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Involvement of the endothelial DDAH/ADMA pathway in nitroglycerin tolerance: The role of ALDH-2

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Abstract

Previous studies have shown that nitroglycerin (GTN) tolerance is closely related to an oxidative stress-induced decrease in activity of mitochondrial isoforms of aldehyde dehydrogenase (ALDH-2), and prolonged GTN treatment causes endothelial dysfunction. Asymmetric dimethylarginine (ADMA), a major endogenous NO synthase (NOS) inhibitor, could inhibit NO production and induce oxidative stress in endothelial cells. ADMA and its major hydrolase dimethylarginine dimethylaminohydrolase (DDAH) have recently been thought of as a novel regulatory system of endothelium function. The aim of the present study was to determine whether the DDAH/ADMA pathway is involved in the development of GTN tolerance in endothelial cells. Tolerance, reflected by the decrease in cyclic GMP (cGMP) production, was induced by exposure of human umbilical vein endothelial cells (HUVECs) to GTN (10 μM) for 16 h. While the treatment increased reactive oxygen species (ROS) production/malondialdehyde (MDA) concentration and decreased ALDH-2 activity as well as cGMP production, it markedly increased the level of ADMA in culture medium and decreased DDAH activity in endothelial cells. Exogenous ADMA significantly enhanced ROS production/MDA concentration and inhibited ALDH-2 activity, and overexpression of DDAH2 could significantly suppress GTN-induced oxidative stress and inhibition of ALDH-2 activity, which is also attenuated by L-arginine. Therefore, our results suggest for the first time that the endothelial DDAH/ADMA pathway plays an important role in the development/maintenance of GTN tolerance.

Keywords: Nitroglycerin tolerance; Asymmetric dimethylarginine; Dimethylarginine dimethylaminohydrolase; Oxidative stress; Aldehyde dehydrogenase

Introduction

Nitroglycerin (GTN) has been one of the most widely used antiischemic drugs for more than a century. GTN is thought to be converted in the vasculature to nitric oxide (NO), which activates guanylate cyclase and subsequently elevates cyclic GMP (cGMP) levels, resulting in vasodilatation (Murad, 1999). However, long term administration of GTN can cause the development of tolerance. The mechanisms responsible for tolerance are not yet clearly understood. Also, endothelial dysfunction can be observed in patients during prolonged GTN therapy, which may be related to abnormalities of NOS function and decreases in vascular NO bioavailability (Caramori et al., 1998; Gori et al., 2001; Schulz et al., 2002).

Asymmetric dimethylarginine (ADMA), a major endogenous inhibitor of NOS, can inhibit NO production and induce endothelial dysfunction both in vivo and in vitro (Leiper and Vallance, 1999; Jiang et al., 2006a). Most of the ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine (Vallance and Lieper, 2004). The key role of DDAH in the regulation of ADMA levels was directly shown through overexpression of the DDAH gene and the blocking of DDAH

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activity (MacAllister et al., 1996; Dayoub et al., 2003). The DDAH/ADMA system is thought of as a novel pathway to modulate NO production and endothelial function (Jiang et al., 2006b). Interestingly, it was reported that NO donors could inhibit DDAH activity via S-nitrosylation of its active site and consequently lead to ADMA accumulation (Leiper et al., 2002).

Mitochondrial aldehyde dehydrogenase (ALDH-2), which is the main enzyme responsible for GTN bioactivation, has been considered to play a key role in cGMP-mediated, GTN-induced vasorelaxation (Chen et al., 2002; Sydow et al., 2004). The concept was supported by several experimental studies using ALDH-2 deficient mice (ALDH-$2^{-/-}$) and several clinical reports based on Asian subjects with a point-mutated, dysfunctional ALDH-2 (Chen et al., 2005; Mackenzie et al., 2005; Li et al., 2006). It is well known that nitrate tolerance is closely related to oxidative stress induced by increased production of reactive oxygen species (ROS). Our recent study and other previous reports documented that the decrease in ALDH-2 activity in endothelial cells was related to oxidative stress after long-term GTN treatment and contributed to the status of nitrate tolerance (Sydow et al., 2004; Daiber et al., 2004, 2005; Chen et al., 2007). Previous studies demonstrated that ADMA could directly increase intracellular ROS production via NOS-independent pathway in endothelial cells (Böger et al., 2000; Jiang et al., 2006a). Thus, it is likely that the high ADMA level can inhibit ALDH-2 activity via induction of oxidative stress.

Based on the data mentioned above, we speculated that the DDAH/ADMA pathway might be involved not only in the blunted vascular response to GTN, but also in endothelial dysfunction in nitrate tolerance. Therefore, the aim of the present study was to investigate the potential role of the DDAH/ADMA pathway in the development of nitrate tolerance in cultured endothelial cells.

Materials and methods

Materials

ADMA, *N*-acetylcysteine and L-arginine were obtained from Sigma (St. Louis, MO, USA). GTN was purchased from Beijing Yiming Pharmaceutical Factory (Beijing, PR China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco. A radioimmunoassay kit for the measurement of cGMP was purchased from the Shanghai University of Chinese Medicine (Shanghai, PR China). ROS detection kits and BCA protein assay kits were purchased from Beyotime Company (Jiangsu, China). MDA detection kits were purchased from Jiancheng Biological Product Company (Nanjing, China).

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs, ATCC, CRL-2480) were cultured in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were placed into 6-well culture dishes and serum-starved for 24 h in DMEM containing 1% FBS when the cells had reached subconfluence. Nitrate tolerance in endothelial cells was induced by treatment with GTN (10 μM) for 16 h, as described in our previous study (Chen et al., 2007). In the other groups, the cells were pretreated with antioxidant *N*-acetylcysteine (10 mM) for 0.5 h or NOS substrate L-arginine (1 mM) for 1 h, and then exposed to GTN (10 μM) for 16 h in the presence of *N*-acetylcysteine or L-arginine.

Determination of ADMA concentration

The ADMA content was measured by high-performance liquid chromatography (HPLC) as described previously, with some modifications (Chen et al., 1997). HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with Shimadzu SCL-6A system controller and Shimadzu SIC-6A autosampler. *O*-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by pre-column mixing were monitored using a model RF 530 fluorescence detector set at $\lambda^{ex}=338$ nm and $\lambda^{em}=425$ nm on a Resolve C_{18} column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 M (pH 6.8) sodium acetate–methanol–tetrahydrofuran (81:18:1, v:v:v), and mobile phase B composed of 0.05 mM sodium acetate–methanol–tetrahydrofuran (22:77:1, v:v:v) at a flow-rate of 1 ml/min.

DDAH-activity assay

The activity of DDAH in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme. In an ice bath, cell lysates were divided into two groups, and ADMA was added (final concentration 500 μM). To inactivate DDAH, 30% sulfurosalicylic acid was immediately added to one experimental group. 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 previously described. The difference in ADMA concentration between the two groups reflected the DDAH activity. For every experiment, DDAH activity of endothelial cells exposed to normal conditioned medium was defined as 100% and DDAH activity under other conditions was expressed as a percentage of the ADMA metabolized compared with the control.

Overexpression of DDAH2

Human DDAH2-expressing plasmid was prepared via cloning *hDDAH2*cDNA (oligonucleotide primers: P1: 5′-CTA*AGATC-T*ATGGGGACGCCGGGGGAG-3′; P2: 5′-CTG*GAATTC*T-CAGCTGTGGGGGCGTGT-3′) into a pEGFP-C1 expression vector. For the transfection experiments, the test groups were transfected with *hDDAH2* plasmid and the control groups were exposed to the transfection reagent in the presence of the vector backbone. Transfection experiments were performed using Lipofectamine 2000 from Invitrogen. Transfection efficiency for each experiment was determined by the percentage of cells that expressed EGPF under a fluorescent microscope. Culture dishes with 70% or more transfection rates were used for the experiments 72 h after transfection.

Determination of ROS generation

Changes in intracellular ROS level were determined by measuring the oxidative conversion of cell permeable 2′,7′-

dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in a fluorospectrophotometer (F4000, Japan). The cells were washed with D-Hank's and incubated with DCFH-DA at 37 °C for 20 min. Then DCF fluorescence distribution of 20,000 cells was detected by fluorospectrophotometer analysis at an excitation wavelength of 488 nm and an emission wavelength of 535 nm (Jia et al., 2005).

Determination of malondialdehyde (MDA) concentrations

Lipid peroxidation was determined by the thiobarbituric acid reaction using a commercial kit according to the manufacturer's recommendations as previously described (Xia et al., 2003). Briefly, 100 μl samples of cell lysate were incubated in 1.5 ml of reaction buffer and 1.4 ml of 0.2 M Tris–0.16 M KCl (pH 7.4) at 37 °C for 30 min, followed by the addition of 1.5 ml thiobarbituric acid reagent. The mixture was then heated in a boiling water bath for 10 min. After cooling with ice, 3.0 ml pyridine: *n*-butanol (3:1, v/v) and 1.0 ml 1 M NaOH were added and mixed by shaking. The absorbance was read at 548 nm. MDA levels were expressed as nmol/mg protein.

ALDH-2 activity assay

Cells were collected with ice-cold phosphate-buffered saline (PBS) and their wet weight was determined. A 16.7% (w/vol) cell suspension with 20 mM ice-cold phosphate buffer (deoxygenated with nitrogen gas, pH 7.4) containing 250 mM sucrose was prepared. Cells were homogenized and centrifuged at 900 *g* for 5 min to collect supernatant. The supernatant was further centrifuged at 10,000 *g* for 10 min to collect the precipitated mitochondria. The mitochondria were dispersed in 5 vol ice-cold aqueous 30 mM potassium phosphate buffer (deoxygenated with nitrogen gas), pH 7.5. The protein concentration was determined with BCA protein assay reagent. ALDH activity in the supernatant was monitored at room temperature by following NADH formation at 340 nm. The assay mixture (1 ml) contained: 100 mM Tris–HCl (pH 8.5), 1 mM NAD^+, 1 mM propionaldehyde, and 1 mM 4-methylpyrazole (Chen et al., 2002; Sydow et al., 2004; Ohsawa et al., 2003).

Determination of cellular cGMP-LI levels

Cells in six-well plates were washed twice with D-Hank's buffer and then were treated with 100 μM GTN for 1 min. The reaction was stopped by addition of 5% ice-cold trichloroacetic acid. cGMP was extracted and measured according to the manufacturer's protocol (Dikalov et al., 1998; Chen et al., 2007).

Statistical analysis

Results are expressed as means ± SEM. All data were analyzed by ANOVA followed by the unpaired Student's t-test for multiple comparisons. The significance level was chosen as $P<0.05$.

Results

Role of oxidative stress and ALDH-2 in GTN tolerance

We determined the relationship between GTN concentration and cGMP formation. The results are shown in Fig. 1A. Coincubation of endothelial cells with GTN at 1 μM, 10 μM, and 100 μM resulted in the dose-dependent generation of cGMP, and in the 100 μM concentration, there was a maximal generation of cGMP.

Fig. 1B shows that GTN tolerance could be established in HUVECs: pretreatment with GTN (10 μM) for 16 h significantly decreased the cGMP formation induced by subsequent acute treatment with GTN (100 μM). GTN tolerance was markedly reversed by pretreatment with either *N*-acetylcysteine (10 mM) or L-arginine (1 mM). However, *N*-acetylcysteine or L-arginine itself had no effect on the GTN-induced cGMP formation.

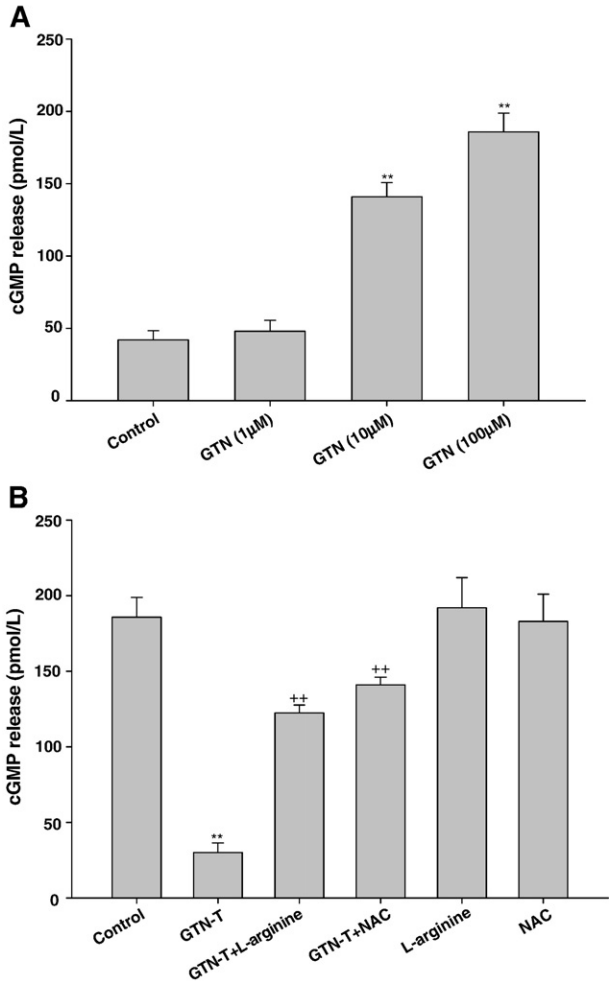

Fig. 1. Effects of GTN on the intracellular cGMP level. A: Relationship between GTN concentration and cGMP formation. GTN: endothelial cells were treated with GTN (1 μM, 10 μM, 100 μM) for 1 min, respectively. Values are expressed as means ± SEM ($n=4$). $^{**}P<0.01$ vs. control. B: GTN-T: endothelial cells were treated with GTN (10 μM) for 16 h, and then stimulated with GTN (100 μM) for 1 min; GTN-T + L-arginine: endothelial cells were treated with GTN (10 μM) for 16 h and L-arginine (1 mM) for 17 h, and then stimulated with GTN (100 μM) for 1 min; GTN-T + NAC: endothelial cells were treated with GTN (10 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h, and then stimulated with GTN (100 μM) for 1 min; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h, and then stimulated with GTN (100 μM) for 1 min; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h, and then stimulated with GTN (100 μM) for 1 min. Values are means ± SEM ($n=4$). $^{**}P<0.01$ vs. control; $^{++}P<0.01$ vs. GTN-T.

Based on the key role of oxidative stress in the GTN tolerance, we determined the intracellular ROS production after treatment with GTN. Exposure of endothelial cells to GTN (10 μM) for 16 h significantly increased the intracellular level of ROS, which was greatly attenuated by pretreatment with *N*-acetylcysteine (10 mM) or L-arginine (1 mM). However, neither *N*-acetylcysteine nor L-arginine had an effect on the intracellular level of ROS (Fig. 2A).

Furthermore, oxidative stress in the endothelial cells treated with GTN was evaluated by MDA content, an index reflecting lipid peroxidation. Similar to the effects on ROS, GTN (10 μM, 16 h) significantly increased the MDA concentration, and this effect was attenuated by pretreatment with *N*-acetylcysteine (10 mM) or L-arginine (1 mM). However, *N*-acetylcysteine or L-arginine per se had no effect on the MDA concentration (Fig. 2B).

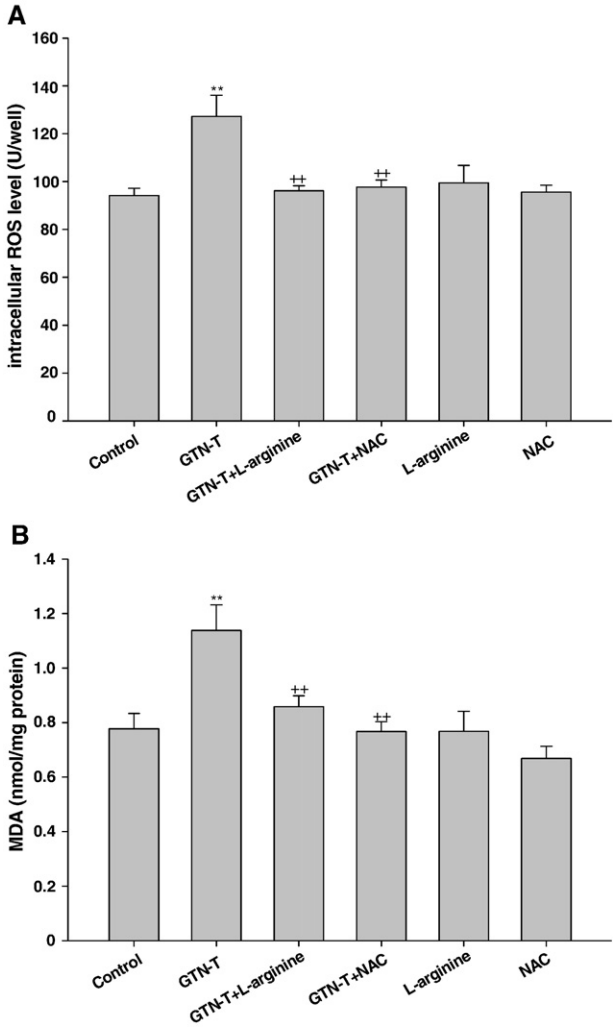

Fig. 2. Effects of GTN on intracellular ROS production (A) and MDA concentration (B) in endothelial cells. GTN-T: endothelial cells were treated with GTN (10 μM) for 16 h; GTN-T+L-arginine: endothelial cells were treated with GTN (10 μM) for 16 h and L-arginine (1 mM) for 17 h; GTN-T+NAC: endothelial cells were treated with GTN (10 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h. Values are means±SEM (n=4). $^{**}P<0.01$ vs. control; $^{++}P<0.01$ vs. GTN-T.

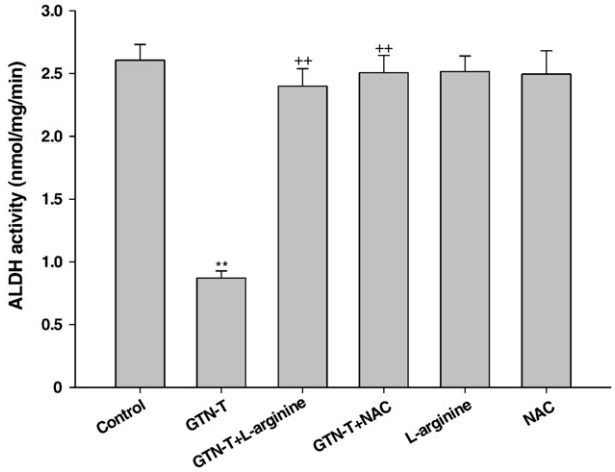

Fig. 3. Effects of GTN on the intracellular ALDH activity. GTN-T: endothelial cells were treated with GTN (10 μM) for 16 h; GTN-T+L-arginine: endothelial cells were treated with GTN (10 μM) for 16 h and L-arginine (1 mM) for 17 h; GTN-T+NAC: endothelial cells were treated with GTN (10 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h. Values are means±SEM (n=4). $^{**}P<0.01$ vs. control; $^{++}P<0.01$ vs. GTN-T.

Following our previous study showing that the inhibition of ALDH-2 activity could result in GTN tolerance, we measured the intracellular ALDH-2 activity after the treatment of GTN (10 μM) for 16 h in the present study. As shown in Fig. 3, ALDH-2 activity was significantly decreased in endothelial cells treated with GTN. *N*-acetylcysteine (10 mM) or L-arginine (1 mM) markedly attenuated the decreased activity of ALDH2. However, *N*-acetylcysteine or L-arginine per se had no effect on the activity of ALDH2.

Effect of GTN tolerance on DDAH/ADMA pathway in HUVECs

As shown in Fig. 4, treatment with GTN (10 μM) for 16 h could markedly increase the concentration of ADMA in culture medium of HUVECs. Considering the fact that ADMA is mostly degraded by DDAH, we determined the effect of GTN on intracellular activity of DDAH in HUVECs. The activity of DDAH was significantly reduced after incubation of HUVECs with GTN (10 μM) for 16 h (Fig. 5).

Effects of DDAH/ADMA pathway on ALDH-2 activity

To define the relationship between elevated ADMA level and GTN tolerance, we observed the effect of exogenous ADMA on oxidative stress and ALDH-2 activity. As shown in Fig. 6A and B, treatment with ADMA (1 μM) for 16 h could markedly increase intracellular ROS production and MDA concentration, which could be reversed by pretreatment with *N*-acetylcysteine (10 mM) or L-arginine (1 mM). However, *N*-acetylcysteine or L-arginine per se had no effect on the intracellular level of ROS and MDA concentration. Treatment with ADMA (1 μM) for

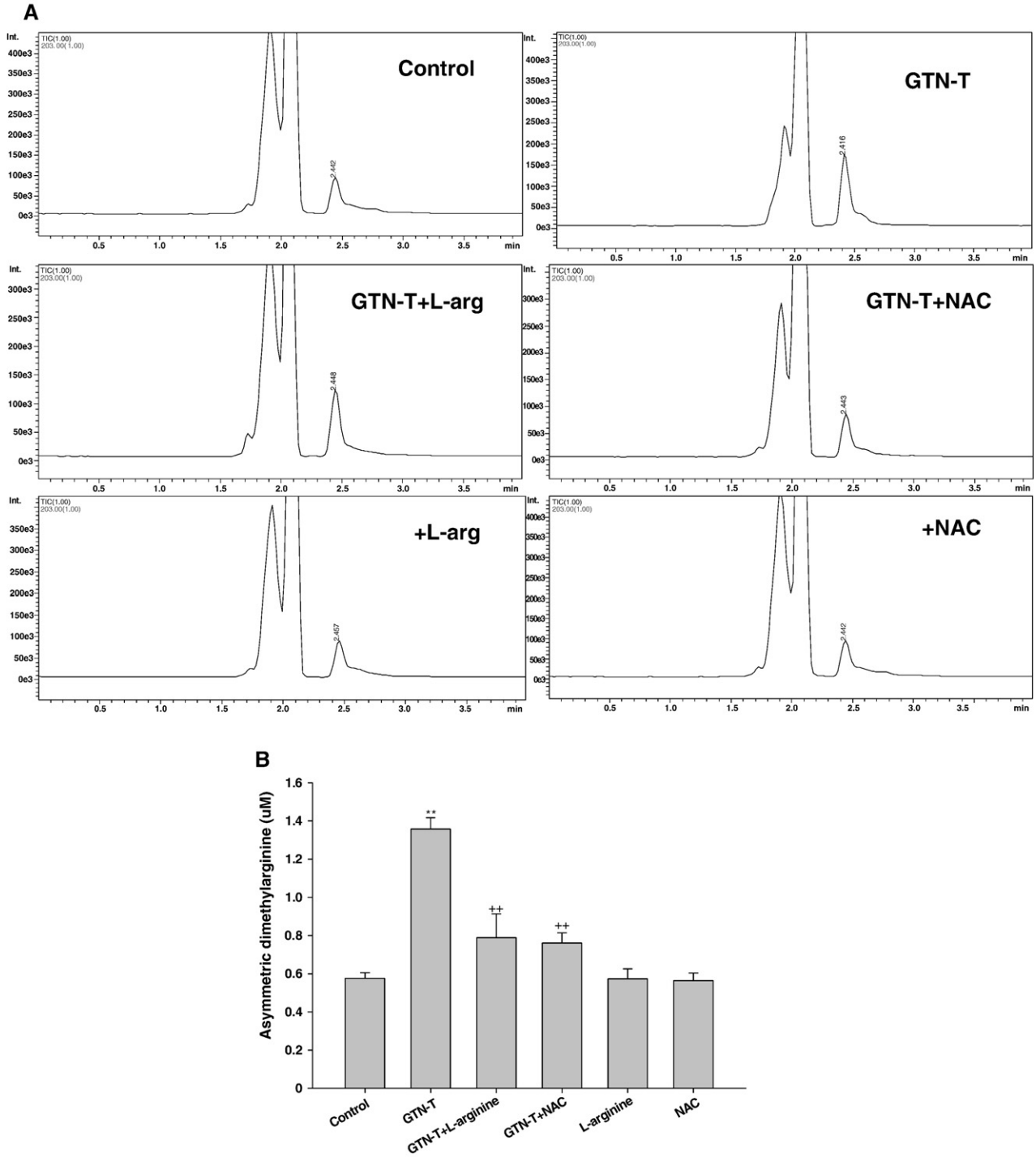

Fig. 4. Effects of GTN on the ADMA concentration. Representative chromatograms graph (A). Statistical graph (B). GTN-T: endothelial cells were treated with GTN (10 μM) for 16 h; GTN-T+L-arginine: endothelial cells were treated with GTN (10 μM) for 16 h and L-arginine (1 mM) for 17 h; GTN-T+NAC: endothelial cells were treated with GTN (10 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h. Values are means±SEM (n=4). **P<0.01 vs. control; ^{++}P<0.01 vs. GTN-T.

16 h markedly inhibited ALDH-2 activity in cultured endothelial cells (Fig. 6C).

Furthermore, we observed the role of the DDAH/ADMA pathway on GTN tolerance in DDAH2-overexpressing endothelial cells. We transfected the hDDAH2 plasmid into HUVECs, resulting in overexpression of the DDAH2 protein. Compared to the control groups, the protein expression of DDAH2 in endothelial cells transfected with the hDDAH2 plasmid increased about three-fold (Fig. 7A,B). There were no differences in ROS production and ALDH-2 activity between the control and DDAH2-overexpressing endothelial cells (Fig. 7C). However, GTN-induced increases in intracellular

ROS production and decreases in ALDH-2 activity were markedly attenuated in cells with DDAH2-overexpression compared with those in control cells (Fig. 7D).

Discussion

Extending our previous study showing that ROS-induced inhibition of ALDH-2 activity plays a crucial role in GTN tolerance (Chen et al., 2007), the present study further established that: (1) the ADMA concentration was greatly increased in endothelial cells treated with GTN (10 μM) for 16 h, which was concomitant with the decrease in activity of DDAH; (2) exogenous ADMA could markedly induce oxidative stress and inhibit ALDH-2 activity in HUVECs; and (3) the overexpression of DDAH2 could attenuate a GTN-induced increase in intracellular ROS production and a decrease in ALDH-2 activity in endothelial cells.

Recently, much attention has been focused on the effects of ADMA (the major endogenous inhibitor of NOS) on endothelial dysfunction. Many studies have reported that ADMA concentration-dependently increased intracellular ROS production via "uncoupling of NOS activity" in endothelial cells (Böger et al., 2000; Jiang et al., 2006a). ADMA could competitively and nonselectively inhibit all three isoforms of NOS and decrease NO production both in vitro and in vivo (Leiper and Vallance, 1999). Elevation of circulating ADMA levels was associated with endothelial dysfunction in some cardiovascular diseases (Böger, 2003a,b). Moreover, ADMA has been found to induce apoptosis and senescence as well as pro-thrombotic phenotype in endothelial cells (Bode-Böger et al., 2005; Jiang et al., 2006a,b; Xin et al., 2007), and it is a novel risk factor contributing to endothelial dysfunction. It has been found that prolonged GTN therapy could cause endothelial dysfunction, which has been thought to be related to abnormal NOS function and a decrease in

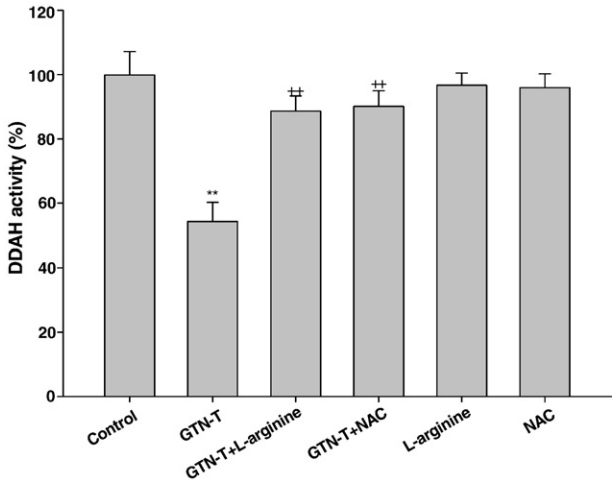

Fig. 5. Effects of GTN on the DDAH-2 activity. GTN-T: endothelial cells were treated with GTN (10 μM) for 16 h; GTN-T+L-arginine: endothelial cells were treated with GTN (10 μM) for 16 h and L-arginine (1 mM) for 17 h; GTN-T+NAC: endothelial cells were treated with GTN (10 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h. Values are mean ±SEM ($n=4$). $^{**}P<0.01$ vs. control; $^{++}P<0.01$ vs. GTN-T.

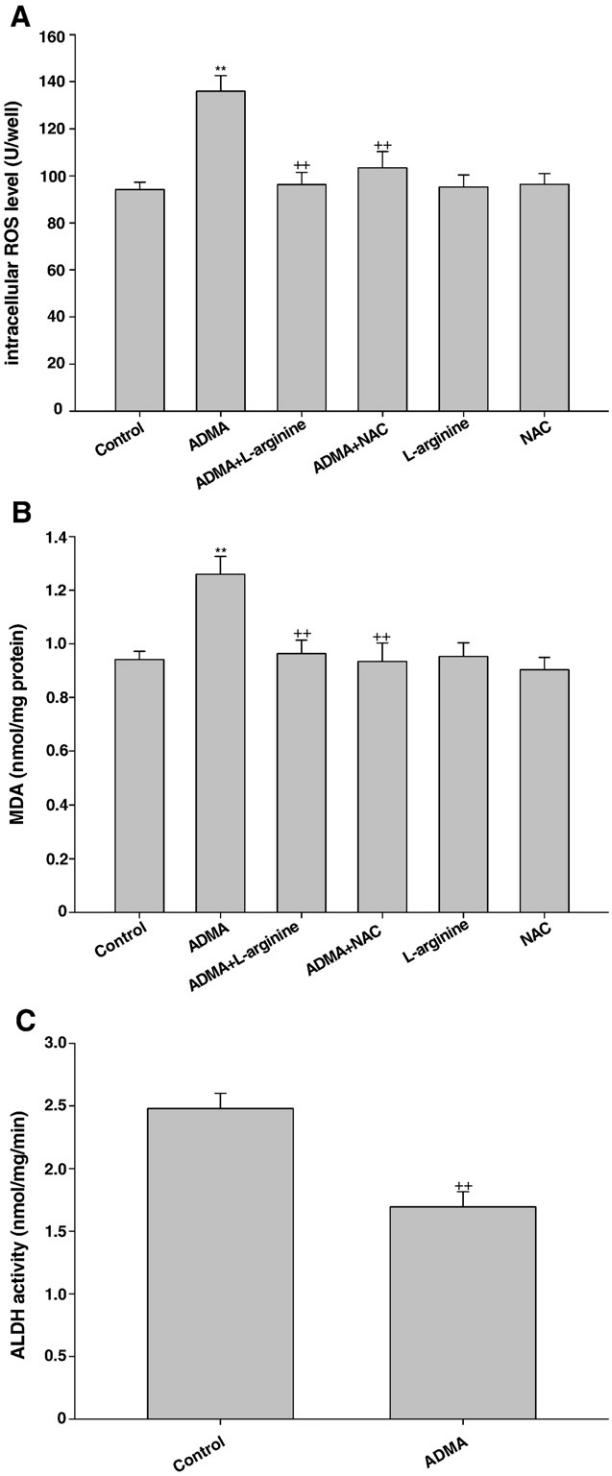

Fig. 6. Effect of ADMA on the intracellular ROS production (A) and MDA concentration (B) and ALDH activity (C) ADMA: endothelial cells were treated with ADMA (1 μM) for 16 h; ADMA+L-arginine: endothelial cells were treated with ADMA (1 μM) for 16 h and L-arginine (1 mM) for 17 h; ADMA+NAC: endothelial cells were treated with ADMA (1 μM) for 16 h and *N*-acetylcysteine (10 mM) for 16.5 h; L-arginine: endothelial cells were treated with L-arginine (1 mM) for 17 h; endothelial cells were treated with *N*-acetylcysteine (10 mM) for 16.5 h. Values are means±SEM ($n=4$). $^{**}P<0.01$ vs. control; $^{++}P<0.01$ vs. ADMA.

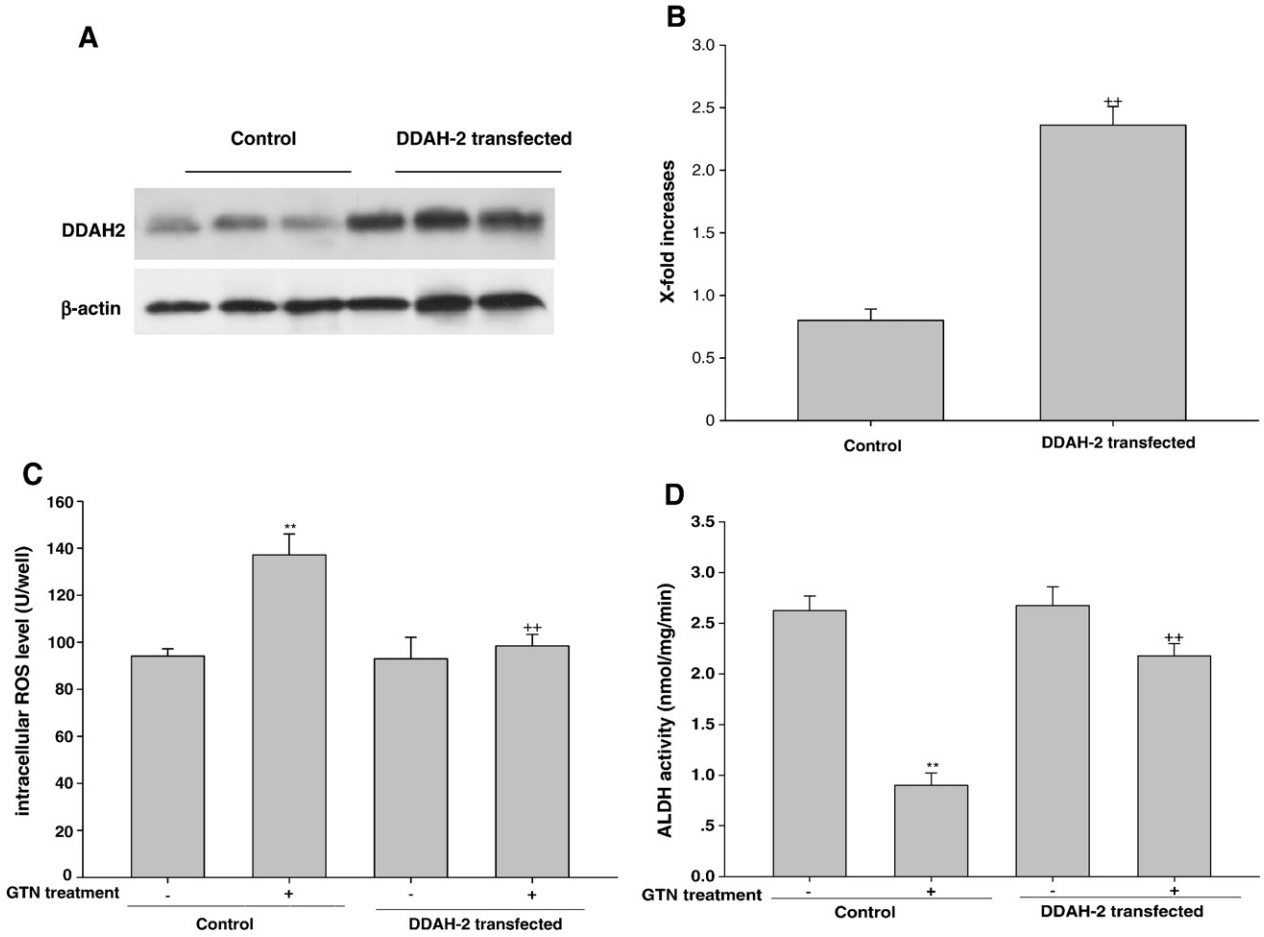

Fig. 7. Effects of DDAH2 overepression on GTN tolerance induced ROS production and ALDH-2 activity in HUVECs. Protein expression of DDAH2 was significantly increased in endothelial cells transfected with DDAH2 expression vector (A) data are shown by three independent experiments (B) DDAH overexpression significantly attenuated GTN-induced increase in ROS production (C) and decrease of ALDH-2 activity (D) in endothelial cells. HUVECs with DDAH2 expression vector or vector backbone were treated with GTN (10 μM) for 16 h. Values are means±SEM, $n=6$ in three independent experiments. $^{**}P<0.01$ vs. control cells. $^{++}P<0.01$ vs. GTN-treated control cells.

vascular NO bioavailability (Caramori et al., 1998; Gori et al., 2001; Schulz et al., 2002). In the present study, we are the first to observe that treatment of endothelial cells with GTN (10 μM) for 16 h increases the accumulation of endogenous ADMA, which suggests that the elevation of ADMA levels may contribute to GTN-induced endothelial dysfunction.

ADMA is synthesized by protein arginine methyltransferase-I, which utilizes S-adenosylmethionine as the methyl group donor, and is degraded by DDAH, which hydrolyzes ADMA to L-citrulline and dimethylamine. So far, two isoforms of DDAH have been characterized: DDAH1 and DDAH2. DDAH1 is typically found in tissues expressing neuronal NOS, whereas DDAH2 predominates in tissues containing eNOS (Leiper et al., 1999). Experiments using overexpression of DDAH gene and DDAH inhibition showed that DDAH plays a key role in the regulation of ADMA levels (MacAllister et al., 1996; Dayoub et al., 2003). It has been shown that elevation of ADMA levels is related to the decrease of DDAH activity in cultured endothelial cells treated with various factors and is decreased by pharmacological interventions targeted at improving DDAH activity (Ito et al., 1999; Jiang et al., 2003, 2006b; Maas, 2005). In the present study, we found that treatment with GTN could decrease the activity of DDAH in endothelial cells. These results are consistent with a previous report by Leiper et al. (2002) that demonstrated that NO donors could inhibit DDAH activity via S-nitrosylation of Cys-249 in its active site, which further results in ADMA accumulation in endothelial cells. Furthermore, it has been reported that DDAH activity is post-translationally regulated by oxidative modification of Cys-249 (Ito et al., 1999). As a donor of NO-like species, long-term GTN treatment is known to increase the intracellular ROS production; thus, it is likely that GTN tolerance could inhibit DDAH activity via both nitrosative stress and oxidative stress.

Recent studies identified the key role of ALDH-2 in GTN metabolism and tolerance (Chen et al., 2002, 2005; Mackenzie et al., 2005; Li et al., 2006). These conclusions were based on experiments in vitro and in vivo showing that the inhibitors and some substrates of ALDH-2 (cyanamide, acetaldehyde and chloral hydrate) induced a marked shift of the GTN dose-response relationship to the right, while relaxations to the NO donor SNP were unaltered. To define the role of the DDAH/ADMA pathway in GTN tolerance, we examined the effect of

exogenous ADMA on intracellular ALDH-2 activity. In the present study, treatment with ADMA could markedly inhibit the ALDH-2 activity in cultured endothelial cells, which could be reversed by pretreatment with L-arginine. Moreover, the overexpression of DDAH2 could markedly attenuate GTN-induced decreases in ALDH-2 activity. These results further confirm that the DDAH/ADMA pathway contributes to the development of GTN tolerance.

The loss of ALDH-2 activity was associated with or was secondary to mitochondrial ROS formation upon long-or short-term challenges with GTN in vitro and in vivo (Daiber et al., 2004; Sydow et al., 2004). It has been demonstrated that increased oxidative stress within mitochondria from mice with heterozygous for Mn-SOD deficiency (Mn-SOD$^{+/-}$) predisposes vascular tissue to developing tolerance as well as cross-tolerance (endothelial dysfunction) in response to in vitro and in vivo GTN challenges (Daiber et al., 2005). Also, there is a growing body of evidence that ADMA could stimulate ROS production in cultured endothelial cells via induced eNOS uncoupling or other mechanisms (Böger et al., 2000; Jiang et al., 2006a).

In accordance with previous reports, our current data show that GTN treatment could increase intracellular ROS production and lipid peroxidation (reflected by MDA concentration). Furthermore, we found that ADMA significantly enhanced ROS production and MDA concentration in endothelial cells, and L-arginine or *N*-acetylcysteine could attenuate such effects of GTN or ADMA on ROS production and ALDH activity. Moreover, the overexpression of DDAH2 could markedly attenuate GTN-induced oxidative stress. These results suggest that the effect of the DDAH/ADMA pathway on the development of GTN tolerance might involve the regulation of oxidative stress and ALDH-2 activity.

In conclusion, our data have revealed for the first time that the DDAH/ADMA pathway plays an important role for the development/maintenance of nitrate tolerance in endothelial cells. These data provide a novel therapy target for the amelioration of GTN tolerance.

Acknowledgement

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