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 (NH 4Cl)-induced acidosis. Serum adiponectin was determined before and after NH 4Cl 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 NH 4Cl, 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

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
Table 1. Arterial blood gas analysis at baseline and on Day 3 of NH4Cl ingestion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline (n = 5)</th>
<th>NH4Cl (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.44 ± 0.01</td>
<td>7.32 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
<td>104.6 ± 12.7</td>
<td>112.7 ± 9.8</td>
<td>0.058</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>36.5 ± 5.1</td>
<td>28.8 ± 3.2</td>
<td>0.057</td>
</tr>
<tr>
<td>HCO3 (mEq/L)</td>
<td>24.8 ± 3.4</td>
<td>15.0 ± 1.7</td>
<td>0.008</td>
</tr>
<tr>
<td>Total CO2 (mEq/L)</td>
<td>25.9 ± 3.5</td>
<td>15.9 ± 1.8</td>
<td>0.008</td>
</tr>
</tbody>
</table>

*Calculated from 24 × pCO2/HCO3.

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 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).

Adiponectin mRNA expression by quantitative real-time RT–PCR

Total RNA was isolated and reverse transcribed into cDNAs using oligo (dT) primer and M-MLV reverse transcriptase in a two-step RT–PCR reaction. Quantitative real-time RT–PCR was performed in a multiplexed reaction in triplicate on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA, USA). The primer and probe sequences for adiponectin and beta-actin (reference gene) (Biosearch Technologies, Novato, CA, USA) are as follows: adiponectin: forward 5′-ccccaggaagtgaagagagggatt-3′, reverse 5′-gggctcaacatggaatcaaa-3′, probe 5′-FAM-tctcagacgacgccagcttgat-3′; and beta-actin: forward 5′-tgctcgacagatgacaa-3′, reverse 5′-gcctcgacagatgagca-3′, probe 5′-CAL-Flour-Orange-560-gaccacagaattctggactgctctcc-3′. Relative expression levels of adiponectin normalized by beta-actin were calculated. The average values of delta Cts from each sample were used for statistical analysis.

Adiponectin protein secretion, and triglyceride and protein content of hMSC-derived adipocytes

At the end of the incubation period, the conditioned media was collected and aliquoted at ~80°C for further analysis of total adiponectin by ELISA (Zen-Bio, Research Triangle Park, NC, USA). Adherent cells were lysed in cold cell lysis 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.

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, 1 mM EDTA and 1 mM mercapto) 400 μL was added to each well. The plates were frozen at ~80°C for at least 12 h. Subsequently, the cell lysate was thawed at room temperature for 15 min, transferred to cold microcentrifuge tubes and centrifuged at 4°C to precipitate lipid. The supernatant was diluted further 1:10, and 100 μL was used in enzyme kinetic assay. GPDH converts dihydroxyacetone phosphate (DHAP) through oxidation of NADH that can be monitored by absorbometry at 340 nm. One hundred microlitres of assay solution containing 0.334 mM β-NADH and 0.4 mM DHAP in 0.1 M triethanolamine, 2.5 mM EDTA and 0.1 mM mercapto was pre-warmed to 37°C in temperature-controlled ELISA reader. The supernatant was transferred into the assay solution, and the absorbance was read immediately at a 30-s interval for a duration of 3 min with shaking prior to each reading. The maximum slope that represented enzyme activity was calculated. 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 acidemia. In a preliminary study of five healthy female volunteers, ingestion of NH₄Cl resulted in a significant decline 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]. Circulating adiponectin at baseline and after NH₄Cl ingestion is shown in Figure 2. Serum adiponectin decreased significantly after a 7-day course of NH₄Cl-induced acidosis. The reduction in adiponectin concentration persisted after correction for the changes in BMI. Analysis of the association between the percentage changes of adiponectin from baseline to other biochemical parameters revealed

<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>25.6 ± 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).
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 3. pH and gas composition of culture media

<table>
<thead>
<tr>
<th>HCl (μL/mL)</th>
<th>pH</th>
<th>$pCO_2$ (mmHg)</th>
<th>$HCO_3$ (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 acidemia. 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].

Fig. 4. (A) Adiponectin mRNA expression (mean ± SE of delta Cts), (B) adiponectin protein secretion from adipocytes, (C) adipocyte triglyceride content and (D) adipocyte protein content. Results are representative of 3–5 separate experiments (n = 32–52 culture wells for each condition).

Fig. 5. Adipocyte GPDH enzyme activity. There was no significant difference in the enzyme activity between pH 7.4 and 6.9 (n = 12–16 culture wells for each condition; *P < 0.01 vs. 2 h; **P < 0.001 vs. 2 h).
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 calcuria 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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