The association of adipose-derived dimethylarginine dimethylaminohydrolase-2 with insulin sensitivity in experimental type 2 diabetes mellitus

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Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS), which can be hydrolyzed by dimethylarginine-dimethylaminohydrolase (DDAH). It has been reported that adipocytes can produce DDAH/ADMA, but its role remains unknown. In the present study, we examined the effects of adipocyte-derived DDAH/ADMA on insulin sensitivity using animal and cell models. Results showed that in adipose tissue of high fat diet-fed diabetic rats, as well as in high glucose (25 mM) plus insulin (100 nM)-treated 3T3-L1 adipocytes, expression levels of insulin receptor substrate-1 (IRS-1), glucose transporter-4 (GLUT-4), and DDAH isoform-2 (DDAH-2) were down-regulated compared with control, although DDAH-1 expression showed no significant changes. We also observed that nitric oxide bioavailability, DDAH and NOS activities were subsequently decreased, while the local ADMA content was elevated in diabetic adipose tissue. Transfection of human DDAH-2 gene into high glucose- and insulin-treated 3T3-L1 adipocytes significantly ameliorated DDAH activity, reduced ADMA contents, and up-regulated the mRNA expression levels of IRS-1 and GLUT-4. These findings suggested that in the development of type 2 diabetes mellitus, local DDAH-2 in adipocytes might play an important role in regulating insulin sensitivity.

Keywords dimethylarginine dimethylaminohydrolase; insulin resistance; diabetes mellitus; adipocyte

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Introduction

Asymmetric dimethylarginine (ADMA) is widely accepted as a predictor of endothelium dysfunction. It can impair nitric oxide (NO) bioavailability, promote oxidative stress, and induce inflammation [1,2]. Previous studies showed that plasma concentrations of ADMA were increased in some metabolic diseases, such as diabetes mellitus and polycystic ovary syndrome [3–5]. It was positively correlated with body mass index and homeostatic model assessment of insulin resistance (HOMA-IR). Moreover, weight loss was accompanied with reduced ADMA level [6]. These studies indicated that ADMA might play a role in the development of insulin resistance (IR).

It is difficult to directly regulate ADMA at gene level, because it is only a kind of metabolite of arginine. Dimethylarginine-dimethylaminohydrolase (DDAH), including DDAH-1 and DDAH-2 isoforms, is specifically responsible for hydrolyzing ADMA. Up-regulation of DDAH expression or activity significantly reduces the ADMA level [7]. Interestingly, it has been reported that DDAH also has ‘non-hydrolase function’ besides its ‘hydrolase function’ in hydrolyzing ADMA. DDAH can promote angiogenesis by regulating vascular endothelial growth factor (VEGF) expression, which might be independent of ADMA [8]. Therefore, studies which focus on DDAH itself have increased recently.

It is widely accepted that adipose tissue is not only an energy-storage organ, but also an important endocrine organ, which is able to secrete abundant adipokines regulating insulin sensitivity [9–11]. Since DDAH is expressed in human adipocytes [12], we are interested in finding the role of adipocyte-derived DDAH/ADMA in IR. Moreover, it has been reported that there are notable differences in the distribution of DDAH-1 and DDAH-2, and the DDAH isoforms exert different contributions to plasma concentrations of ADMA and NO [13–16]. Therefore, we also examined the expression levels of DDAH isoforms in adipose tissue.
Materials and Methods

Materials
Male Sprague–Dawley rats (180 ± 20 g) were obtained from Animal Services of Central South University (Changsha, China). Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium, streptozotocin (STZ), insulin, oil red O, 3-isobutyl-1-methyl xanthine (IBMX), and dexamethasone were obtained from Sigma (St Louis, USA). Nitric oxide synthase (NOS) and NO assay kits were purchased from Jian-Chen Biological Medical Engineering Institute (Nanjing, China). Trizol reagent and Superscript III first-strand were from Invitrogen (Carlsbad, USA). The PerfectShot Taq kit was from TaKaRa (Dalian, China). Anti-DDAH-1 and -DDAH-2 antibodies and all secondary antibodies were purchased from Santa Cruz (Santa Cruz, USA).

Animal experiment
Rats were housed in an air-conditioned room at 22 ± 2°C under controlled lighting conditions (12/12 h, light/dark cycle) and acclimatized for 1 week on a standard diet (3.6 kcal/g, including 13% fat, 60% carbohydrate, and 26% protein). Then the rats were randomly divided into two groups: standard diet group and high fat diet group (79% standard diet, 10% egg yolk powder, 10% lard, and 1% cholesterol). Four weeks later, the high fat-fed rats received a single intraperitoneal injection of STZ (35 mg/kg) after fasting for 12 h and fed again with high fat diet for 2 months. This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of Central South University. The protocol was approved by the Committee on the Ethics of Animal Experiments of School of Pharmaceutical Sciences of Central South University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Identification of diabetes
The experimental model of IR-related type 2 diabetes was identified by HOMA-IR. Fasting plasma insulin (FPI) was determined by enzyme-linked immunosorbent assay using an Ultra-sensitive rat insulin kit (R&D, Minneapolis, USA). HOMA-IR was calculated from the values of fasting blood glucose (FBG) and FPI to estimate IR using the formulae HOMA-IR = FBG (mM) × FPI (mIU/l)/22.5. The levels of triglycerides (TGs), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were also measured using commercial reagents (Rsbio, Shanghai, China).

Immunohistochemistry
To examine DDAH-1 and DDAH-2 proteins in rat visceral adipose tissue (mainly from greater omentum and perirenal fat tissue), immunohistochemistry was performed by routine methods. In brief, adipose tissue sections were incubated with 0.3% H2O2 for 30 min to block endogenous peroxidase. After being washed twice with phosphate-buffered saline (PBS), 10% goat serum was used to prevent non-specific binding of secondary antibody. Then anti-DDAH1 or anti-DDAH2 primary antibody was added at a dilution of 1 : 150. After incubation at 37°C for 2 h, the tissue sections were washed and incubated with secondary antibody (1 : 150) for 15 min. The reactions were visualized by diamino-benzidine (DAB) staining using an assay kit (Boster, Wuhan, China).

Cell preparation
3T3-L1 cell line (ATCC® Number: CL-173) was a gift from the Second Xiangya Hospital of Central South University (Changsha, China). 3T3-L1 fibroblasts were cultured in DMEM/F12 medium containing 10% fetal bovine serum in an atmosphere of 5% CO2 and 95% O2 at 37°C to confluence. Cell differentiation was induced by treatment with DMEM/F12 containing 0.5 mM IBMX, 1 μM dexamethasone, 10 μg/ml insulin, and 10% fetal bovine serum for 48 h [17]. Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum for the next 8-10 days. When at least 95% cells showed adipocyte phenotype identified by oil red O stain, cells were used for the subsequent experiments. IR was induced according to Renströ¨m et al. [18] with some modifications. In brief, treatment with 25 mM glucose in combination with 100 nM insulin for 72 h was considered as IR group, while treatment with 5.5 mM glucose and 1 nM insulin was considered as control group (Con).

Transfection
The cDNA of human DDAH-2 was cloned, and the polymerase chain reaction (PCR) product was sub-cloned into the pEGFP-C1 plasmid. Transfection of the pEGFP-C1-hDDAH2 expression vector was performed using Lipofectamine 2000 from Invitrogen. Reverse transcription-PCR (RT-PCR) was carried out to identify the over-expression of human DDAH-2 in 3T3-L1 adipocytes. In brief, total RNA was converted into cDNA using Superscript III first-strand kit. PCR was performed using the following primers: 5′-gacctcctctcacacc-3′ (forward) and 5′-agcggcaatgactccta-3′ (reverse). The linear exponential phase for hDDAH-2 was 28 cycles, and the melting temperature (Tm) was 58°C. RT-PCR products were loaded on 1.5% agarose gels, staining with ethidium bromide to visualize the specific product of hDDAH-2.
Biochemical testing
Homogenate from adipose tissue of rats or from 3T3-L1 cells was centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was used to measure DDAH, NOS, ADMA, and NO. As described previously, ADMA contents were measured by high-performance liquid chromatography, and DDAH activity was evaluated by determining the amount of hydrolyzed ADMA [19]. Total NOS activity was measured by determining the conversion of L-arginine to L-citrulline [19]. NO production was determined by the Griess method (Jian-cheng Bioengineering Institute). NO production was measured by determining the production of NO.

Real-time PCR analysis
Total RNA (2 µg) was reverse-transcribed in 20-µl reactions with Superscript III first-strand, and the resulting cDNA was amplified using the PerfectShot Taq kit containing a brilliant SYBR green QPCR master mix buffer and a passive diluted reference dye (ROX). Sense and antisense primers for real-time PCR were shown in Table 1. The reactions were performed in a volume of 25 µl using 7300 real-time PCR system (Applied Biosystems, Foster City, USA). The thermal cycling program was 10 s at 95 °C for enzyme activation, and 45 cycles of denaturation and annealing (5 s at 95 °C for denaturation, then 31 s at 60 °C for annealing). Melting curve analysis was performed to confirm the specificity of real-time PCR products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′→3′)</th>
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<tbody>
<tr>
<td>Rat GAPDH</td>
<td>tggcctccaaaggtaagaac (F) ggcctetcttgctctcgattc (R)</td>
</tr>
<tr>
<td>Rat GLUT4</td>
<td>cgttgccgactgggactcg (F) gcctetgtccagcacttag (R)</td>
</tr>
<tr>
<td>Rat IRS-1</td>
<td>aagcggggtacctcagaag (F) ggcccttcgccgattag (R)</td>
</tr>
<tr>
<td>Rat DDAH1</td>
<td>aaggactacgacctcaccag (F) cagcatctgctgaaactc (R)</td>
</tr>
<tr>
<td>Rat DDAH2</td>
<td>aagcgcgtcaggggcatg (F) cgcttcgaggggtcaga (R)</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>cgaggtgttgatggggaatg (F) cgcagatcctcctcctg (R)</td>
</tr>
<tr>
<td>Mouse GLUT4</td>
<td>aegtagtgacacacactttgtg (F) cgccctcgctgctcagag (R)</td>
</tr>
<tr>
<td>Mouse IRS-1</td>
<td>ctggagtagattagaggacag (F) cgccatgaggcaagtgt (R)</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.

Table 1 Primers for real-time PCR analysis

Table 2 Profiles of diabetic rats (DM) compared with control rats (Con)

<table>
<thead>
<tr>
<th>Profiles</th>
<th>Con</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>490.5 ± 26.8</td>
<td>345.6 ± 23.3**</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>1.55 ± 0.16</td>
<td>9.43 ± 0.54**</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.39 ± 0.01</td>
<td>4.06 ± 0.15**</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>0.81 ± 0.02</td>
<td>0.47 ± 0.01**</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>4.55 ± 0.12</td>
<td>7.98 ± 0.15**</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5.08 ± 0.19</td>
<td>18.9 ± 2.48**</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>19.57 ± 1.76</td>
<td>10.52 ± 0.94**</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.43 ± 0.42</td>
<td>8.61 ± 1.12*</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM. n = 6.

*P < 0.05 vs. control (Con).

**P < 0.01 vs. Con.

Results

Identification of diabetes and IR
As shown in Table 2, in high fat diet and STZ-induced diabetic rats, plasma levels of TC, TG, and LDL-cholesterol were increased, and HDL-cholesterol level was notably decreased. FBG of diabetic rats was markedly elevated, whereas FPI was reduced when compared with control. To estimate whether IR existed in this animal model, we calculated HOMA-IR by formula FBG/FPI/22.5, and found that HOMA-IR was higher in the diabetic group. Moreover, mRNA expression levels of insulin receptor substance-1 (IRS-1) [Fig. 1(A)] and glucose transporter-4 (GLUT-4) [Fig. 1(B)], two key genes in insulin-stimulated glucose uptake, were significantly down-regulated in adipose tissue of diabetic rats, indicating impairment of insulin signal transduction.

Changes of DDAH/ADMA/NOS/NO in adipose tissue of diabetic rats
In adipose tissue of diabetic rats, the activities of DDAH and NOS, and the production of NO were decreased, while ADMA contents were increased (Table 3). Real-time PCR analysis (Fig. 1) and immunohistochemistry (Fig. 2) revealed that DDAH-2 expression was notably down-regulated, while the expression of DDAH-1 showed no changes both at mRNA (P = 0.637) and at protein (P = 0.541) levels compared with control.

Involvement of adipose-derived DDAH-2 in regulating insulin signal
Treatment of high glucose in combination with high insulin also significantly inhibited DDAH-2 mRNA expression, while DDAH-1 mRNA expression showed a slight increase but without statistical significance (P = 0.125) (Fig. 3).
To further explore the effects of DDAH-2, we performed over-expression of hDDAH-2 gene in 3T3-L1 adipocytes, resulting in amelioration of cellular DDAH activity and decrease of ADMA content (Fig. 4). We observed that hDDAH-2 over-expression significantly up-regulated expressions of IRS-1 and GLUT-4 mRNA (Fig. 5).

Discussion

Many studies reveal that dysfunction of adipocyte is crucially important in the development of metabolic disorders. In our previous study, we demonstrated that exogenous ADMA at concentrations of 3–10 μM could inhibit the expression and phosphorylation of IRS-1, and decrease GLUT-4 expression and translocation in 3T3-L1 adipocytes, which might relate to reduced basic and insulin-stimulated 2-[3H]DG uptake [19]. DDAH is specific for hydrolyzing ADMA and regulating intracellular ADMA contents. Because DDAH genes (including DDAH-1 and DDAH-2) exist in adipocytes, we design the present study to examine adipocyte-derived DDAH in diabetes mellitus using animal and adipocyte models. Our results shows that adipocyte-derived DDAH/ADMA participates in the development of diabetes, and DDAH-2 might play a protective role in reserving insulin sensitivity.

IR is the central feature of various metabolic disorders. Many pathological factors, such as hyperinsulinemia and hyperglycemia, can cause IR by impairing insulin signal transduction [20–24]. Studies have demonstrated that elevated plasma ADMA level is closely associated with the
impairment of insulin sensitivity in metabolic disorders including type 2 diabetes mellitus. For example, animal experiment shows that insulin sensitivity index is higher in DDAH transgenic mice than that in wild-type control, accompanying with decreased plasma ADMA and increased NO level. Treatment with exogenous ADMA inhibits glucose utilization in skeletal muscle [25], which is similar to what we have observed in cultured 3T3-L1 adipocytes.

It has been reported that plasma levels of ADMA in humans and rats are in the ranges of 0.3–0.5 μM [7], which may be far below those required to have significant effects in cultured adipocytes. So how does ADMA influence adipocytes in vivo? It has been reported that intracellular ADMA is much higher than that in plasma [26], so we postulate that adipocyte-derived DDAH/ADMA might play an important role in regulating insulin sensitivity of adipocytes.

Figure 2 Immunolocalization of DDAH-1 and DDAH-2 in adipose tissue of diabetic rats

Immunohistochemistry analysis was performed to examine the protein expression in adipose tissue of control and diabetic rats. Positive protein expression was stained brown by DAB agent. Treatment with PBS instead of primary antibodies was used as negative control. Magnification, ×100. Results of immunohistochemical scoring were expressed as the mean ± SEM. n = 6. *P < 0.05, **P < 0.01 vs. control.

Figure 3 Expressions of DDAH isoforms in insulin-resistant 3T3-L1 adipocytes

Insulin resistance (IR) was induced by treatment with high concentrations of glucose (25 mM) in combination with insulin (100 nM) for 72 h. Treatment with glucose (5.5 mM) plus insulin (1 nM) was considered as control (Con). The mRNA expressions of mouse DDAH-1 (A) and DDAH-2 (B) were quantified by real-time PCR using β-actin as the internal standard. Results were expressed as the mean ± SEM. n = 3. *P < 0.05, **P < 0.01 vs. Con.
In the present study, we found that intracellular DDAH activity was decreased both in insulin-resistant animal and cell models, accompanying with increased local ADMA contents and decreased NO level. Interestingly, the expression of DDAH-2 was prominently down-regulated both in adipose tissue of diabetic rats and in high glucose plus high insulin-treated adipocytes, while DDAH-1 expression showed no significant change. Previous reports demonstrate that changes of the two DDAH isoforms are not always the same in some diseases. For example, in rats underwent renal ischemia-reperfusion, DDAH-1 expression is decreased after reperfusion, whereas DDAH-2 is increased \[15\]. But in hearts with congestive heart failure, DDAH activity and DDAH-2 content are decreased, whereas DDAH-1 is increased \[12\]. Reasons for this discrepancy are not quite clear. It has been reported that DDAH-2 can be down-regulated by high concentrations of glucose and reactive oxygen species, and DDAH-1 can be up-regulated by interleukin-1β \[7\]. Since both oxidant stress and inflammation participate in the development of diabetes, they may be responsible for the discrepant expressions of DDAH-2 and DDAH-1.

We also observed that the expressions of IRS-1 and GLUT-4 mRNA were down-regulated in diabetic adipose tissue as well as in high glucose and insulin-treated adipocytes. Inhibition of IRS-1 and/or GLUT-4 might lead to defect in

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**Figure 4** Identification of hDDAH-2 over-expression in 3T3-L1 adipocytes  
Insulin resistance was induced as mentioned in (Fig. 3) (Con and IR meant control and insulin-resistant adipocytes without transfection, respectively). Transient transfection was performed to over-express hDDAH-2 in both control adipocytes (Con + DDAH) and insulin-resistant adipocytes (IR + DDAH). The mRNA expression of hDDAH-2 was examined by RT-PCR and visualized by gel imaging system (A). The activity of DDAH (B) and contents of ADMA (C) in adipocytes were also examined. Results were expressed as the mean ± SEM. \( n = 3 \). *\( P < 0.05 \), **\( P < 0.01 \) vs. Con; *\( P < 0.05 \), **\( P < 0.01 \) vs. IR.

**Figure 5** Effect of hDDAH-2 over-expression on IRS-1 or GLUT-4 mRNA expression in 3T3-L1 adipocytes  
Insulin resistance was induced as described in (Fig. 3) (Con and IR meant control and insulin-resistant adipocytes without transfection, respectively). Transient transfection was performed to over-express hDDAH-2 in both control adipocytes (Con + DDAH) and insulin-resistant adipocytes (IR + DDAH). The mRNA expression levels of mouse insulin receptor substance-1 (IRS-1) (A) and glucose transporter-4 (GLUT-4) (B) were quantified by real-time RT-PCR using β-actin as the internal standard. Results were expressed as the mean ± SEM. \( n = 3 \). *\( P < 0.05 \), **\( P < 0.01 \) vs. control (Con); *\( P < 0.05 \), **\( P < 0.01 \) vs. IR.
insulin signaling, so we further explored the relationship between DDAH-2 and the two insulin signal molecules.

We constructed pEGFP-C1-hDDAH2 expression vector and transfected into adipocytes. Results showed that over-expression of hDDAH-2 increased intracellular DDAH activity, decreased ADMA contents, and ameliorated insulin sensitivity by up-regulating IRS-1 and GLUT-4 expression. Since defect of insulin signal in adipocytes has been confirmed to be crucially important in metabolic diseases [27,28], we have reason to postulate that local DDAH might be crucially important in metabolic diseases [27,28].

However, in this study, we do not explore more mechanisms of DDAH than hydrolyzing ADMA and promoting NO bioavailability. As mentioned above, DDAH-2 gene is able to regulate VEGF expression in endothelial cells through transcriptional factor Sp1 [8]. Previous reports have demonstrated that endothelial NOS (eNOS) promoter also has a cis-element for Sp1 binding [29]. In the present study, we also find that NOS activity is impaired in diabetic adipose tissue. Whether DDAH-2 can up-regulate eNOS expression by promoting Sp1 or other transcriptional factors in adipose tissue needs further study. Moreover, the role of DDAH-1 in this procedure still remains unknown, and we plan to study it in the future.

In summary, we disclose that the expression of DDAH isoform-2, not DDAH isoform-1, is down-regulated both in adipose tissue of diabetic rats and in high glucose plus high insulin-treated adipocytes. The over-expression of DDAH-2 improves DDAH activity and up-regulates insulin signal molecules, indicating that adipose-derived DDAH-2 might be involved in the regulation of insulin sensitivity.

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