Metabolic acidosis lowers circulating adiponectin through inhibition of adiponectin gene transcription

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Abstract

Background. Metabolic acidosis (MA) adversely affects protein and lipid metabolism as well as endocrine function. Adipose tissue communicates with the rest of the body through synthesis and release adipokines, such as leptin, adiponectin and TNF-alpha. Adiponectin enhances insulin sensitivity and possesses anti-atherogenic and anti-inflammatory properties. Circulating adiponectin correlates inversely with cardiovascular events. It is possible that MA negatively regulates adiponectin contributing to poor patient outcome. The present study investigates the effect of MA on adiponectin in vivo and in vitro.

Methods. Twenty healthy female volunteers underwent a 7-day course of oral ammonium chloride (NH₄Cl)-induced acidosis. Serum adiponectin was determined before and after NH₄Cl ingestion. Adipocytes were differentiated from their precursors, human mesenchymal stem cells (hMSCs), in culture. Concentrated HCl was added to the media to lower pH. Adiponectin mRNA and protein were determined at 48 and 96 h by real-time RT–PCR and ELISA, respectively.

Results. After a 7-day course of NH₄Cl, serum bicarbonate decreased significantly associated with the increase in urine ammonium and titratable acid. Adiponectin decreased significantly from 10 623 to 9723 pg/mL (P < 0.005). MA suppressed adiponectin mRNA in hMSC-derived adipocytes at 48 and 96 h (P < 0.01). The amount of adiponectin released into the culture media declined corresponding to the mRNA levels (P < 0.001). MA did not affect adipocyte triglyceride or protein content.

Conclusions. MA lowered circulating adiponectin through inhibition of adiponectin gene transcription in adipocytes.

Keywords: adipocyte; adipokine; adiponectin; mesenchymal stem cell; metabolic acidosis

Introduction

Metabolic acidosis (MA) complicates later stages of chronic kidney disease (CKD) and occurs as a primary consequence of disorder in acid secretion, such as renal tubular acidosis (RTA), or secondary to other conditions, for example medication and ketoacidosis. MA results in various metabolic consequences, including protein-energy malnutrition, osteoporosis, impaired endocrine function and altered lipid metabolism [1–6]. The increase in mortality was observed in pre-dialysis CKD patients with MA [7]. Adipose tissue, a site where excess energy is stored as triglyceride, communicates with the rest of the body by synthesizing and releasing adipokines, such as leptin, adiponectin, TNF-alpha and interleukin-6 (IL-6) [8]. Leptin controls food intake and energy expenditure and modulates immune response. Previously, MA has been shown to lower serum leptin in uraemic rats through suppression of leptin secretion from adipocytes [6]. Adiponectin is a 30-kDa adipokine exclusively expressed in mature adipocytes. Adiponectin possesses insulin-sensitizing, anti-atherogenic and anti-inflammatory properties [9]. Adiponectin levels are inversely associated with obesity-induced insulin resistance and the development of hypertension and coronary artery disease [10,11]. Several studies indicated the importance of adiponectin as diagnostic–prognostic value in cardiovascular diseases [12,13]. The effect of MA on adiponectin has never been investigated. Its negative impact on adiponectin might as well participate in poor patient outcome. The present study examined the effect of MA on serum adiponectin in human volunteers and the direct effect of MA on adiponectin mRNA and protein in cultured human adipocytes.

Materials and methods

Subjects and protocol

Twenty healthy female volunteers were given ammonium chloride (NH₄Cl) 187.3 mg/kg/day in four-divided dose for 7 days. Fasting blood
and urine samples were collected prior to and after a 7-day course of NH₄Cl. Serum samples were aliquoted and stored at −20°C for further measurement of adiponectin. Significant metabolic acidemia produced by this protocol was confirmed in a preliminary study of five healthy female volunteers by arterial blood gas analysis obtained at baseline and between the doses of NH₄Cl on the third day of ingestion (Table 1). The study protocol was approved by the ethical committee for research involving human subjects of Ramathibodi Hospital, Mahidol University and conducted according to the Declaration of Helsinki. Written informed consents were obtained from all subjects.

Biochemical analysis

Blood and urine chemistries were determined by Technicon automatic analyser (Dade Behring, Marburg, Germany). Urinary ammonium (NH₄⁺) and titratable acid (TA) were determined by standard titrimetry. Serum total adiponectin was determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Reagents

Tissue culture media and accessories were obtained from Hyclone (Waltham, MA, USA). Other chemical reagents (unless specified otherwise) were supplied by Sigma (Saint Louis, MO, USA). Reagents used in quantitative real-time RT–PCR were purchased from Jena Bioscience (Jena, Germany).

Adipocyte differentiation from human mesenchymal stem cells (hMSCs)

Isolation and characterization of hMSCs in our lab have been described in details previously [14]. Briefly, bone marrow samples were obtained from healthy bone marrow donors. Bone marrow mononuclear cells were separated by density gradient centrifugation. A total of 2 × 10⁶ cells/mL were expanded in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PCN/Strep). The detached haematopoietic cells were discarded every 4 days. Upon 90% confluence, hMSCs were trypsinized and passaged for the next expansion. The purity of hMSCs was confirmed by the presence of cell surface markers (CD105+CD44−CD73−CD34−HLA-DR−CD115−) using flow cytometry. Cells between passages 3 and 5 were used for adipocyte differentiation according to the previously published protocols with some modifications [15–17]. hMSCs in DMEM:DMEM/F-12 1:1, 10% FBS, 1% PCN/Strep and 2.5 μg/mL amphotericin B were plated in multi-well tissue culture plates at an initial density of 4 × 10⁴ cells/cm² and 1% PCN/Strep and 2.5 μg/mL streptomycin (PCN/Strep). The detached haematopoietic cells were expanded in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PCN/Strep). The detached haematopoietic cells were discarded every 4 days. Upon 90% confluence, hMSCs were trypsinized and passaged for the next expansion. The purity of hMSCs was confirmed by the presence of cell surface markers (CD105+CD44−CD73−CD34−HLA-DR−CD115−) using flow cytometry. Cells between passages 3 and 5 were used for adipocyte differentiation according to the previously published protocols with some modifications [15–17]. hMSCs in DMEM:DMEM/F-12 1:1, 10% FBS, 1% PCN/Strep and 2.5 μg/mL amphotericin B were plated in multi-well tissue culture plates at an initial density of 4 × 10⁴ cells/cm² and 1% PCO₂ incubator. One day post-confluency, troglitazone and 3-isobutyl-1-methylxanthine 25 μg/mL were added to induce adipocyte differentiation. The media was changed twice a week, and the supernatant was further diluted 1:6 and used for protein assay by Bradford. The rest of the cell lysate was heated to 80°C for 5 min and the heat block and allowed to cool to room temperature. The heating and cooling were repeated twice, and the supernatant was used for triglyceride assay by triglyceride reagent. Assays were performed in duplicate.

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity

This was performed according to the previously published protocol with some modifications [18]. Differentiated adipocytes in 24-well plates were washed three times with PBS. Ice-cold GPDH enzyme extraction buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA and 5% Triton-X) followed by three freeze-thaw cycles at −80°C and 37°C. The cells were diluted 1:10 in cold 20 mM Tris pH 7.4 and 150 mM NaCl, scraped off the plate, transferred to microcentrifuge tubes and homogenized through 23-gauge needles 25 times on ice followed by centrifugation. The supernatant was further diluted 1:6 and used for protein assay by Bradford. The rest of the cell lysate was heated to 80°C for 5 min in the heat block and allowed to cool to room temperature. The heating and cooling were repeated twice, and the supernatant was used for triglyceride assay by triglyceride reagent. Assays were performed in duplicate.

Statistical analysis

Results are presented as mean ± SD unless specified otherwise. Student’s t-test was used to compare group means. Relationships between two continuous variables were evaluated by Pearson’s correlation. The difference was considered significant at P-value <0.05.
Results

Effect of NH₄Cl-induced acidosis on adiponectin in healthy female volunteers

In order to study the effect of MA on circulating adiponectin, oral NH₄Cl ingestion was used to induce metabolic acidosis. In a preliminary study of five healthy female volunteers, ingestion of NH₄Cl resulted in a significant decrease in arterial pH from 7.44 to 7.32 associated with an increase in H⁺ ion concentration and a reduction in HCO₃⁻ on Day 3 (Table 1). This protocol was applied to induce MA in 20 healthy female volunteers for 7 days. Biochemical parameters and serum adiponectin were evaluated before and after a 7-day course of NH₄Cl ingestion. Only female subjects were chosen because of the significant difference between fat mass and circulating adipokine levels between male and female [19]. At the study completion, the body weight (BW) and body mass index (BMI) decreased slightly but significantly. The presence of MA was confirmed by the decline in serum bicarbonate in concomitant with an increase in chloride (Table 2). Marked increase in 24-h urine NH₄⁺ and TA as well as urinary wasting of potassium, calcium and phosphate that commonly occurs during MA were observed in our subjects (Table 2; Figure 1) [20,21].

Table 2. Biochemical parameters of the subjects before and after 7 days of NH₄Cl-induced acidosis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before (n = 20)</th>
<th>After (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32 ± 6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (kg)</td>
<td>53.4 ± 9.9</td>
<td>52.6 ± 10</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.6 ± 2.9</td>
<td>21.3 ± 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>140.2 ± 2.4</td>
<td>139.2 ± 2.3</td>
<td>0.23</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>3.9 ± 0.3</td>
<td>3.8 ± 0.3</td>
<td>0.24</td>
</tr>
<tr>
<td>Cl (mmol/L)</td>
<td>103 ± 2.5</td>
<td>107 ± 2.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Total CO₂ (mmol/L)</td>
<td>25.8 ± 2.5</td>
<td>18.6 ± 3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.42</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.1 ± 0.4</td>
<td>8.8 ± 0.5</td>
<td>0.08</td>
</tr>
<tr>
<td>PO₄ (mg/dL)</td>
<td>4 ± 0.7</td>
<td>3.7 ± 0.8</td>
<td>0.14</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>45.8 ± 3.4</td>
<td>45.4 ± 4.5</td>
<td>0.67</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>67.3 ± 26.6</td>
<td>61.9 ± 30.8</td>
<td>0.43</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>82.1 ± 6.3</td>
<td>81.4 ± 7.3</td>
<td>0.62</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.53 ± 0.31</td>
<td>0.51 ± 0.31</td>
<td>0.69</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dL)</td>
<td>0.17 ± 0.05</td>
<td>0.15 ± 0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Urine K (mEq/day)</td>
<td>34.1 ± 16.3</td>
<td>42.4 ± 22.7</td>
<td>0.04</td>
</tr>
<tr>
<td>Urine Calcium (mg/day)</td>
<td>116 ± 59</td>
<td>162 ± 62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine PO₄ (mg/day)</td>
<td>495 ± 24</td>
<td>890 ± 39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine NH₄ (mEq/day)</td>
<td>17.6 ± 8.5</td>
<td>40.9 ± 15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urine titratable acid (mEq/day)</td>
<td>35 ± 30.6</td>
<td>124 ± 82</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Fig. 1. Twenty-four-hour urine collection before and after 7 days of NH₄Cl-induced acidosis expressed as fold change from baseline. (A) Urine NH₄⁺ and titratable acid (TA). (B) Urine potassium (K), calcium (Ca) and phosphate (PO₄).

Fig. 2. (A) Serum adiponectin before and after 7 days of NH₄Cl-induced acidosis and (B) after correction for the changes in BMI (n = 20).
an inverse correlation with the changes of fasting plasma glucose ($r = -0.785$, $P < 0.001$) and serum direct bilirubin ($r = -0.491$; $P = 0.03$).

Adiponectin mRNA and adiponectin protein secretion from cultured adipocytes

Isolated adipocytes cultured directly on the surface normally float on top of the medium and undergo cell lysis within 72 h; therefore, in most studies, adipocytes were differentiated from the precursor cells, such as MSCs isolated from bone marrow, peripheral blood or adipose tissue [17,22,23]. MSCs are multi-potent cells that can differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon and muscle under appropriate condition [24]. The characterization process for hMSCs in our lab has been described previously [14]. In the present study, confluent hMSCs were able to differentiate into mature adipocytes up to 60–90% (Figure 3A and B). To simulate MA in vitro, mature adipocytes were incubated in the presence of HCl. The pH and gas composition of the media are shown in Table 3. MA suppressed adiponectin mRNA and protein up to 70% at 48 and 96 h (Figure 4A and B). MA has been shown to promote triglyceride accumulation of cultured adipocytes and inhibit cell proliferation in long-term culture [6,14]. In the present study, there was no significant change in the amount of triglyceride or protein content of adipocytes cultured in acid media up to 96 h (Figure 4C and D). To ensure that MA had no significant effect on adipocyte differentiation during the study period, GPDH enzyme activity was monitored. GPDH is an enzyme necessary for triglyceride synthesis. It is up-regulated in matured adipocytes and used as a marker for terminal adipocyte differentiation [18,25]. Although GPDH enzyme activity declined slightly during the 96-h study period, MA did not have a significant effect on enzyme activity when compared with neutral pH (Figure 5). Adipocyte morphologies on light microscopy, e.g. the number of differentiated cells or the size of lipid droplets, appeared similar at the end of the incubation period (Figure 3C and D).

<table>
<thead>
<tr>
<th>HCl (μL/mL)</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
<th>HCO₃ (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.4</td>
<td>34.8</td>
<td>24.1</td>
</tr>
<tr>
<td>2</td>
<td>6.9</td>
<td>30.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Discussion

MA affects several systems in the body ranging from enhanced protein breakdown to osteoporosis and abnormal endocrine function [1,3,4]. The increased mortality was observed in pre-dialysis CKD patients with MA [7]. Adiponectin, an adipokine expressed exclusively in adipocytes, possesses insulin-sensitizing, anti-atherogenic and anti-inflammatory properties [9]. Adiponectin levels are inversely associated with insulin resistance and coronary artery disease [10,11]. The present study is the first to illustrate the effect of MA on adiponectin in vivo and in vitro. MA lowers circulating adiponectin possibly through a direct suppression of adiponectin mRNA expression and adiponectin secretion from adipocytes.

**Effect of MA on circulating adiponectin**

Previously, MA has been shown to lower serum leptin in an animal model of uraemic acidosis [6]. In CKD patients, infusion of NaHCO₃ augmented serum leptin, and in distal RTA, alkaline therapy restored circulating leptin towards normal [26,27]. The effect of MA on circulating adiponectin has never been reported in animal or human. In the present study, oral NH₄Cl was used to induce MA in healthy subjects. The presence of MA was confirmed by the decline in serum bicarbonate and the increase in urine NH₄⁺ and TA. The augmented kaliuria, calciuria and phosphaturia ensure significant acidaemia. MA lowered serum adiponectin significantly when compared with baseline. The loss of weight among the subjects might have affected adipose tissue mass and adiponectin. However, after correction for BMI, the decline in adiponectin remained significant. Moreover, circulating adiponectin is known to have an inverse relationship with BW and BMI, and weight reduction positively influenced serum adiponectin [10].
Therefore, it is unlikely that changes in the BW were utterly responsible for the decline in adiponectin. The biological relevance of the changes of adiponectin by MA was demonstrated by the inverse relationship with the changes of plasma glucose, which might indicate the possibility of impaired insulin sensitivity. A properly designed study to evaluate insulin sensitivity will be required to confirm this observation. Recently, a protective effect of adiponectin on hepatic steatosis was suggested [28,29]. The inverse relationship between the changes of adiponectin and serum direct bilirubin supported the role of adiponectin in hepatic metabolism. Considering the importance of adiponectin as a cardioprotective protein with significant impact on metabolic syndrome and cardiovascular disease, further study regarding long-term outcome of the patients is warranted. In CKD patients, adiponectin accumulates, and adiponectin levels increase as GFR declines. The association of low circulating adiponectin with cardiovascular disease was preserved after adjusting for CKD stage [30–32].

The increase in cardiovascular mortality is well known among CKD patients; therefore, the negative impact of MA on adiponectin may contribute to the poor patient outcome.

**Effect of MA on adiponectin mRNA and protein secretion from cultured adipocytes**

Previous evidence suggested the effect of MA on several systems in the body occurred through the alteration of gene expression [1,14,33,34]. In the present study, MA markedly suppressed adiponectin mRNA and protein secretion from cultured adipocytes. MA has been shown to inhibit leptin secretion by post-translational mechanism [35]. Up-regulation of ubiquitin and proteasome mRNAs resulted in an increase in muscle proteolysis during MA [1]. In bone, acidosis induced increase in bone resorption through up-regulation of TNF-alpha as well as altered the expression of several osteoblastic genes [14,36–38]. The mechanism responsible for inhibition of adiponectin mRNA and protein could be direct or mediated through other mediators. For example, MA augments the release of several cytokines such as TNF-alpha, IL-6 and IL-10 [39]. Chronic exposure of adipocytes to TNF-alpha and IL-6 suppressed adiponectin secretion [40]. TNF-alpha and IL-6 are adipokines, and locally increased concentration as a result of acidosis may contribute to attenuated adiponectin expression. In end-stage renal disease, the expression of adiponectin mRNA in adipose tissue was reduced when compared with healthy controls [41]. MA may be involved in the down-regulation of adiponectin in this regard.

**Limitation of the study**

Arterial pH was not obtained during the study period; however, to ensure significant acidemia produced by NH4Cl ingestion, arterial blood gas analysis was analysed in a preliminary study that included a small number of healthy female subjects. During the study, significant MA was ascertained by the marked increase in urine NH4+ and TA as well as the presence of increased calcium and phosphaturia. Adiponectin exists in several different isoforms with variable biological activities. Insulin-sensitizing action appeared to be related to the presence of high-molecular-weight isoform [42]. In the present study, total adiponectin was measured, and the effect of MA on different adiponectin isoforms was not examined. The signalling mechanism by which MA alters adiponectin mRNA and protein was not elucidated and will necessitate further study. The long-term effect of MA on the body is complex. The degree varies, and several systems are affected. Experiments with short-term induction of MA may not, in all respects, represent the long-term consequences.

In conclusion, MA lowers circulating adiponectin through inhibition of adiponectin gene transcription in adipocytes. The impact of such finding on patient outcome in various conditions will require further studies.

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**Conflict of interest statement.** None declared.

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