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Asymmetric Dimethylarginine Impairs Glucose Utilization via ROS/TLR4 Pathway in Adipocytes: an Effect Prevented by Vitamin E

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Key Words

Asymmetric dimethylarginine • Glucose • Adipocytes • Oxidative stress • Toll-like receptor 4

Abstract

Background: Asymmetric dimethylarginine (ADMA), the inhibitor of nitric oxide synthase (NOS), has been reported to be associated with glucose metabolism, but its mechanisms remain unknown. Methods: In 3T3-L1 adipocytes, we measured the effects of ADMA on glucose transport process under basal or insulin-induced condition, and examined the production of nitric oxide (NO), reactive oxygen species (ROS) and tumor necrosis factor alpha (TNF-α), and the expression of toll-like receptor 4 (TLR4). Results: ADMA significantly impaired basal or insulin-stimulated 2-deoxy- [^{3}H] glucose uptake, and decreased the expression of insulin receptor substrate-1 (IRS-1) and glucose transporter-4 (GLUT4). Phosphorylated protein of IRS-1 and translocation of GLUT4 with insulin-stimulation were also inhibited by ADMA. NO decreased, while production of ROS and TNF-α, and expression of TLR4 increased after ADMA treatment. Vitamin E reduced the effects of ADMA on glucose transport system, and on NO, ROS and TLR4. Moreover, vitamin E decreased ADMA contents by up-regulating dimethylarginine dimethylaminohydrolase (DDAH) activity in adipocytes. Though L-arginine also increased NO level, but failed to reduce the effects of ADMA. Conclusion: ADMA significantly impairs both basal and insulin-stimulated glucose transport in adipocytes, which may relate to activation of the ROS/TLR4 pathway.

Introduction

Nitric oxide (NO) is an important modulator in cardiovascular system, which is formed by nitric oxide synthase (NOS) converting the substrate L-arginine to NO and L-citrulline. Previous studies have documented that in multiple cardiovascular diseases, endothelial dysfunction caused by impaired NO bioavailability is closely associated with the elevation of endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) [1-3], which is structurally similar to L-arginine. ADMA can competitively inhibit the activity of all three isoforms of NOS including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), while addition of L-arginine can reverse the effect of ADMA on NO.

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ADMA is hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH) and DDAH over-expression can decrease ADMA content [4].

Recently, growing evidence supports that ADMA is involved in regulation of glucose metabolism and insulin sensitivity. Sydow et al found that human dimethylarginine dimethylaminohydrolase (hDDAH-I) transgenic mice have a blunted increase in plasma insulin and glucose levels after glucose challenge than wild-type control and exogenous ADMA markedly suppresses insulin-induced glycogen synthesis in fast-twitch skeletal muscle [5]. In women with previous gestational diabetes mellitus, elevated concentrations of asymmetric dimethylarginine are associated with deterioration of glucose tolerance [6], and some hypoglycemic agents, such as metformin [7] and insulin [8], can decrease plasma level of ADMA. But till now, how ADMA influences glucose metabolism remains unknown.

Adipocytes are quite important in whole body energy homeostasis, and insulin is a major hormone regulating its differentiation and function. In the cascade of intracellular insulin signaling events in adipocytes, tyrosine phosphorylation of insulin receptor substrates 1 (IRS-1) is important for activation of the glucose transport system. The final step is the translocation of glucose transporter 4 (GLUT-4) from intracellular compartments to the plasma membrane, facilitating the glucose uptake process. Glucose transport is considered to be the rate-limiting step in glucose metabolism under most physiological conditions [9].

The energy metabolism of adipocytes is regulated by endogenous substances, such as NO. Constructive production of NO has been reported to be "*good*" in maintaining glucose homostasis. But growing evidence also shows that over-production of NO by activation of iNOS will be harmful to adipocytes [10]. Moreover, NO seems to have no effect on insulin-stimulated glucose transport process [11-12]. Based on these, the contribution of ADMA to the impairment of glucose utilization in adipocytes is questionable if it only acts through NO pathway.

Recently, it has been reported that ADMA participates in oxidative stress [13-14] or inflammatory reactions [15]. TLR4 is the receptor for LPS and plays a critical role in innate immunity. Stimulation of TLR4 activates pro-inflammatory pathways and induces the expression of many inflammatory cytokines, such as TNF-alpha and IL-6, in a variety of cells. It has been demonstrated that TLRs are inducible in adipose tissues [16]. Evidence further indicates that both oxidative stress [17-19] and toll-like receptor 4 (TLR4)-mediated inflammation [20-21] are involved in the development of adipocyte dysfunction. Asehnoune et al have also found that proximal events in TLR4 signaling are oxidant-dependent [22]. Anti-oxidant agents, such as vitamin E (Vit E), are able to reduce insulin resistance [23]. So in the present study, we aimed to examine the effects of ADMA on glucose transport in adipocytes, and to explore the involvement of ROS/TLR4 pathway in its mechanisms.

Materials and Methods

Materials

3T3-L1 cell line was a gift from The Second Xiang-Ya Hospital of Central South University (Changsha, China). DMEM/F12 medium, ADMA, vitamin E, insulin, 3-isobutyl-1-methyl xanthine (IBMX), cytochalasin B and dexamethasone were obtained from Sigma Chemical Co (St. Louis, MO, USA). NO assay kit was purchased from Jian-Chen Biological Medical Engineering Institute (Nanjing, China). ROS assay kit was from Beyotime Institute of Biotechnology (Jiangsu, China). Anti-IRS-1 and anti-phospho-tyrosine (P-Tyr-100) antibodies were purchased from Cell Signaling Inc. Anti-TLR4 antibody (blocking antibody) and rat IgG2a isotype control were from eBiosience (San Diego, CA). Antibodies of GLUT4 and all secondary antibodies were obtained from Santa Cruz (CA, USA). ECL western blot kit was from Amersham Biosciences (Buckinghamshire, UK). TRIzol reagent and Superscript™ III First-Strand were from Invitrogen (Carlsbad, CA, USA). The PerfectShot Taq kit was from Takara Biomedicals (Dalian, China).

Cell preparation

3T3-L1 fibroblasts were cultured in DMEM /F12 medium containing 10% fetal bovine serum in an atmosphere of 5% CO_2 and 95% O_2 at 37°C to confluency. Differentiation was induced by treatment of cells with DMEM /F12 containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/ml insulin and 10 % fetal bovine serum for 48 h. Then the cells were cultured in DMEM/F12 medium supplemented with 10 % fetal bovine serum for the next 8-10 days. When at least 95% of the cells showed an adipocyte phenotype identified by oil red O stain, the mature adipocytes were used for the experiments.

Cell viability

Cell viability was determined by MTT assay. 3T3-L1 cells were planted into 96-well plates and incubated in medium containing 5 mg/ml MTT (200 μl) at 37°C for 4 h. The medium was gently aspirated, and then 150 μl DMSO was added to each well. The optical density of each sample was immediately measured in an ELISA microplate reader (ELX800, USA) at 490 nm.

Glucose transport

Glucose transport was measured by 2-deoxyglucose (2-DG) uptake as described previously with some modifications [24]. Briefly, after incubation of 3T3-L1 adipocytes in KRH buffer (NaCl, 120 mM; KCl, 6 mM; Mg_2SO_4, 1.2 mM; $CaCl_2.2H_2O$, 1 mM; Na_2HPO_4, 0.6 mM; NaH_2PO_4, 0.4 mM and HEPES, 30 mM, pH 7.4) in the absence or presence of insulin (1, 10, 100 nM) for 30 min at 37 °C, 2-[^{3}H] deoxy-D-glucose (Amersham Biosciences) was added at a final concentration of 0.5 μCi /ml. Ten minutes later, the reaction was stopped by the addition of 0.5 M NaOH for 2 h. Nonspecific glucose uptake was determined in the presence of 10 μM cytochalasin B and was subtracted for each determination. All values were normalized for protein content.

Real-time RT-PCR analysis

Total RNA of 2 μg from 3T3-L1 adipocytes was reverse-transcribed in 20-μl reactions with Superscript™ III First-Strand. The real-time quantitative PCR analysis (QPCR) was then performed according to Fu et al [25]. In brief, 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 designed as below: mouse β-Actin were 5'-CCA GGG TGT GAT GGT GGG AAT G-3' (F) and 5'-CGC ACG ATT TCC CTC TCA GCT G-3' (R); mouse TLR4 were 5'-AAA CTT GCC TTC AAA ACC TGG C-3' (F) and 5'-ACC TGA ACT CAT CAA TGG TCA CAT C-3' (R); mouse GLUT4 were 5'-AGC GTA GGT ACC AAC ACT TTC TTG T-3' (F) and 5'-CCG CCC T TA GTT GGT CAG AAG-3' (R); mouse IRS-1 were 5'-CCT GGA GTA TTA TGA GAA CGA G-3' (F) and 5'-CGG CAA TGG CAA AGT GT-3' (R). The reactions were performed in a volume of 25 μl using a 7300 real-time PCR system (Applied Biosystems, USA). The thermal cycling program was 10 second at 95°C for enzyme activation, 45 cycles of denaturation for 5 second at 95°C and 31 second of annealing at 60°C. Melting curve analysis was performed to confirm the real-time QPCR products. The amplified products were denatured and re-annealed at different temperature points to detect their specific melting temperature.

Examination of tyrosine-phosphorylated protein of IRS-1

After being treated with ADMA (at concentrations of 1, 3, 10 μM, respectively) for 48 h and subsequently with insulin (1nM) for 30 min, 3T3-L1 adipocytes were lysed with lysis agents (Beyotime Institute of Biotechnology, Jiangsu, China) on ice and centrifuged at 12,000g for 30 min. The protein concentration was measured using BCA assay kits (Pierce, Rockford, IL, USA), and then immunoprecipitation and immunoblotting were performed as previously described with some modifications [26]. In brief, after an overnight-incubation of the lysates with anti-phospho-tyrosine antibody at 4°C, the immune complexes were collected on protein A-Sepharose for 4 h at 4°C followed by washing with lysis buffer and boiled in SDS sample buffer (50 mM Tris, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). Samples were electrophoresed on 7.5% SDS-polyacryamide gels and transferred to a poly-vinylidine difluoride membrane. The membrane was probed with anti-IRS-1-antibody at 4°C overnight and incubated with secondary antibody for 1 h at room temperature. The reactions were visualized by enhanced chemiluminescence using ECL western blot kit and quantified by densitometry.

GLUT4 translocation

Adipocytes were treated with ADMA at given concentrations mentioned above for 48 h and then treated with insulin (1nM) for 30 min at 37°C. GLUT4 translocation was examined by immunoblotting assay. Firstly, adipocytes were fractionated by ultracentrifugation to separate the cytosol and plasma membrane (PM) contents according to the method described by Kabayama et al [27] with some modifications. In brief, cell lysate was centrifuged at 700 g for 10 min to remove nuclei and large cellular debris. The supernatant was centrifuged at 13,000 g for 20 min to pellet the plasma membrane and mitochondria. This supernatant contained high-density microsomal fraction, cytosol fraction and other intracellular contents. The crude plasma membrane (PM) was added to 2.5 ml sucrose solution (sucrose, 1.12 M; EDTA, l mM; Tris, 20 mM; PH 7.4) and centrifuged at 200,000g for 60 min. The supernatant was again centrifuged at 60,000g for 45 min to further purify the plasma membrane. After that, intracellular expression of GLUT4 protein (C-GLUT4) and expression of GLUT4 protein in plasma membrane (PM-GLUT4) were determined by immunoblotting using anti-GLUT4 antibody and visualized by enhanced chemiluminescence. The ratio of PM-GLUT4 to C-GLUT4 represents the translocation of GLUT4 from intracellular region to plasma membrane.

Determination of ADMA, NO, ROS and TNF-α

ADMA in adipocytes were measured by high-performance liquid chromatography (HPLC) and standardized for protein concentrations. NO production was measured by Griess method. The levels of intracellular ROS were determined by measuring the oxidative conversion of cell permeable 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in DTX 880 multimode detector (Beckman Coulter, USA), which had been previously described [28]. TNF-α production in the medium was determined by ELISA assay (Jingmei BioTech Co, China).

Determination of DDAH activity

DDAH activity assay was performed according to Lin et al [29]. In brief, cell lysates were divided into two groups, and ADMA was added at a final concentration of 500 μM. 30% sulfurosalicylic acid was immediately added to one experimental group to inactivate DDAH, and this group provided a baseline of 0 % DDAH activity. The other lysate was incubated at 37°C for 2 h before the addition of 30 % sulfurosalicylic acid. The ADMA level in each group was measured by HPLC as described above. The difference in ADMA concentrations between the two groups reflected the DDAH activity. For every experiment, DDAH activity in control group was defined as 100 %, and DDAH activities in other groups were expressed as percentages of the ADMA metabolized compared with the control.

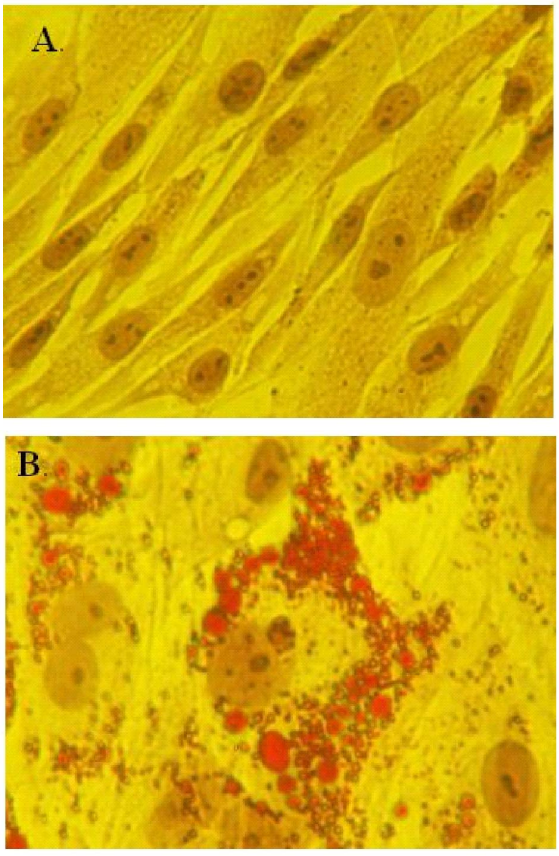

Fig. 1. Identification of differentiated 3T3-L1 adipocytes. Oil Red O staining was performed 10 days after the induction of differentiation. Briefly, Oil Red O (0.1 mg/ml) was dissolved in isopropyl alcohol. Cells were fixed with 10% formaldehyde for 1h and then stained with Oil Red O solution for 2 h at room temperature. Lipid produced by the differentiated 3T3-L1 adipocytes was stained red (B), while un-differentiated adipocytes showed no changes because of little production of lipid (A). Magnification 10×40.

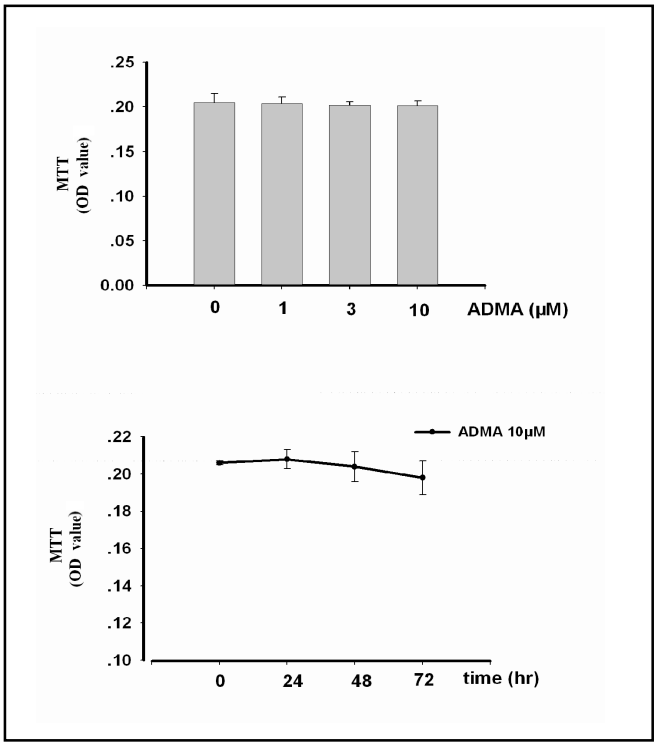

Fig. 2. ADMA had no effect on cell viability. 3T3-L1 adipocytes were treated with ADMA (1, 3, 10 μM) for 48 h or ADMA (10 μM) for 24, 48, 72 h, respectively. MTT assay was used to measure cell viability. Data were expressed as means ± S.E.M. n =6.

Statistical Analysis

All the results were expressed as means ± S.E.M. Differences among different groups were analyzed by one-way ANOVA followed by *t*-test for multiple comparisons as implicated in the software SPSS 10.0. Statistical significance was regarded as $p < 0.05$.

Results

Effect of ADMA on glucose transport

Mature adipocytes (identified by oil red O stain, as shown in Fig. 1B) were treated with exogenous ADMA. MTT assay showed that ADMA at given concentrations (1, 3, 10 μM) had no effect on cell viability (Fig. 2). Fig. 3a and Fig. 3b showed the dose- and time-dependent inhibition of ADMA in basal 2-DG uptake in 3T3-L1 adipocytes. Fig. 3c showed that incubation of insulin (1, 10, 100 nM) for 30 min dose-dependently increased 2-DG uptake, and the insulin-responsive curve was shifted nonparallelly to right after pretreatment with ADMA (10 μM) for 48 h, indicating the inhibitory role of ADMA in insulin-stimulated 2-DG uptake.

Effects of ADMA on expression of IRS-1 and GLUT4

After exposure of 3T3-L1 adipocytes to ADMA at concentrations of 3 or 10 μM for 48 h, GLUT4 (Fig. 4a) or IRS-1 (Fig. 5a) mRNA expression under basal condition was down-regulated. ADMA also significantly decreased the protein expression of GLUT4 (Fig. 4b) and phosphorylated protein of IRS-1 (Fig. 5b) in the presence of insulin. GLUT4 protein expression in plasma membrane decreased more significantly than that in intracellular region, for the ratio of PM-GLUT4 to C-GLUT4 (PM-GLUT4/C-GLUT4) markedly decreased (Fig. 4b).

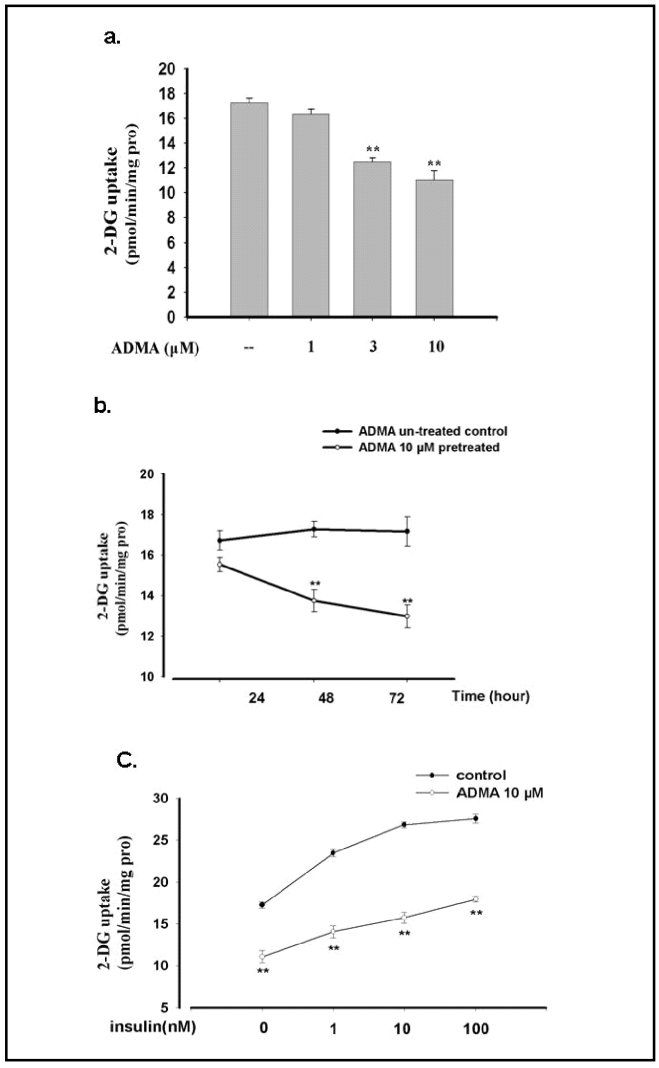

Fig. 3. Effect of ADMA on glucose transport in 3T3-L1 adipocytes. 2-DG uptake of 3T3-L1 cells without insulin-stimulation was assayed after incubation with ADMA (0, 1, 3, 10 μM) for 48 h (a) or with ADMA (10 μM) for 24, 48, 72 h, respectively (b). After pretreatment with ADMA (10 μM) for 48 h, cells were again incubated with insulin (1, 10, 100 nM) for 30 min to examine the effect of ADMA on insulin-responsive 2-DG uptake (c). Results were expressed as means ± S.E.M, n =6, **p* <0.05, ***p* <0.01 *vs* control.

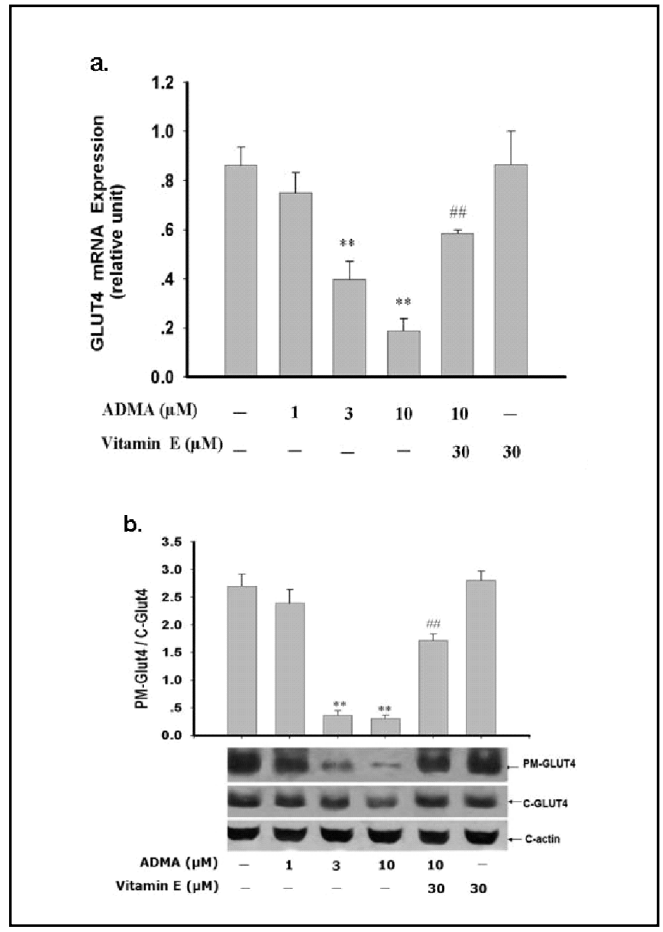

Fig. 4. Effects of ADMA on expression and insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Cells were treated with ADMA for 48 h in the absence or presence of vitamin E, then mRNA expression of GLUT4 was quantified by real-time RT-PCR using β-actin mRNA as the internal standard (a). For examination of insulin-induced translocation of GLUT4, cells were stimulated with 1nM insulin for 30 min after ADMA pretreatment and immunoblotting analysis was performed. The ratio of GLUT4 protein expression in plasma membrane (PM-GLUT4) to that in intracellular region (C-GLUT4) represented the translocation of GLUT4, and the expression of house-keeper gene beta-actin in intracellular region (C-actin) was also examined (b). Results were expressed as means ± S.E.M. n= 3. **p* <0.05, ***p* <0.01 *vs* control; #*p* <0.05, ##*p* <0.01 *vs* ADMA 10 μM.

Effects of ADMA on ROS/TLR4 pathway

Besides its typical role in inhibiting NO availability (Fig. 6a), ADMA at a concentration of 3 or 10 μM was able to promote the production of ROS in 3T3-L1 adipocytes (Fig. 6b). Fig. 7a suggested that anti-TLR4 antibody (10 μg/ml) pretreatment reduced the effect of ADMA on basal or insulin-stimulated 2-DG uptake, while equal concentration of rat IgG control showed no effects (Fig. 7b). Furthermore, ADMA increased TNF-α level (Fig. 7c) and up-regulated mRNA expression of TLR4 (Fig. 7d) in adipocytes.

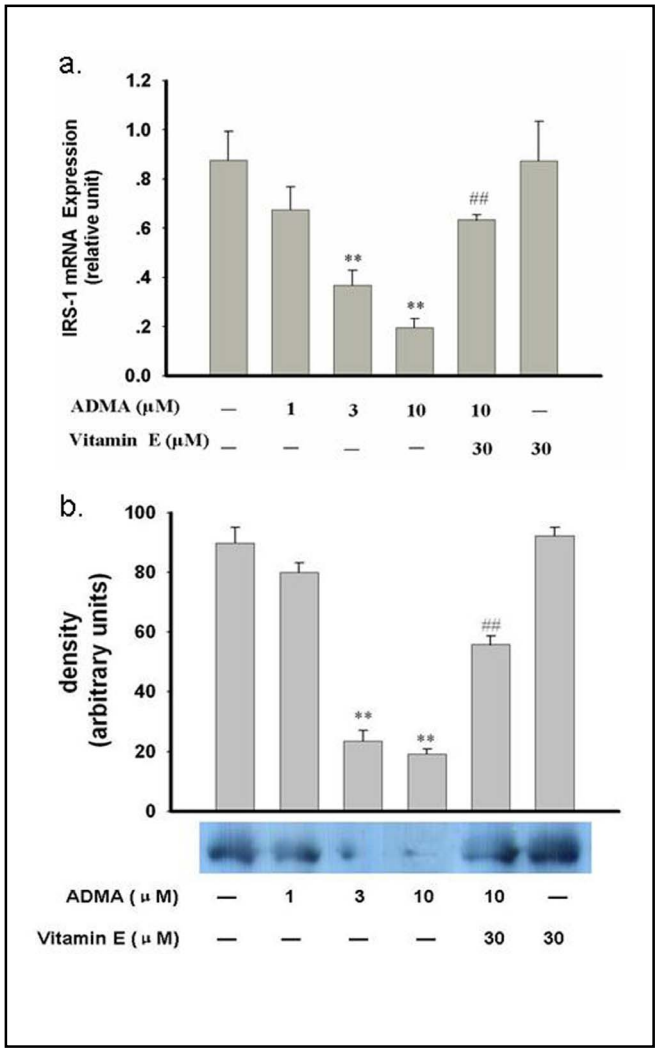

Fig. 5. Effects of ADMA on expression of IRS-1 in 3T3-L1 adipocytes. After treatment with ADMA for 48 h in the absence or presence of vitamin E, the mRNA expression of IRS-1 in 3T3-L1 cells was quantified by real-time RT-PCR using β-actin mRNA as the internal standard (a). For determination of tyrosine-phosphorylated protein of IRS-1, cells were stimulated with 1nM insulin for 30 min after ADMA treatment and immunoprecipitation analysis was performed (b). Results were expressed as means ± S.E.M. n= 3. **p* <0.05, ***p* <0.01 *vs* control; #*p* <0.05, ##*p* <0.01 *vs* ADMA 10 μM.

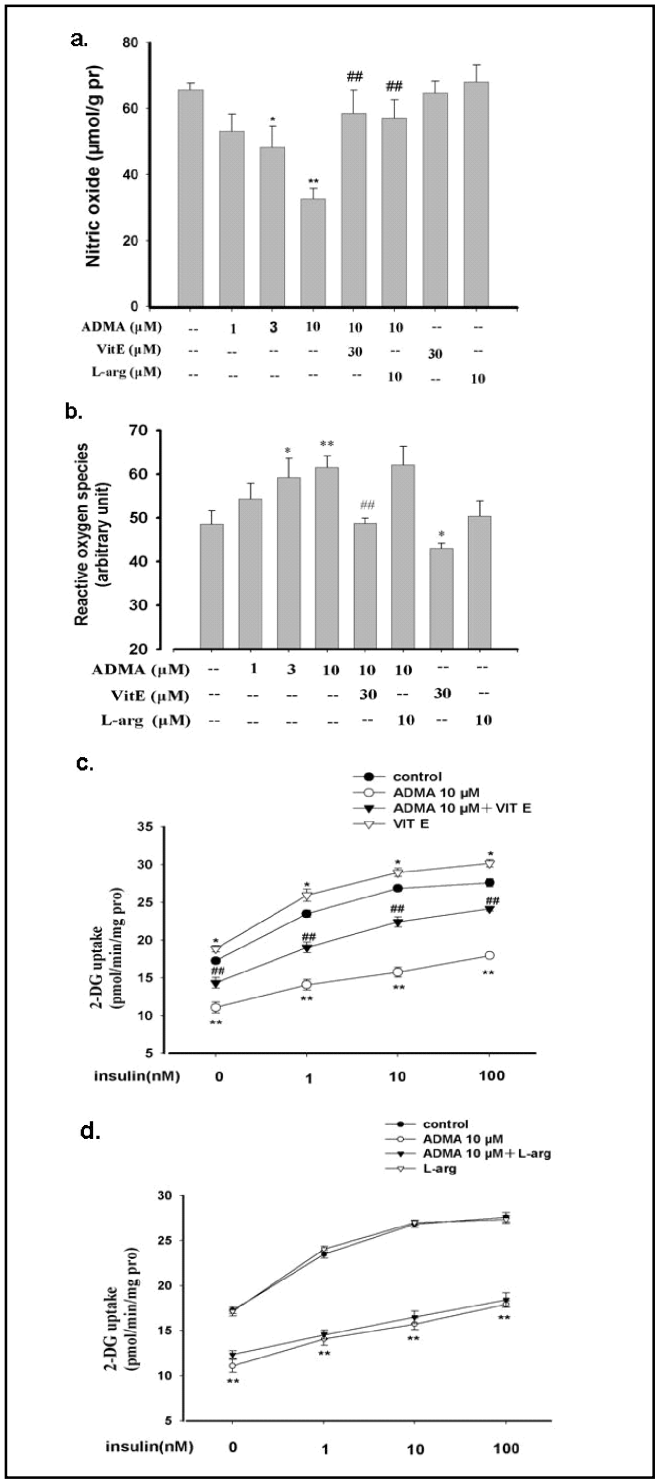

Fig. 6. Effects of vitamin E or L-arginine on NO, ROS and 2-DG uptake in 3T3-L1 adipocytes. After pretreatment with vitamin E (30 μM) or L-arginine (10 μM) for 30 min, 3T3-L1 cells were again co-incubated with ADMA (10 μM) for 48 h and the production of NO (a) and ROS (b) were examined. We also measured the effects of vitamin E (c) or L-arginine (d) pretreatment on both basal and insulin-stimulated 2-DG uptake. Results were expressed as means ± S.E.M, n =6, **p* <0.05, ***p* <0.01 *vs* control; #*p* <0.05, ##*p* <0.01 *vs* ADMA 10 μM.

Vitamin E, not L-arginine, reduced the effects of ADMA

Vitamin E (30 μM) pretreatment attenuated the effects of ADMA on GLUT4 (Fig. 4), IRS-1 (Fig. 5), ROS (Fig.6b), 2-DG uptake (Fig. 6c), and on TLR4 expression (Fig. 7d). Though both vitamin E and L-arginine reversed the effect of ADMA on NO (Fig. 6a), L-arginine had no effect on 2-DG uptake (Fig. 6d). Moreover, vitamin

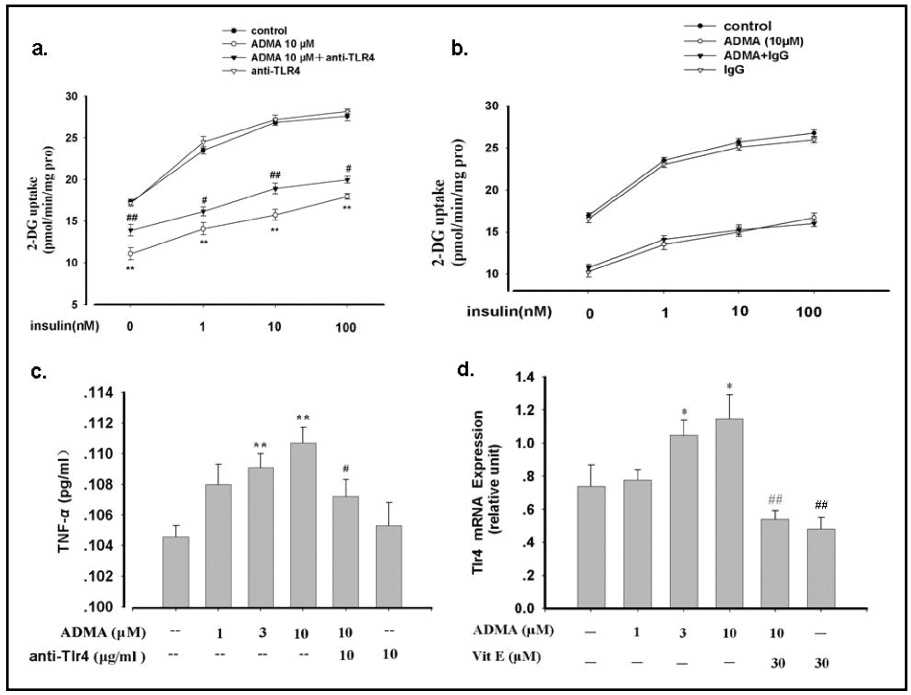

Fig. 7. TLR4-mediated inflammation was involved in the effects of ADMA. Fig 7a and Fig 7b: After pretreatment with anti-TLR4 antibody 10 μg/ml (Fig 7a), or rat IgG isotype control 10 μg/ml (Fig 7b) for 30 min, 3T3-L1 cells were co-incubated with ADMA (10 μM) for 48 h and subsequently with insulin (0, 1, 10, 100 nM) for 30 min. Fig 7c: Adipocytes were incubated with ADMA (1, 3, 10 μM) with or without anti-TLR4 antibody (10 μg/ml) for 48 h and the contents of TNF-α in the conditioned medium were measured. Fig 7d: Cells were treated with ADMA (1, 3, 10 μM) for 48 h with or without vitamin E pretreatment, and TLR4 mRNA expression was quantified by real-time RT-PCR using beta-actin mRNA as the internal standard. Data were expressed as means ± S.E.M, n =6 (in Fig 7d, n=3), *p <0.05, **p <0.01 vs control; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 *vs* ADMA 10 μM.

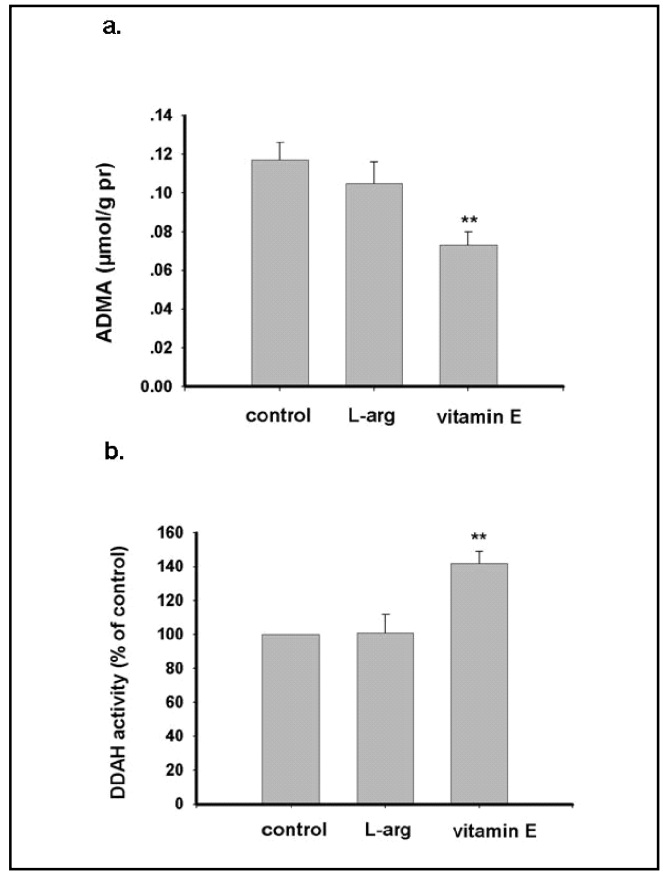

Fig. 8. Effects of vitamin E or L-arginine on ADMA production and DDAH activity in 3T3-L1 adipocytes. After treatment with vitamin E (30 μM) or L-arginine (10 μM) for 48 h, the contents of ADMA (a) as well as the activity of DDAH (b) in adipocytes were examined. Results were expressed as means ± S.E.M, n =6, *p <0.05, **p <0.01 *vs* control; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 *vs* ADMA 10 μM.

E decreased ADMA contents in adipocytes (Fig. 8a) by up-regulating DDAH activity (Fig. 8b), while L-arginine showed no effects.

Discussion

The relationship between ADMA and dysglycemia has been discovered by recent studies, but details on how ADMA influences glucose metabolism are unclear. The main contribution of our present study was to initially explore the involvement of oxidant/TLR4 pathway in the impairment of both basal and insulin-stimulated glucose transport by ADMA.

ADMA impaired glucose transport in adipocytes

In the present study, we observed that ADMA impaired mRNA expression of IRS-1 and GLUT4 in 3T3-L1 adipocytes, responsible for inhibited glucose uptake under basal condition. Since ADMA impaired insulin-stimulated translocation of GLUT4 and expression of tyrosine-phosphorylated protein of IRS-1, the dose-dependent increase in 2-DG uptake after insulin-stimulation was also inhibited by ADMA, for the insulin-

responsive curve shifted nonparallelly to right. These results indicated that ADMA might participate in the development of glucose metabolism disorders.

Possible mechanisms responsible for ADMA

Studies suggest that constitutive NO production in adipocytes is mainly formed by eNOS, which is beneficial in maintaining glucose homeostasis and insulin sensitivity [10]. But the regulatory role of NO in glucose metabolism seems to be limited and quite different under various conditions. It has been reported that NO-stimulated glucose uptake might be insulin-independent, for exogenous NOS inhibitor L-NAME has no effect on insulin-stimulated glucose transport though it blocks the exercise-stimulated transport [11]. Similarly, Kaddai et al found that NO donator NCX 4016-stimulated glucose transport did not involve the insulin-signaling cascade required to stimulate glucose transport, but through a novel mechanism possibly involving S-nitrosylation [12]. Moreover, over-production of NO by iNOS will react with superoxide anions and form the powerful oxidant peroxynitrite which is harmful to cells. In the present study, we also found that L-arginine at a concentration of 10 μM could neither reduce the effect of ADMA on basal glucose transport nor on insulin-responsive glucose uptake though it improved the NO production. Therefore, inhibition of NO availability failed to fully explain the impairment of 2-DG uptake by ADMA in 3T3-L1 adipocytes.

Studies have proved that enhanced oxidative stress can impair glucose transport system and cause insulin resistance. Differentiated adipocytes exposed to oxidative stress become insulin resistant, exhibiting decreased expression of genes including GLUT4 [17]. A recent study in the literature shows that in adipocytes, oxidative stress affects the expression of C/EBP alpha and delta, resulting in altered C/EBP-dimer composition potentially occupying the GLUT4 promoter [18]. Others find that pSer307-IRS1 and pSer632-IRS1 may define two minimally overlapping pools of IRS-1 in response to oxidative stress, contributing differentially to the inhibition of tyrosine phosphorylation of IRS-1 [19].

Recent studies demonstrate that increased concentration of ADMA contributes to increased ROS production via *uncoupling* of NOS [30]. In such uncoupled state, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-arginine, resulting in production of superoxide rather than NO. The eNOS-derived superoxide/ROS formation bears consequences opposite to "normal" NOS function [31]. Though shortage of L-arginine could theoretically lead to uncoupling of eNOS, studies demonstrate that in diabetic rats, ROS production is increased after the addition of L-arginine and a shortage of L-arginine does not cause eNOS-dependent ROS production in bEnd.3 cells. In fact, increasing concentration of L-arginine up to 500 μM enhances VEGF-induced ROS production [32]. Previous studies also indicate that the activity of PRMTs or DDAH is redox-sensitive [31]. Thus, promoted oxidative stress will result in increased ADMA concentration that significantly inhibits eNOS activity or even uncouples the enzyme, which would further increase superoxide production in a positive feedback fashion. Based on these, we postulated that oxidative-stress might be involved in the mechanisms responsible for ADMA.

In our study, we observed that traditional anti-oxidant agent vitamin E pretreatment reduced the effects of ADMA on both basal and insulin-responsive glucose transport process, and decreased ROS level. Interestingly, we found that vitamin E decreased ADMA contents by up-regulating DDAH activity in normal adipocytes, while L-arginine showed no effects. Though how vitamin E up-regulates DDAH activity remains unknown, these results at least indicate that the anti-oxidant effect of vitamin E possibly involves DDAH/ADMA pathway.

More importantly, Asehnoune et al [22] find that LPS-induced inflammation reaction, including activation of NF-κB and production of TNF-α, is inhibited by pretreatment with the antioxidants *N*-acetylcysteine or vitamin E in neutrophils, which indicates that ROS can modulate NF-κB-dependent transcription through their involvement in early TLR4-mediated cellular responses. As the role of TLR4 in regulation of energy balance and insulin resistance is well established [33-35], we also examined the involvement of TLR4-mediated inflammation in the present study. We found that after exogenous ADMA treatment, both mRNA expression of TLR4 and production of TNF-alpha were increased. Anti-TLR4 antibody pretreatment was able to improve 2-DG uptake and decrease TNF-alpha level. Furthermore, vitamin E significantly inhibited TLR4 mRNA. These results indicated that ADMA might activate TLR4-inflammation pathway by promoting ROS production.

Limitation of the present study

(1) Previous studies indicate that decreased oxidative stress will result in inhibition of ADMA production and/or promotion of ADMA degradation. In the present study,

we find that vitamin E also decreases ADMA level under normal conditions. The question is that, which one should be considered as the up-stream mediator in the anti-oxidant procedure of vitamin E, ADMA or ROS?

(2) The present study lacks of direct proofs to establish the potential ADMA/oxidant/TLR4/inflammation pathway. Moreover, we also observed that as compared with vitamin E, anti-TLR4 antibody only partly reversed the effect of ADMA on 2-DG uptake. Since ROS impairs cell structure and function through multiple mechanisms, for example, it can directly attack the polyunsaturated fatty acids of membrane lipids or cause breakage of DNA, the present study only indicates that TLR4 may be one of the down-stream executors in the ADMA/oxidant pathway. Thus, more convincing methods rather than pretreatment with anti-TLR4 antibody are needed to identify the importance of TLR4 in the action of ADMA, such as the silence of TLR4 gene.

Contribution of the present study

In summary, we are the first to document that ADMA impairs both basal and insulin-stimulated glucose utility in adipocytes, and the effect of ADMA involves the oxidant/TLR4 pathway. Moreover, vitamin E is beneficial in glucose uptake by modulating ADMA/oxidant/TLR4 pathway in 3T3-L1 adipocytes.

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