In fact, an increase in plasma fructosamine is indicative of the development of insulin resistance. In addition, a possible relationship between these changes and oxidative stress was also investigated.

**Methods and materials**

**Chemicals**

Casein (technical grade), Comassie brilliant blue G, 2,4-dinitrophenylhydrazine, HCl, sodium sulphate dodecyl (SDS), heptane, acetate, ethanol, reduced glutathione, malondialdehyde (MDA) and thiobarbituric acid (TBA) were obtained from Sigma, (St. Louis, MO., USA). Mono- and dibasic potassium phosphate, acetic acid, ascorbic acid, ortho-phosphoric acid, tris buffer (tris[hydroxymethyl]aminomethane) and trichloroacetic acid were obtained from Merck (Rio de Janeiro, Brazil). Hydrochlorothiazide, cornstarch, lard, bone meal, wheat bran, soybean oil, vitamin and mineral complex were obtained from various commercial sources.

**Animals and diets**

Adult male Wistar rats (two months old), weighing 250-300 g, were used for the experiments. The animals were kept in a room where the temperature was regulated to 21-25 °C and humidity at roughly 56%, and with a 12 h light/12 h dark cycle, and free access to food and water. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (23081.002435/2007-16).

Rats were randomly divided into eight experimental groups, with five animals per group and fed for 16 weeks with: (1) control diet (CT); (2) CT plus HCTZ (0.4 g/kg of diet); (3) CT plus HCTZ (1.0 g/kg of diet); (4) CT plus HCTZ (4.0 g/kg of diet); (5) high-fat diet (HF); (6) HF plus HCTZ (0.4 g/kg of diet); (7) HF plus HCTZ (1.0 g/kg of diet) and (8) HF plus HCTZ (4.0 g/kg of diet). The composition of the diets is shown in **table 1**. Diets were prepared weekly and stored at 4 °C. Body weight was measured every week.

**Table 1.** Composition of the diets (g/kg).

<table>
<thead>
<tr>
<th>Components</th>
<th>High-fat diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>-</td>
<td>280</td>
</tr>
<tr>
<td>Casein</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Lard</td>
<td>280</td>
<td>-</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Bone flour</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>188</td>
<td>188</td>
</tr>
<tr>
<td>Mineral mixture 1</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mixture 2</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

1 The mineral mixture contained (g/kg): bone meal (449); NaCl (38); KCl (134.2); MgSO4 (20); ZnCl2 (0.4); CuSO4 (0.175); MnSO4 (1.2); FeSO4 (2), and cornstarch (355).

2 The vitamin mixture (mg or IU/g) was composed of vitamin A, 2000 IU; vitamin D, 200 IU; tocopherol, 10 IU; menadione, 0.5 mg; choline, 200 mg; folic acid, 0.2 mg; p-aminobenzoic acid, 1.0 mg; inositol, 10 mg; calcium D-panthotenate, 4.0 mg; riboflavin, 0.8 mg; thiamine-hydrochloride, 0.5 mg; pyridoxine-hydrochloride, 0.5 mg; niacinamide, 0.3 mg; and biotin, 0.04 mg.
Blood samples and tissue preparation

At the end of the experimental period, and after 12 h of fasting, the animals were sacrificed by decapitation under anesthesia. Blood was collected by cardiac puncture in heparinized tubes for magnesium, potassium and fructosamine determination. The samples of liver were quickly removed, rinsed with saline, weighed, placed on ice and homogenized in 10 volumes (w/v) in cold 50 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 4,000 × g at 4°C for 10 min to yield a low-speed supernatant fraction (S1) that was used for biochemical assays, except for measurement of protein carbonyl content (PCO), which was determined in samples of the homogenate.

Biochemical analysis

The plasma concentration of magnesium, potassium and fructosamine were measured using commercial Kits (Labtest, Minas Gerais, Brazil).

Lipid peroxidation (LPO) levels

Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBARS), and was expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al. [21], in which MDA, an end-product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. In brief, samples were incubated at 100°C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate, 1.27 mol/L acetic acid/270 mmol/L HCl, pH 3.5, and 0.8% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

Protein carbonyl (PCO) content

The PCO content was determined as described by Levine et al. [22] with some modifications. Briefly, homogenates were diluted to 750-800 μg/mL of protein in each sample, and 1 mL aliquots were mixed with 0.2 mL of 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.2 mL HCl (2 M). After incubation at room temperature for 1 h in a dark, ambient environment, 0.6 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.8 mL of heptane (99.5%) and 1.8 mL of ethanol (99.8%) were sequentially added, mixed with vortex agitation for 40 sec and centrifuged for 15 min. The protein isolated from the interface was washed twice with 1 mL of ethyl acetate/ethanol 1:1 (v/v), and suspended in 1 mL of denaturing buffer. Each DNPH sample was read at 370 nm in a spectrophotometer against the corresponding HCl sample (blank), and total carbonylation calculated using a molar extinction coefficient of 22,000/M.cm. Protein concentration was measured using Bradford’s method (1977), with bovine serum albumin as standard [23].

Vitamin C concentration

Total content of vitamin C (ascorbic acid) in liver was determined using the method of Jacques-Silva et al. [24]. Proteins were precipitated with 1 volume of cold, 10% trichloroacetic acid followed by centrifugation. An aliquot of 300 μL of the supernatants was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL), CuSO₄ (0.075 mg/mL) and trichloroacetic acid 13.3% (final volume 1 mL), and incubated for 3 h at 37°C. Then, 1 mL of H₂SO₄ 65% (v/v) was added to the medium. Ascorbic acid concentration was measured spectrophotometrically at 520 nm, and calculated using a standard curve (1.5-4.5 μmol/L ascorbic acid freshly prepared in sulfuric acid).

Statistical analysis

All values obtained are expressed as mean ± standard error. Data were analyzed by one-way, two-way or three-way ANOVA analyses of variance followed by Duncan’s multiple range tests when appropriate. Differences between groups were considered to be significant when p<0.05.

Results

Body weight

Three-way ANOVA (two diets x 4 HCTZ x 16 sampling times) revealed significant HCTZ x time interaction (figure 1, p<0.05), suggesting that HCTZ treatment caused a reduction in the body weight gain rate.
Figure 1. Effect of control (A, open symbols) or high-fat diet (B, closed symbols) supplemented with hydrochlorothiazide on body weight gain of rats. Data are expressed as means of five animals. The results of three-way ANOVA are indicated in the text. "*" Indicates a significant difference from the respective control group using Duncan’s multiple range test comparisons at the specified time points (p at least <0.05).
Fructosamine concentration

Two-way ANOVA for fructosamine data revealed a significant effect of the diet (F(1,32) = 32.23, p < 0.05). In fact, ingestion of the HF diet increased fructosamine concentration in all groups (table 2). In a previous study with the same rats used here, we observed that HCTZ and an HF diet increased blood glucose concentrations [25].

Magnesium and potassium determination

Two-way ANOVA of magnesium concentration revealed a significant effect of diet [F(1,32) = 17.23, p < 0.05], and a significant effect of HCTZ [F(3,32) = 8.34, p < 0.05]. Post hoc treatment using Duncan’s multiple range tests revealed that an HF diet caused a significant reduction in magnesium levels. Analyses also demonstrated that HCTZ decreased plasma magnesium levels in control and HF diets (table 2). Of particular importance, negative correlations were found between the magnesium and glucose concentrations (r = -0.51, p < 0.05), as well as between magnesium and fructosamine concentrations (r = -0.37, p < 0.05).

Two-way ANOVA of potassium concentration revealed a significant effect of the diet (F(1,32) = 20.8, p < 0.05) and of the HCTZ treatment [F(3,32) = 10.99, p < 0.05] and a tendency for significant diet x HCTZ interaction [F(3,32) = 2.44, p < 0.10]. Post hoc comparisons indicated that HCTZ and HF caused a significant decrease in potassium concentrations when compared to the CT group (table 2).

Protein carbonyl group content

No significant difference among the groups was observed in PCO levels in the liver (table 3, p > 0.05).

Vitamin C concentration

Two-way ANOVA revealed that the intake of the HF diet [F(1,32) = 50.96, p < 0.05] and HCTZ treatment [F(3,32) = 21.16, p < 0.05] caused a significant increase in the vitamin C concentrations in the liver. However, the increase was proportionally higher in the HF groups treated simultaneously with HCTZ as highlighted by a significant diet x HCTZ treatment interaction [F(3,32) = 8.85, p < 0.05] (table 3) (p > 0.05).

Discussion

It has been shown that long-term consumption of an HF diet, and HCTZ treatment, are important factors for the development of certain metabolic changes related to insulin resistance. In the

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fructosamine (mmol/L)</th>
<th>Magnesium (mg/dL)</th>
<th>Potassium (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>0.99 ± 0.03</td>
<td>1.44 ± 0.08</td>
<td>6.8 ± 0.16</td>
</tr>
<tr>
<td>CT+HCTZ (0.4 g/kg)</td>
<td>1.04 ± 0.04</td>
<td>1.35 ± 0.06</td>
<td>5.2 ± 0.22*</td>
</tr>
<tr>
<td>CT+HCTZ (1.0 g/kg)</td>
<td>1.10 ± 0.04</td>
<td>1.10 ± 0.05*</td>
<td>5.2 ± 0.19*</td>
</tr>
<tr>
<td>CT+HCTZ (4.0 g/kg)</td>
<td>1.01 ± 0.04</td>
<td>1.10 ± 0.04*</td>
<td>5.3 ± 0.18*</td>
</tr>
<tr>
<td>HF</td>
<td>1.62 ± 0.03*</td>
<td>1.21 ± 0.09*</td>
<td>5.3 ± 0.12*</td>
</tr>
<tr>
<td>HF+HCTZ (0.4 g/kg)</td>
<td>1.42 ± 0.04*</td>
<td>1.01 ± 0.07*</td>
<td>4.7 ± 0.16*#</td>
</tr>
<tr>
<td>HF+HCTZ (1.0 g/kg)</td>
<td>1.43 ± 0.04*</td>
<td>0.9 ± 0.04*#</td>
<td>4.5 ± 0.21*#</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.M. of five animals. * Indicates a significant difference from CT or # from HF, p < 0.05.

Table 2. Effect of hydrochlorothiazide associated with a control or high-fat diet on plasma biochemical parameters.
Table 3. Effect of hydrochlorothiazide associated with a control or high-fat diet on hepatic thiobarbituric acid reactive substances (TBARS), protein carbonyl (PCO) and vitamin C levels.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (nmol/g tissue)</th>
<th>Protein carbonyl (nmol/mg protein)</th>
<th>Vitamin C (g/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>67.8 ± 0.9</td>
<td>23.2 ± 1.3</td>
<td>498.0 ± 20.0</td>
</tr>
<tr>
<td>CT+HCTZ (0.4 g/kg)</td>
<td>75.2 ± 0.8*</td>
<td>18.8 ± 1.8</td>
<td>717.6 ± 32.4*</td>
</tr>
<tr>
<td>CT+HCTZ (1.0 g/kg)</td>
<td>77.4 ± 0.7*</td>
<td>20.1 ± 1.6</td>
<td>721.1 ± 55.7*</td>
</tr>
<tr>
<td>CT+HCTZ (4.0 g/kg)</td>
<td>73.3 ± 0.7*</td>
<td>20.7 ± 1.4</td>
<td>696.0 ± 50.9*</td>
</tr>
<tr>
<td>HF</td>
<td>75.1 ± 0.8*</td>
<td>17.7 ± 1.0</td>
<td>684.0 ± 17.4*</td>
</tr>
<tr>
<td>HF+HCTZ (0.4 g/kg)</td>
<td>75.3 ± 0.6*</td>
<td>19.1 ± 0.7</td>
<td>811.4 ± 69.1*</td>
</tr>
<tr>
<td>HF+HCTZ (1.0 g/kg)</td>
<td>76.0 ± 0.9*</td>
<td>19.1 ± 1.4</td>
<td>842.8 ± 59.7*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.M. of five animals. *indicates a significant difference from CT or # from HF, p<0.05.

present study we saw that an HF diet, associated or not with HCTZ, enhanced fructosaminemia, which is compatible with the development of insulin resistance. Literature data have reported that chronic intake of an HF diet [10], in addition to hyperglycemia are linked to oxidative stress, and that increased concentrations of ROS are involved in the development of insulin resistance [8, 10, 11]. However, data about the potential facilitating effects of HCTZ in promoting insulin resistance and oxidative stress in animal models are scarce or lacking in the literature.

As regards oxidative stress, we have assessed its potential occurrence in hepatic tissue following treatment with HCTZ and an HF diet by determination of TBARS to estimate LPO concentration. Although TBARS quantification has certain limitations, such as the detection of products that are not derived from lipid peroxidation, as well as the potential interference of factors such as high temperature that may cause artifactual formation of lipid peroxidation products during color development [26], this method has been extensively used as a marker of LPO in biological materials [21, 26-28]. With this in mind, we recently compared the Ohkawa’s colorimetric method [21] with a high performance liquid chromatographic (HPLC) analysis [29]. Results were similar for the two methods and the artifactual increase in TBARS production during color development did not occur as a result of the presence of SDS in medium [29]. Thus, it is possible to assert that TBARS could be used to determine oxidative stress. In this context, we observed that chronic HF consumption caused a significant increase in hepatic TBARS concentration that was potentiated by HCTZ treatment. Accordingly, the HF diet produced an increase in fructosamine and oxidative stress in hepatic tissue, which was potentiated by HCTZ. In general, non-enzymatic glycation of proteins generates highly reactive products that might explain the relationship between hyperglycemia and lipid peroxidation [25]. The concentrations of PCO were not modified by treatment; however, these results must be viewed with caution because we did not include a positive control in the present experiments, and the oxidation of specific types of target proteins cannot be ruled out.

Another aspect that must be considered is that the consumption of the HF diet increased hepatic vitamin C concentration, something that might have occurred as a mechanism of protection against lipid peroxidation. Accordingly, an increase in the antioxidant defense systems has been observed in a variety of experimental models of pathologies, possibly as a compensatory response of the tissues to the presence of oxidative insults [25, 30]. Alternatively, the observed effect may be related to a direct stimulatory effect of HCTZ or some fatty acid in the signaling pathways leading to increased synthesis or to a decrease in vitamin C degradation and not related to oxidative stress at all. Furthermore, HCTZ had a direct effect on vitamin C concentrations that were independent of the HF diet intake.

It has been suggested that the depletion of potassium by thiazide is likely to have a role in impaired glucose metabolism, perhaps by impairing β-cell insulin release [31] and that potassium supplementation can attenuate glucose intolerance induced by thiazides [32]. Intracellular magnesium also seems to play a key role in modulating
insulin-mediated glucose uptake [33, 34]. In fact, studies have demonstrated a relationship between low magnesium concentrations with metabolic diseases such as type 2 diabetes mellitus and hypertension [33-41]. Accordingly, here we found a significant decrease in plasma magnesium and potassium concentrations, as well as an increase in blood fructosamine and hepatic lipid peroxidation in rats fed an HF diet, associated or not with HCTZ. It is important to emphasize that the association of the HF diet with HCTZ (4.0 g/kg of diet) potentiated magnesium depletion and increased lipid peroxidation. The results presented here indicate that magnesium and potassium depletion plays an important role in the metabolic toxicity of HCTZ. Besides, the association of HCTZ and an HF diet caused additional loss of magnesium, which may indicate that this element has a more fundamental role in HCTZ toxicity than potassium. Since biochemical changes were exacerbated by the combined consumption of HCTZ and an HF diet, we can suppose that magnesium depletion has a central role in the metabolic toxicity of HCTZ. The imbalance in these metabolic processes could be compensating for the stimulatory effect of the HF diet on body weight gain.

The data presented here show that the chronic intake of HCTZ or an HF diet causes metabolic changes consistent with the development of insulin resistance, and that the association of the HF diet with HCTZ treatment might exacerbate some of these biochemical alterations, particularly magnesium concentration. We would like to suggest therefore that our experimental model could be used in the study of the metabolic side effects of HCTZ. In view of the fact that HF ingestion aggravated the toxicity of HCTZ, it would be important to investigate whether the incidence of type 2 diabetes in hypertensive patients taking HCTZ is higher in patients who have diets with high levels of fat. Indeed, environmental factors can affect the metabolic response to HCTZ [6], and reinforces the importance of investigating the role of fat ingestion in development of the side effects of HCTZ in hypertensive subjects.

Disclosure

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