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Contribution of Endogenous Inhibitor of Nitric Oxide Synthase to Hepatic Mitochondrial Dysfunction in Streptozotocin-induced Diabetic Rats

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Key Words

Asymmetric dimethylarginine • Diabetes mellitus • Liver • Mitochondrial dysfunction • Mitochondrial biogenesis • Nitric oxide • Oxidative stress • Uncoupling protein 2

Abstract

Aims: Mitochondrial dysfunction plays important roles in the development of diabetes. Elevated nitric oxide (NO) synthase inhibitor asymmetric dimethylarginine (ADMA) has been shown to be closely related to diabetes. But the relationship between them in diabetes has not been determined. This study was to explore the role of ADMA in hepatic mitochondrial dysfunction and its potential mechanisms in diabetic rats and hepatocytes. Methods: Respiratory enzymes activities, mitochondrial transmembrane potential and ATP content were measured to evaluate mitochondrial function. The copy number ratio of mitochondrial gene to nuclear gene was used to represent mitochondrial biogenesis. The activity of superoxide dismutase and malondialdehyde content were detected to reflect oxidative stress. Furthermore, changes in ADMA and NO contents, uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) transcriptions were determined. Results: Elevated ADMA levels in serum of diabetic rats were found to be associated with hepatic mitochondrial dysfunction reflected by reductions of respiratory enzyme activities, mitochondrial membrane potential and ATP contents. Similar mitochondrial dysfunction also occurred in ADMA-treated hepatocytes. The mitochondrial dysfunction observed in diabetic rats or hepatocytes was accompanied with suppressions of mitochondrial biogenesis, PGC-1α transcription and NO synthesis as well as enhances of UCP 2 transcription and oxidative stress. These effects of ADMA could be attenuated by treatments with antioxidant or NO donor. Conclusions: These results indicate that elevated endogenous ADMA contributes to hepatic mitochondrial dysfunction in diabetic rats, and underlying mechanisms may be related to the suppression of mitochondrial biogenesis and mitochondrial uncoupling via inhibiting NO synthesis and enhancing oxidative stress.

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Introduction

There is accumulating evidence that mitochondrial dysfunction plays important roles in the development of diabetes mellitus[1, 2]. Mitochondria are important organelles of energy generation in the form of adenosine triphosphate (ATP) in eukaryocytes. Mitochondrial membrane potential ($\Delta\Psi m$) is a sensitive index to reflect mitochondrial function. Among these respiratory enzymes, complex II (succinate dehydrogenase) and complex IV (cytochrome C oxidase) are also key enzymes in mitochondrial respiratory chain [3]. Furthermore, mitochondrial function is also related to mitochondrial biogenesis [4], which is often mirrored by the copy number ratio of mitochondrial genes such as cytochrome C oxidase subunit I (COX I) to nuclear genes like β-actin indirectly [5]. Most investigations about mitochondrial dysfunction in diabetes mellitus are focused on skeletal muscle [2, 4] and fat tissue [6] while few in liver. In fact, liver is one of the three main targeted tissues of insulin and the place where gluconeogenesis and glycogen synthesis take place as well as the principle organ where insulin and glucagon are inactivated. Accordingly, liver plays an important role in maintenance of blood sugar homeostasis, and it is very significant to investigate the role of hepatic mitochondrial dysfunction in diabetes mellitus.

The mechanisms of mitochondrial dysfunction are not clear. Recent studies have shown that oxidative stress is implicated in mitochondrial dysfunction since mitochondrion is not only a major source of reactive oxygen species (ROS) but also the first target for attacking of ROS [7, 8]. Uncoupling protein 2 (UCP2) up-regulated by ROS plays a vital role in the uncoupling of oxidation and phosphorylation of mitochondria [9, 10]. Furthermore, suppressed mitochondrial biogenesis is also linked to mitochondrial dysfunction [4]. The peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis [11], and its transcription can be upregulated by nitric oxide (NO) [12], which is critically determined by N^G, N^G-asymmetric dimethylarginine (ADMA).

It has been documented that ADMA is an endogenous inhibitor of nitric oxide synthase (NOS) and derived from proteins containing methylated arginine residues via hydrolysis. The major pathway for endogenous ADMA clearance is degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Both endogenous NOS inhibitor ADMA and exogenous NOS inhibitor N^G-Nitro-L-arginine Methyl Ester (L-NAME) can inhibit NO synthesis and increase superoxide generation by means of uncoupling NOS [13]. Many studies have demonstrated that elevated endogenous ADMA is closely related to insulin resistance [14, 15] and diabetes mellitus [16, 17]. Based on the important roles of mitochondrial dysfunction in diabetes mellitus and the close relationship between ADMA and diabetes mellitus, it is necessary to determine whether endogenous ADMA accumulation is involved in mitochondrial dysfunction, especially hepatic mitochondrial dysfunction in diabetes mellitus, because liver is the main organ for ADMA metabolism [18]. Since oxidative stress has been demonstrated to be involved in the pathogenic processes of endogenous ADMA accumulation [13], mitochondrial dysfunction [7] and diabetes mellitus [8], it is very important to determine whether oxidative stress is the central hub among them. In addition, how is the causal relationship between ADMA and mitochondrial dysfunction, whether supplement of NO by NO donor sodium nitroprusside can reverse ADMA-induced mitochondrial dysfunction, all of them are the subjects worth exploring. Accordingly, this study was designed to determine the relationship between elevated endogenous ADMA and hepatic mitochondrial dysfunction in streptozotocin-induced diabetic rats, subsequently to investigate the direct effect of ADMA on mitochondrial function in rat hepatocytes, and simultaneously to investigate the effect of antioxidant pyrrolidine dithiocarbamate (PDTC) or NO donor sodium nitroprusside on ADMA-induced hepatic mitochondrial dysfunction in diabetic rats or cultured hepatocytes. Revealing the important role of endogenous ADMA in hepatic mitochondrial dysfunction and understanding the mechanisms for ADMA-induced mitochondrial dysfunction may provide insights into more effective therapeutic approaches to diabetes mellitus.

Materials and Methods

Reagents

Streptozotocin, ADMA, sodium nitroprusside, L-NAME, PDTC, ferrocytochrome C, ADP, Rhodamine123, oligomycin, rotenone and Coomassie Brilliant Blue were obtained from Sigma (St Louis, MO, USA). Thiobarbituric acid was from Fluka (Milwaukee, WI, USA). DMEM medium, fetal bovine serum (FBS) and TRIzol were purchased from Gibco (Gaithersburg, MD, USA). Avian myeloblastosis virus (AMV) reverse-transcription and polymerase chain reaction (PCR) kits were from Promega (Madison, WI, USA). Specific PCR primers for PGC-1α, UCP2, COX I, GAPDH and β-actin were synthesized by Ying Jun Biotechnologies (Shanghai, China).

Induction of diabetic rat model

The experimental study was approved by the Animal Care and Use Committee of Guangzhou Medical University and Central South University. Male Sprague-Dawley rats (210 ~ 230 g) were obtained from Central South University Animal Services (Changsha, China) and randomly divided into four groups including control, diabetic, PDTC-treated diabetic and PDTC-treated control groups. Diabetic animal model was induced by intraperitoneal injection of streptozotocin (60 mg/kg) to rats as previously described [15]. Diabetes was confirmed by the presence of glycosuria and hyperglycemia after streptozotocin injection. Following the onset of diabetes, rats of PDTC-treated diabetic and PDTC-treated control groups were received PDTC treatment (10 mg/kg/d in drinking water) for 8 weeks. All animals were housed with free access to food and water in an air-conditioned room under a 12 h light/12 h dark cycle.

Blood biochemical assays

At the end of 8 weeks, blood samples were collected from anaesthetized rats for separation of plasma or serum, and then the liver was quickly excised to freeze in liquid nitrogen and store at -70°C for the determination of gene transcription and biochemical indexes. Plasma glucose was measured by glucose oxidase-peroxidase method within 1 h. Serum ADMA was measured by high-performance liquid chromatography (HPLC) method as previously described [19].

Cell culture

Rat hepatoma cell line H4IIE cells (ATCC, Manassas, USA) were cultured in DMEM medium supplemented with 10% FBS. After reaching subconfluence, cells were divided into six groups and treated with ADMA (30 μmol/L), L-NAME (30 μmol/L), ADMA plus sodium nitroprusside1 (10 μmol/L), ADMA plus PDTC (10 μmol/L), PDTC alone and without any drug, respectively. After treatment for 48 h, the cells were harvested for the isolation of mitochondria and measurements of mitochondrial function, gene transcription, and enzyme activities. The conditional media were collected for the assays of malondialdehyde and nitrite/nitrate contents.

Measurement of mitochondrial function

Preparation of mitochondria. Rat liver tissue (0.5 g) was homogenized in 4.5 ml ice-cold liver homogenization media (0.2 mol/L mannitol, 50 mmol/L sucrose, 10 mmol/L KCl, 1 mmol/L Na_2EDTA, 10 mmol/L HEPES, pH 7.4) to prepare 10% liver homogenates. Hepatic mitochondria were isolated by conventional differential centrifugation as according to a established method [20].

Cell mitochondria were extracted according to the method as previously reported [21]. Briefly, harvested cells were resuspended in hypotonic buffer (10 mmol/L HEPES, 10 mmol/L $MgCl_2$, and 42 mmol/L KCl) and then centrifuged at 1000 g for 5 min at 4°C to remove unlysed cells and nuclei. The supernatant was further centrifuged at 20000 g for 15 min at 4°C. The yielded mitochondrial pellets were resuspended with mitochondrial suspension medium I (0.2 mol/L, mannitol, 50 mmol/L sucrose, 10 mmol/L KCl, 1 mmol/L Na2EDTA and 10 mmol/L HEPES). Isolated mitochondria or whole homogenates were used for the determination of mitochondrial function described below.

Measurements of respiratory enzyme activities. Succinate dehydrogenase activity of whole homogenates was determined according to the method as previously described [22]. One unit of the enzyme activity was defined as the amount that catalyzed the reduction of 2, 6-DPIP per min. The activity of cytochrome C oxidase was assayed by the spectrophotometric method described by Morin et al [23]. One unit of the enzyme activity was defined as the amount that catalyzed the formation of 1 μmol of oxidized ferrocytochrome C per min at 37°C. The protein content of the suspension was measured by the Bradford assay and used to normalize activities of succinate dehydrogenase and cytochrome C oxidase.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potentials ($\Delta\Psi$) of isolated mitochondria were determined by rhodamine123 fluorescence with fluorospectro-photometer (F4000, Japan) at an excitation wavelength of 503 nm and an emission wavelength of 527 nm at 37°C [24]. Mitochondrial membrane potentials (negative inside) were calculated by the electrochemical Nernst–Guggenheim relationship: $\Delta\Psi$=59 log ([Rh-123] in / [Rh-123] out).

Measurement of ATP content. Since the main function of mitochondria is to generate ATP, which accounts for about 90% intracellular ATP, the content of ATP in whole homogenates is an important index of evaluating mitochondrial function. ATP concentrations were detected by bioluminescence assay based on the reaction of ATP with recombinant firefly luciferase and its substrate luciferin [25]. Rat liver (0.5 g) was homogenized in 1:10 wt/vol of ice-cold lysis buffer or cultured hepatocytes were lysed in an ice-bath and then centrifuged at 12,000 g for 5 ~ 10 min (4°C), the resulting supernatant was used immediately for the analysis of ATP content, which was normalized to supernatant protein concentration.

Measurement of Mitochondrial biogenesis. Mitochondrial biogenesis is often mirrored by the ratio of mitochondrial genes such as cytochrome C oxidase subunit I (COX I) to nuclear genes like β-actin copy numbers [5]. The total genomic DNA in rat liver or cells was extracted as previous described [5]. Mitochondrial DNA was determined by PCR analysis using the isolated total genomic DNA as templates and specific primers of mitochondrial gene COX I and nuclear gene β-actin (Table 1) at the reaction condition of PCR (Table 2). The PCR products were electrophoresed on a 1% agarose gel, and visualized with a UV transilluminator. The ratio of copy numbers of COX I to β-actin was determined by optical densities scanning of DNA bands to reflect mitochondrial DNA content.

RT-PCR

Reverse transcription-PCR (RT-PCR) was employed to detect transcriptions of PGC-1α and UCP2 genes with specific primers of rat PGC-1α and UCP2 genes (Table 1) at the reaction condition, respectively (Table 2). The amplified fragments were then separated on 1% agarose gels and visualized by ethidium bromide staining. The optical densities (OD) of mRNA bands were quantified with Gel-pro analyzer (Media Cybernetics, Inc. Bethesda, MD, USA) and normalized to GAPDH as an internal control.

Determinations of DDAH & NOS activities and nitric oxide content

The activities of DDAH in liver homogenates or cell lysates were measured by the conversion of ADMA to L-citrulline [19]. One unit of DDAH activity was defined as the amount that catalyzed formation of 1 μmol L-citrulline from ADMA per min at 37°C. NOS activity was also determined by the conversion of L-arginine to NO as previously reported [19]. One unit of NOS activity was defined as the amount that catalyzed formation of 1 nmol/L NO from L-arginine per min at 37°C. The contents of nitrite/nitrate, the stable end product of NO, in the supernatants of liver homogenates or in conditional media were measured to reflect the production of NO as previously described [19] and normalized to the protein concentrations of the suspension of liver homogenates or cell lysates.

Assays of malondialdehyde content and superoxide dismutase activity

The content of malondialdehyde, derived from lipid peroxidation, in liver homogenates or conditional media was determined as previously described [26]. The activity of superoxide dismutase (SOD) in the supernatants of liver homogenates or cell lysates was assayed by monitoring the inhibition of the autoxidation of hydroxylamine as previously described [19]. One unit of the enzyme activity was defined as the amount that inhibited autoxidation of hydroxylamine by 50%. The activity of lactate dehydrogenase in conditional media of hepatocytes was also measured by the conversion of lactic acid to pyruvic acid [27] to evaluate the cell viability or the degree of cell injury after treatments. Both malondialdehyde contents and enzyme activities were normalized to the protein concentrations of the suspensions of liver homogenates or cell lysates.

Statistical analysis

Results are expressed as mean ± *S.E.M.*, Differences between groups were tested for statistical significance by ANOVA followed by Newman-Keuls' test. Pearson's correlation coefficients were calculated to assess possible correlation between serum ADMA concentrations and the parameters reflecting hepatic mitochondrial function of rats. $P<0.05$ was considered statistically significant.

Genes	Primer sequence		PCR Products
COX I	Sense	5'-GCCTAGATGTAGACACCCGAGCC-3'	430 bp
COX I	Antisense	5'-CGACGAGGT ATCCCTGCTAATCC-3'	
β-actin	Sense	5'-AGCCATGTACGTAGCCATCC-3'	227 bp
β -actin	Antisense	5'- TCTCAGCTGTGGTGGTGAAG-3'	
PGC-1α	Sense	5'-AAGGTCCCCAGGCAGTAGAT -3'	325 bp
PGC-1 α	Antisense	5'-GCGGTCTCT CAGTTCTGTCC-3'	
UCP2	Sense	5'-TCTTCTGGGAGGTAGCAGGA-3'	367 bp
UCP2	Antisense	5'-GCTCTGAGCCCT TGGTGTAG -3'	
GAPDH	Sense	5'-AACTT TGGCATTGTGGAAGG -3'	500 bp
GAPDH	Antisense	5'-TGTGAGGGAGATGCTCAGTA -3'	

Table 1. The primers used in the study

Genes	Initial denature	Cycle parameters	Final extension	Cycle number
COX I	94ºC 5 min	94ºC 30 s; 60 ºC 30 s; 72ºC 45 s	72ºC 10 min	25
PGC-1α	94ºC 5 min	94ºC 30s; 64ºC 45 s; 72ºC 45 s	72ºC 10 min	30
UCP2	94ºC 5 min	94ºC 30s; 55ºC 30 s; 72ºC 1 min	72ºC 10 min	30

Table 2. The reaction condition of polymerase chain reaction

Groups	Glucose (mmol/L)	ADMA (μmol/L)	DDAH activity (U/g protein)	NOS activity (U/mg protein)	Nitrite/nitrate (μmol/g protein)
Con	8.22 ± 0.85	1.14 ± 0.12	0.026 ± 0.003	1.25 ± 0.03	0.82 ± 0.02
DM	30.59 ± 1.48*	2.18 ± 0.23*	0.016 ± 0.003*	0.75 ± 0.03**	0.49 ± 0.03**
PDTC+DM	11.66 ± 1.43$^{\#}$	1.14 ± 0.14$^{\#}$	0.029 ± 0.003$^{\#}$	1.29 ± 0.13$^{\#\#}$	0.77 ± 0.01$^{\#\#}$
PDTC	8.61 ± 0.88	1.12 ± 0.10	0.026 ± 0.004	1.29 ± 0.02	0.84 ± 0.02

Table 3. Changes in plasma glucose and DDAH/ADMA/NOS/NO pathway in diabetic rats. The concentrations of plasma glucose and serum N^G, N^G-asymmetric dimethylarginine (ADMA), activities of dimethylarginine dimethylaminohydrolase (DDAH) and nitric oxide synthase (NOS) as well as the concentrations of nitrite/nitrate in the supernatants of liver homogenates were measured in rats of untreated control (Con), untreated diabetic (DM), pyrrolidine dithiocarbamate(PDTC)-treated diabetic (PDTC+DM) and PDTC-treated control (PDTC). Data are expressed as mean ± *S.E.M.*, n = 5. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* DM.

Results

Changes in plasma glucose and DDAH/ADMA/NOS/NO pathway in diabetic rats

As shown in Table 3 plasma glucose levels were remarkably higher in diabetic rats than those in control rats ($P<0.01$). Treatment with antioxidant PDTC lowered the glucose levels of diabetic rats ($P<0.01$) but not control rats (P=NS).

The activities of hepatic DDAH, the major metabolic enzyme of endogenous ADMA, were decreased, while serum levels of ADMA were significantly elevated in diabetic rats compared to control rats ($P<0.05$). Chronic treatment of diabetic rats with PDTC not only prevented the decease of hepatic DDAH activities but also reversed the elevation of serum ADMA comparing with untreated diabetic rats ($P<0.05$). However, chronic treatment of normal rats with PDTC neither distinctly altered hepatic DDAH activity nor significantly changed serum ADMA levels compared to untreated control rats (P=NS).

In contrast to serum ADMA concentrations, NOS activity and NO production reflected by nitrite/nitrate content were significantly decreased in liver of diabetic

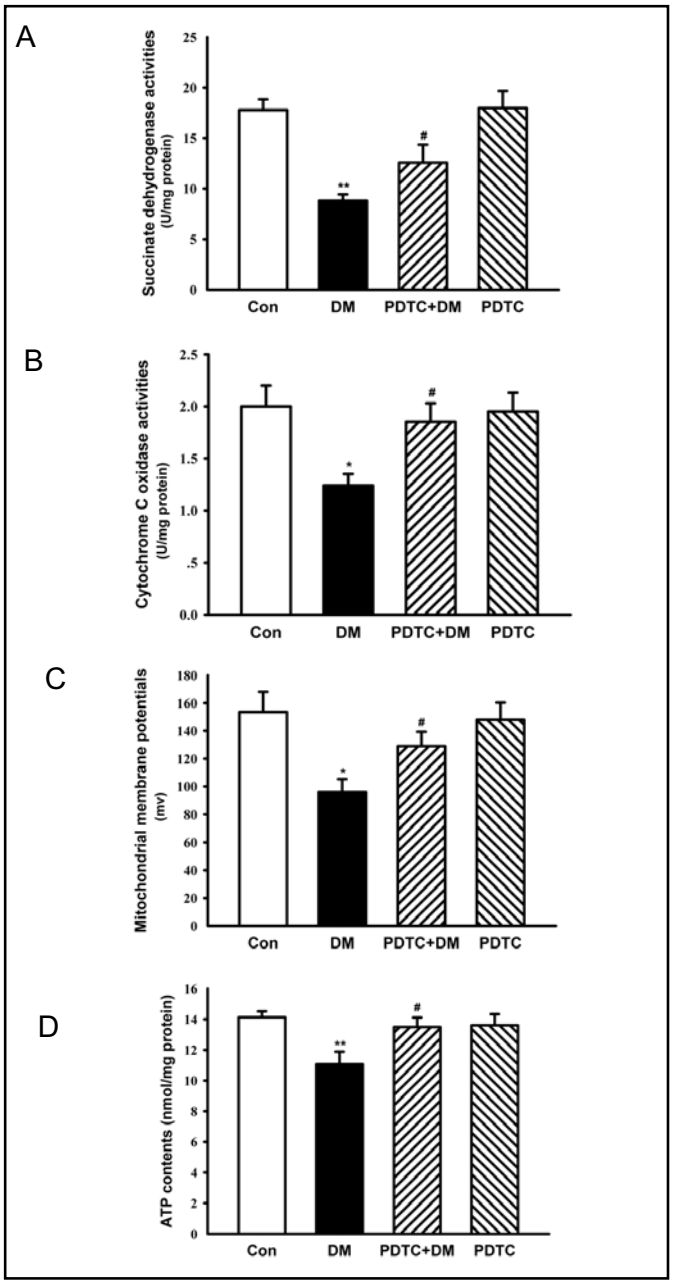


Fig. 1. Impairments of mitochondrial functions in the liver of diabetic rats. The activities of mitochondrial respiratory enzymes succinate dehydrogenase (Fig. A) and cytochrome C oxidase (Fig. B), mitochondrial membrane potentials (Fig. C) as well as ATP contents (Fig. D) were detected in the liver of control (Con), diabetic (DM), PDTC-treated diabetic (PDTC+DM), and PDTC-treated control (PDTC) rats. Data are expressed as mean ± *S.E.M.*, n = 5. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con; $^{\#}P<0.05$ *vs* DM.

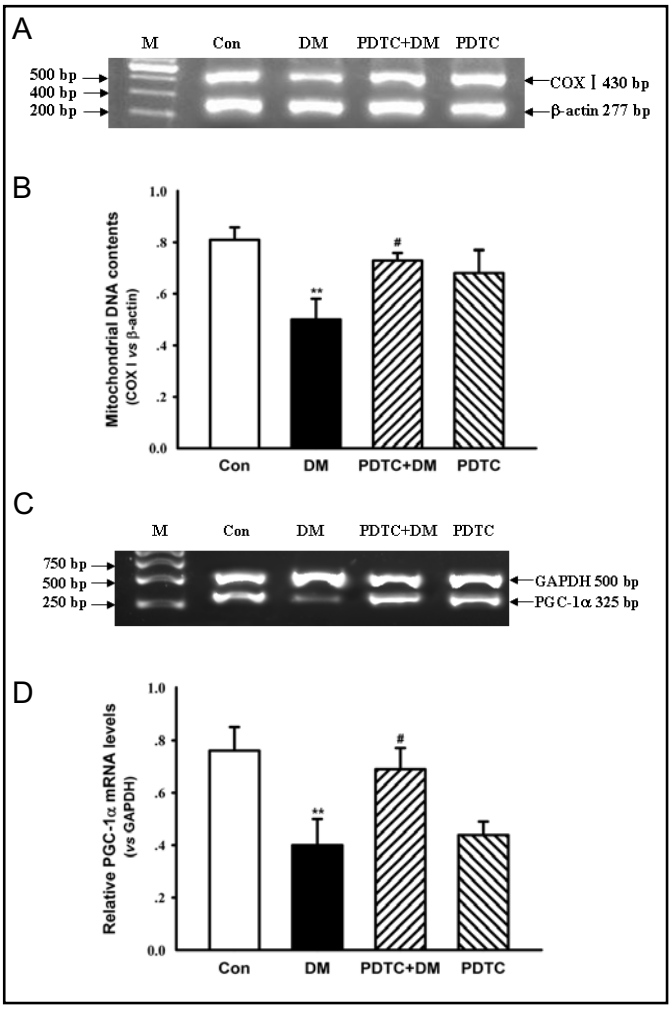


Fig. 2. Suppressions of mitochondrial biogenesis in the liver of diabetic rats. Hepatic mitochondrial biogenesis was evaluated by the content of mitochondrial DNA, which was mirrored by the copy number ratio of mitochondrial genes such as cytochrome C oxidase subunit I (COX I) to nuclear genes like β-actin indirectly and by the transcription of PGC-1α of the liver from control (Con), untreated diabetic (DM), PDTC-treated diabetic (PDTC+DM), and PDTC-treated control (PDTC) rats. Pamel A shows the gel electrophoresis pictures of COX I PCR products from a representative experiment. Panel B shows the mean of quantification ± *S.E.M.* of relative COX I copy numbers *vs* β-actin from 5 independent experiments. Panel C shows the gel electrophoresis picture of PGC-1α PCR products from a representative experiment. Panel D shows the mean of quantification ± *S.E.M.* of relative PGC-1α *vs* GAPDH mRNA levels from 5 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con, $^{\#}P<0.05$ *vs* DM.

rats ($P<0.01$ *vs* control). After treatment with PDTC, both NOS activity and NO production were increased significantly in PDTC-treated diabetic rats ($P<0.01$ *vs* diabetes) but not in PDTC-treated control rats (P=NS).

Impairments of mitochondrial function and mitochondrial biogenesis in diabetic rat liver

Figure 1 demonstrates that the activities of succinate dehydrogenase (Fig.1 A) and cytochrome C oxidase (Fig.1

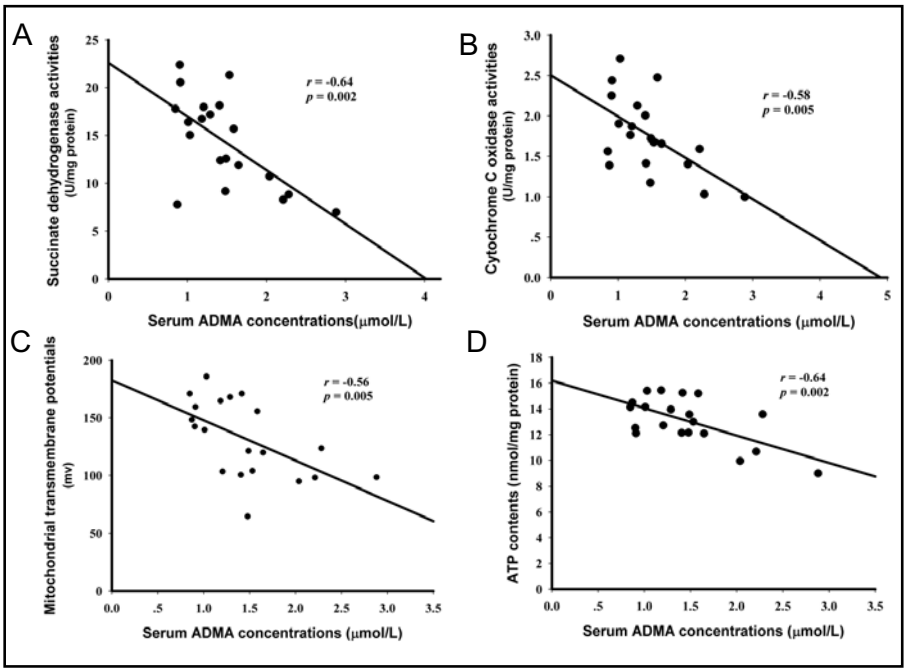


Fig. 3. Linear regression analyses of serum ADMA levels and hepatic mitochondrial functions in control and diabetic rats. To assess possible correlation between endogenous ADMA and hepatic mitochondrial function, the linear regression analyses were performed between serum ADMA concentrations and the parameters reflecting hepatic mitochondrial function including succinate dehydrogenase activities (Panel A), cytochrome C oxidase activities (Panel B), mitochondrial membrane potentials (Panel C) and ATP contents (Panel D) in control and diabetic rats. The Pearson's correlation coeffients were –0.64 (P <0.0025), –0.58 (P <0.005), –0.56 (P< 0.005) and –0.64 (P<0.0025), respectively.

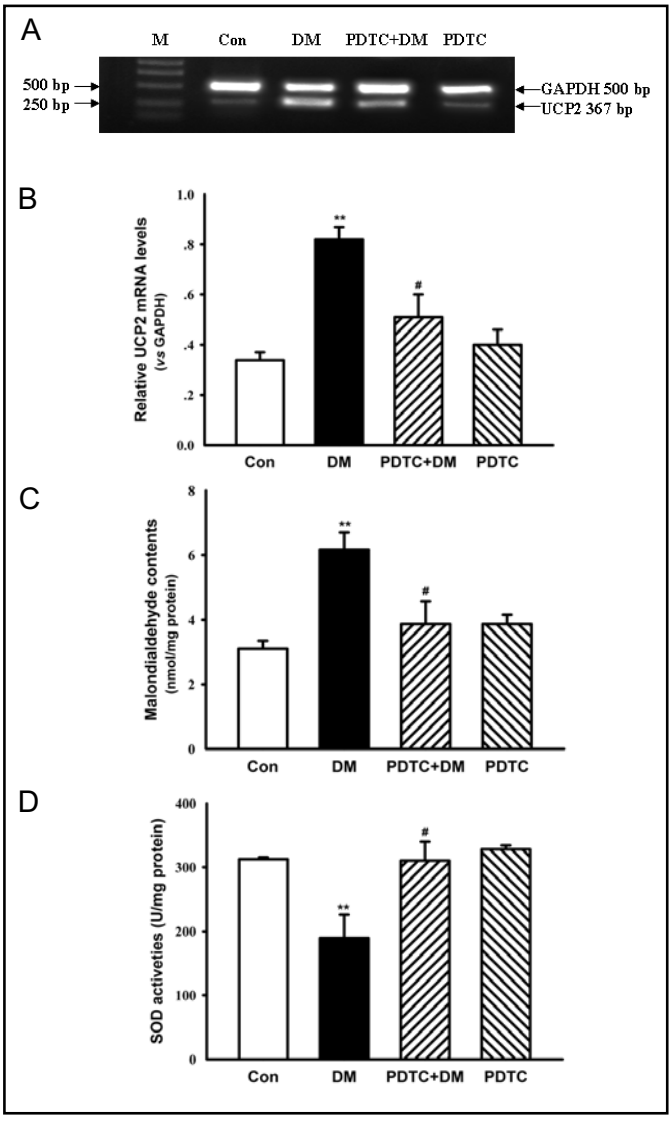


Fig. 4. Enhances of mitochondrial uncoupling and oxidative stress in the liver of diabetic rats. The transcriptions of uncoupling protein 2 (UCP2) were measured by RT-PCR to reflecting mitochondrial uncoupling. The activity of superoxide dismutase (SOD) and content of malondialdehyde (MDA), derived from lipid peroxidation, were assayed to reflect oxidative stress in the liver of untreated control (Con), untreated diabetic (DM), PDTC-treated diabetic (PDTC+DM), and PDTC-treated control (PDTC) rats. Panel A shows the gel electrophoresis picture of UCP2 PCR products from a representative experiment. Panel B shows the mean of quantification ± *S.E.M.* of relative UCP2 *vs* GAPDH mRNA levels from 5 independent experiments. Panel C shows the malondialdehyde levels. Panel D shows the superoxide dismutase activities. Data are expressed as mean ± *S.E.M.*, n = 5. $^{**}P$<0.01 *vs* Con, $^{\#}P$<0.05 *vs* DM.

B) were decreased, mitochondrial membrane potentials were lowered (Fig.1 C), and ATP levels were reduced (Fig.1 D) in the liver of diabetic rats compared to control rats, indicating that hepatic mitochondrial function was impaired in diabetic rats (All P<0.01). Chronic treatment with PDTC could improve hepatic mitochondrial dysfunction of diabetic rats (All P<0.05, Fig. 1 A ~ D) but did not affect hepatic mitochondrial function of control rats (All P=NS, Fig. 1 A ~ D).

As shown in figure 2 A & B, hepatic mitochondrial DNA content reflected by the ratio of COX I to β-actin copy numbers was obviously decreased in the liver of diabetic rats (P<0.01), indicating the suppression of mitochondrial biogenesis. This decrease of hepatic mitochondrial DNA content in diabetic rats could be reversed by PDTC treatment (P<0.05).

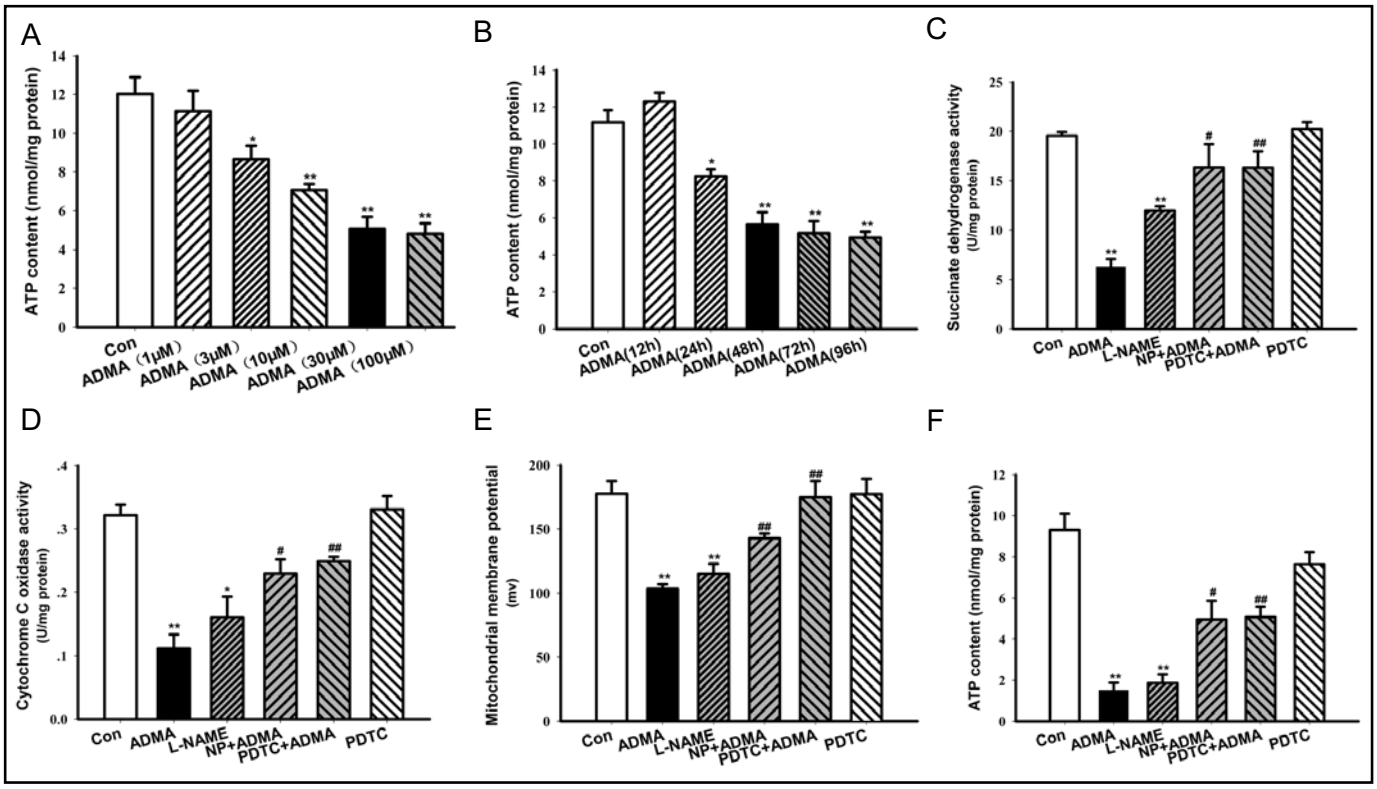


Fig. 5. Suppression of mitochondrial function induced by ADMA in hepatocytes. The concentration-dependent (A) and time-dependent (B) effects of N^G, N^G-asymmetric dimethylarginine (ADMA) on mitochondrial ATP production were measured in H4IIE rat hepatocytes to determine the optimal dose and duration for treatment with ADMA. Panel A shows the results from the concentration-dependent experiment in which hepatocytes were incubated with various concentrations of ADMA (0 ~ 100 μmol/L) for 48 h; Panel B shows the results from the time course experiment in which hepatocytes were incubated with 30 μmol/L ADMA for various period (0 ~ 96 h), and the ATP content after ADMA incubation was determined by spectrophotometry. Panel C shows activities of succinate dehydrogenase; Panel D shows activities of cytochrome C oxidase; Panel E shows mitochondrial membrane potentials; Panel F shows ATP contents. Data are expressed as mean ± *S.E.M.* from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* ADMA.

Since PGC-1α is a master regulator of mitochondrial biogenesis [11], hepatic PGC-1α transcription was checked to reflect the regulation of mitochondrial biogenesis. Figure 2 C & D illustrate that the mRNA level of PGC-1α was declined in the liver of diabetic rats in comparison with control rats ($P<0.05$). Chronic PDTC treatment prevented the decline of PGC-1α mRNA level in the liver of diabetic rats ($P<0.05$) but not in control rats (P=NS).

The correlation of elevated serum ADMA concentrations and mitochondrial dysfunction in the liver of diabetic rats

To assess possible correlation between serum ADMA levels and the parameters reflecting hepatic mitochondrial function, the linear regression analyses were performed, and Pearson's correlation coefficients were also calculated. The scatter plot figures (Fig. 3 A ~ D) show the distribution of every value and a negative linear correlation between serum ADMA concentration and succinate dehydrogenase activity, cytochrome C oxidase activity, mitochondrial membrane potential as well as ATP content, and Pearson's correlation coefficients were –0.64, –0.58, –0.56 and –0.64, respectively (All $P<0.01$).

Up-regulation of UCP2 transcription and oxidative stress in diabetic rat liver

Figure 4A & B show that UCP2 transcription was distinctly up-regulated in liver of diabetic rats comparing with control rats ($P<0.01$). After treatment with PDTC for 8 weeks, this up-regulation of UCP2 transcription was suppressed obviously in the liver of diabetic rats ($P<0.05$). However, PDTC treatment did not affect the hepatic UCP2 transcription of control rats (P=NS).

Figure 4C & D depict that malondialdehyde contents were significantly elevated while SOD activity was

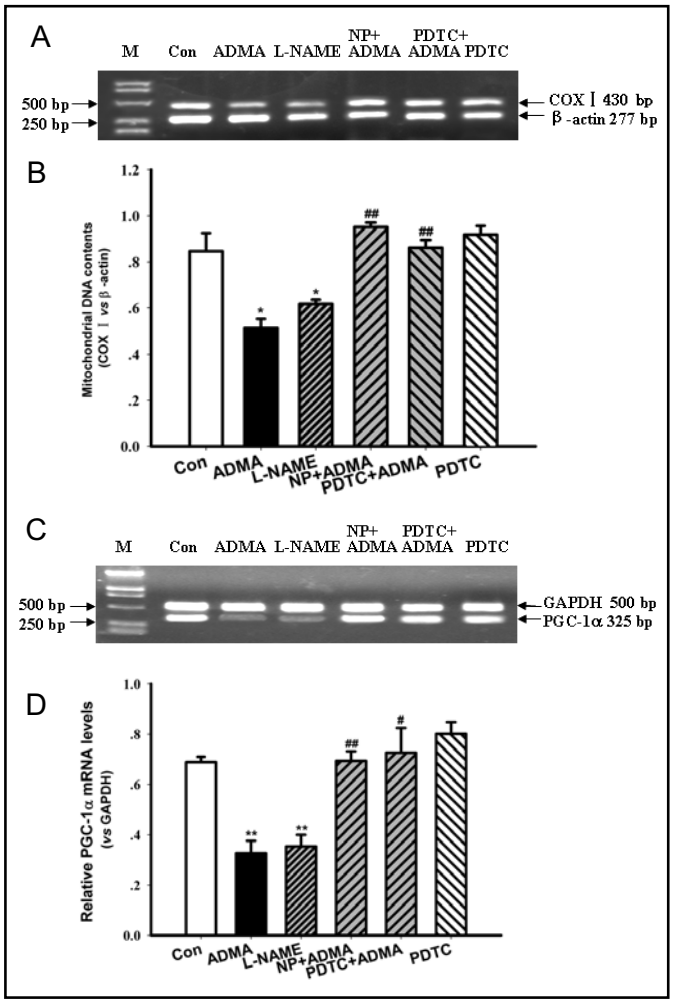


Fig. 6. Suppression of mitochondrial biogenesis induced by ADMA in hepatocytes. The content of mitochondrial DNA, which was mirrored by the copy number ratio of mitochondrial genes such as cytochrome C oxidase subunit I (COX I) to nuclear genes like β-actin, and the transcriptions of PGC-1α gene, a master regulator of mitochondrial biogenesis, were examined to evaluate mitochondrial biogenesis in hepatocytes incubated without (Con) or with 30 μmol/L N^G, N^G-asymmetric dimethylarginine (ADMA) or 30 μmol/L N^G-Nitro-L-arginine Methyl Ester (L-NAME) or 10 μmol/L pyrrolidine dithiocarbamate (PDTC) for 48 h. Some cells were pretreated with 10 μmol/L sodium nitroprusside (NP) or PDTC for 1 h followed by co-incubation with 30 μmol/L ADMA for 48 h. Panel A shows the gel electrophoresis picture of COX I PCR products from a representative experiment. Panel B shows the mean of quantification ± S.E.M. of relative COX I *vs* β-actin copy number from 3 independent experiments. Panel C shows the gel electrophoresis pictures of PGC-1α PCR products from a representative experiment. Panel D shows the mean of quantification ± S.E.M. of relative PGC-1α mRNA expression *vs* GADPH from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* ADMA.

declined in the liver of diabetic rats compared to control rats (Both $P<0.01$), and all these changes could be prevented by PDTC treatment (Both $P<0.05$).

Suppression of mitochondrial function and mitochondrial biogenesis in hepatocytes treated with ADMA

The concentration-response and time course of ADMA on the production of ATP in hepatocytes were performed firstly to ascertain the appropriate dose and incubation time of ADMA applied in the following cellular experiment. After respective incubation of H4IIE hepatocytes with 0, 1, 3, 10, 30, 100 μmol/L ADMA for 0, 12, 24, 48, 72, and 96 h, mitochondrial ATP contents were inhibited in a concentration- and time-dependent manner, especially after exposure to 30 μmol/L ADMA for 48 h (Fig. 5 A ~ B). Accordingly, 30 μmol/L ADMA and 48 h were chosen to apply in the following experiment.

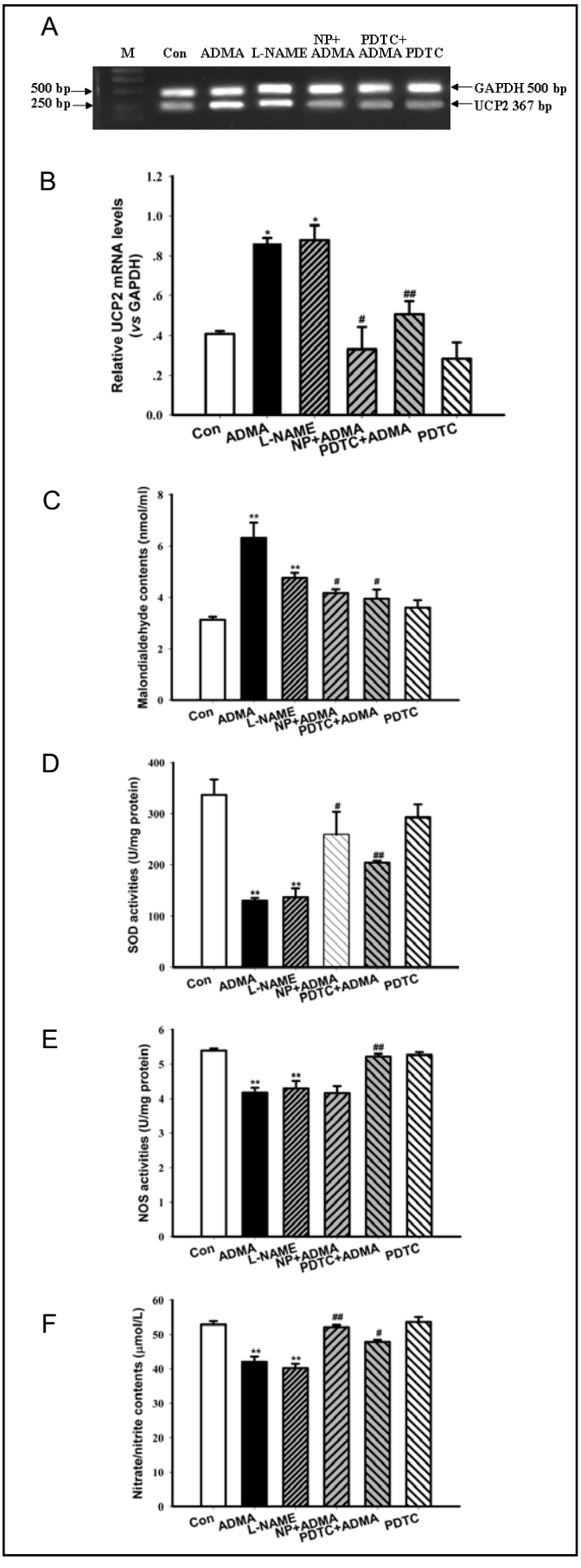
After exposure of hepatocytes to 30 μmol/L ADMA for 48 h, the activities of mitochondrial succinate dehydrogenase (Fig. 5 C) and cytochrome C oxidase (Fig. 5 D) were significantly inhibited, mitochondrial membrane potential (Fig. 5 E) was decreased, and ATP levels (Fig. 5 F) were also reduced (All $P<0.01$). Similar results were achieved by incubation of hepatocytes with another exogenous inhibitor of nitric oxide synthase L-NAME (30 μmol/L) for 48 h (All $P<0.05$). These deleterious effects of ADMA on mitochondrial function were attenuated significantly by pretreatment of hepatocytes with 10 μmol/L of PDTC or NO donor sodium nitroprusside for 1h and then co-treatment with ADMA for another 48h (All $P<0.05$). However, PDTC treatment alone did not affect the mitochondrial function of hepatocytes (P=NS).

Treatment of hepatocytes with ADMA or L-NAME also inhibited mitochondrial biogenesis as shown by the decrease of the copy number ratio of mitochondrial gene COX I to nuclear gene β-actin ($P<0.05$, Fig. 6 A & B) and suppressed transcription of PGC-1α ($P<0.01$, Fig. 6 C & D). Treatment with PDTC or sodium nitroprusside could reverse ADMA inhibition of mitochondrial biogenesis ($P<0.05$) whereas PDTC per se had no effect on mitochondrial biogenesis of hepatocytes (P=NS, Fig. 6 A ~ D).

Effects of ADMA on UCP2 transcription and oxidative stress in hepatocytes

After incubation of hepatocytes with ADMA or L-NAME for 48 h, the transcription of UCP2 was up-regulated compared to control group (Fig. 7 A & B). Treatment with PDTC or sodium nitroprusside could prevent the up-regulation of UCP2 transcription induced

Fig. 7. Up-regulation of mitochondrial uncoupling & oxidative stress and reduction of nitric oxide synthesis in hepatocytes after exposure to ADMA. The transcriptions of uncoupling protein 2 (UCP2) were measured by RT-PCR to represent mitochondrial uncoupling. Panel A shows the gel electrophoresis picture of UCP2 PCR products from a representative experiment. Panel B shows the mean of quantification ± S.E.M. of relative UCP2 mRNA levels *vs* GAPDH from 3 independent experiments. The contents of malondialdehyde (MDA, Panel C), derived from lipid peroxidation, and the activities of superoxide dismutase (SOD, Panel D) were assayed to reflect oxidative stress. The activities of nitric oxide synthase (NOS, Panel E) and the concentrations of nitrite/nitrate, the stable end products of nitric oxide (NO, Panel F) were detected to reflect NO production in hepatocytes treated without (Con) or with 30 μmol/L N^G, N^G-asymmetric dimethylarginine (ADMA) or 30 μmol/L N^G-Nitro-L-arginine Methyl Ester (L-NAME) or 10 μmol/L pyrrolidine dithiocarbamate (PDTC) for 48 h. Other cells were pretreated with 10 μmol/L sodium nitroprusside (NP) or PDTC for 1 h followed by co-incubation with 30 μmol/L ADMA for 48 h. Data are expressed as mean±*S.E.M.* from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Con; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* ADMA.



by ADMA in hepatocytes. However, PDTC alone had no effect on the mRNA levels of UCP2 (Fig. 7 A & B).

In addition, ADMA incubation also enhanced oxidative stress of hepatocytes as expressed by the increase of malondialdehyde contents in condition media (Fig. 7 C) and the decrease of SOD activities in hepatocytes (Fig. 7 C). Similar enhance of oxidative stress was observed after hepatocytes exposure to L-NAME. Pretreatment with PDTC could attenuate the enhance of oxidative stress while PDTC per se did not affect oxidative stress in hepatocytes (Fig. 7 C & D).

Effects of ADMA on NOS activity, NO production and cell viability in hepatocytes

As shown in Fig. 7 E & F, incubation of hepatocytes with ADMA for 48 h significantly inhibited NOS activity and decreased NO production. L-NAME treatment had the similar effects. Treatment with PDTC could increase NOS activity and NO production while treatment with NO donor sodium nitroprusside only enhanced the concentrations of NO metabolites nitrate/nitrite but did not affect the NOS activity. Furthermore, the activities of lactate dehydrogenase were not different in the conditional media of hepatocytes treated with ADMA and other drugs (Data not shown), indicating that all treatments in the hepatocyte experiment did not significantly affect the cell viability.

Discussion

Accumulated evidence indicates that mitochondrial dysfunction plays an important role in the development of diabetes mellitus [1, 2]. Elevated endogenous NOS inhibitor ADMA has been shown to closely relate to insulin resistance [14, 15], diabetes mellitus [16, 17] and diabetic vascular complications [28, 29]. But the relationship between ADMA and mitochondrial dysfunction in diabetes mellitus remains unknown. The present study provided the first evidence that elevated endogenous ADMA in diabetic rats was associated with hepatic mitochondrial dysfunction. The linear regression analysis shows a close correlation between the elevated endogenous ADMA and the parameters reflecting hepatic mitochondrial dysfunction in diabetic rats. To further determine the causal relationship between both, rat hepatoma cell line (H4IIE) was used to investigate direct effects of ADMA on mitochondrial function. Similar mitochondrial dysfunction observed in diabetic rat liver was discovered in ADMA-treated hepatocytes. These results indicate that elevated endogenous ADMA contributes to hepatic mitochondrial dysfunction in diabetic rats.

Concerning the reason for the elevation of endogenous ADMA in diabetes mellitus, inhibition of DDAH activity could be the predominant factor. Because DDAH is the major enzyme for ADMA degradation and its high expression in liver [18]. Furthermore, DDAH can be redox-regulated [30], and decreased DDAH activity as well as enhanced oxidative stress were found in the liver of diabetic rats in the present study. Consistent results were also observed in the aortas of diabetic rats [16]. More importantly, chronic treatment of diabetic rats with antioxidant PDTC not only reversed the decrease of DDAH activity but also prevented the elevation of endogenous ADMA. Collectively, these results indicate that oxidative inactivation of DDAH activity is the key cause for endogenous ADMA accumulation in diabetes mellitus.

Although many factors have been shown to be involved in mitochondrial dysfunction, increasing evidence suggests that reduced mitochondrial biogenesis may be the initial factor responsible for mitochondrial dysfunction, because reduced mitochondrial density and decreased PGC-1α expression were early observed in the skeletal muscle of the non-diabetic offspring of type 2 diabetic patients [2, 31]. Mitochondrial biogenesis is usually represented by mitochondrial DNA content, which is reflected by the copy number ratio of mitochondrial gene cytochrome C oxidase subunit I to nuclear gene β-actin [5]. In this study, the ratio of COX I to β-actin copy numbers was significantly decreased, indicating that mitochondrial biogenesis was suppressed in the liver of diabetic rats and ADMA-treated hepatocytes. It has been recognized that PGC-1α is the master regulator of mitochondrial biogenesis [11] and endothelial NO appears to be an important player in the regulation of PGC-1α expression [12]. We hence speculate that ADMA, as the major endogenous inhibitor of NOS, may inhibit mitochondrial biogenesis by means of reducing NO generations and down-regulating PGC-1α transcription. To address this, we comparatively investigated the effects of exogenous NOS inhibitor L-NAME and NO donor sodium nitroprusside on mitochondrial function in cultured rat hepatocytes. It was found that L-NAME possessed the same effects as endogenous NOS inhibitor ADMA on mitochondrial biogenesis, mitochondrial function, and PGC-1α transcriptions in rat hepatocytes; supplement of NO donor sodium nitroprusside could attenuate the effects of ADMA. Furthermore, down-regulated PGC-1α transcription and suppressed NO production presented by the reduced concentrations of nitrate/nitrite, the stable metabolites of NO and inhibited NOS activity were also observed in both the liver of diabetic rats and ADMA-treated hepatocytes. Consistent results have been reported in the study in which mitochondrial dysfunction was induced by arachidonic acid in rat primary hepatocytes [32]. Moreover, knockout of constitutive NOS has been demonstrated to impair activities of mitochondrial respiratory enzymes in brain, muscle and heart of mice [33]. Taken together, these results strongly support our speculation that the suppression of mitochondrial biogenesis resulting from the inhibition of NO synthesis and down-regulation of PGC-1α transcription is implicated in the mechanisms for hepatic mitochondrial dysfunction induced by ADMA in diabetic rats.

It has been documented that UCP2 plays a vital role in the development of mitochondrial dysfunction [9, 34]. UCP2 may mediate the leaking of protons across mitochondrial inner membrane, resulting in the uncoupling of oxidative metabolism from ATP production [9]. Increased UCP2 expression has been shown to remarkably reduce ATP stored in hepatocytes [34], whereas genetic deficiency of UCP2 has been found to greatly improve ATP production in pancreatic β cells [35]. The current study exhibited an augment of UCP2 transcription in association with mitochondrial dysfunction in the liver of diabetic rats when endogenous ADMA were elevated, suggesting a relationship among UCP2

transcription, hepatic mitochondrial dysfunction and endogenous ADMA accumulation in diabetic rats. Treatments of rat hepatocytes with exogenous ADMA could upregulate UCP2 mRNA levels and induce mitochondrial dysfunction, suggesting that ADMA is the direct cause of stimulating UCP2 transcription resulting in mitochondrial dysfunction. Collectively, it is reasonable to consider that the upregulation of UCP2 transcription is another mechanism under which ADMA induces hepatic mitochondrial dysfunction in diabetic rats.

It is established that UCP2 expression can be stimulated by oxidative stress [10], which is defined the imbalance between the generation of ROS and the antioxidant defense. In addition to stimulating UCP2 expression, oxidative stress can suppress mitochondrial biogenesis [36] and inhibit the activities of respiratory enzymes resulting in mitochondrial dysfunction [37]. Although the content of ROS was not detected directly in this study, the content of malondialdehyde, derived from lipid peroxidation, might reflect the oxidant intensity of ROS indirectly. Additionally, SOD constitutes the first link in the enzyme scavenging system of ROS, and its activity can mirror antioxidative capability properly. The increased malondialdehyde contents and decrease of SOD activity were observed in the liver of diabetic rats, indicating an enhancement of oxidative stress. Interestingly, enhanced oxidative stress and upregulated UCP2 transcription were simultaneously induced by ADMA in cultured rat hepatocytes. Both of them were coordinately attenuated by treatment with antioxidant PDTC either in the liver of diabetic rats or in the ADMA-treated hepatocytes in parallel with the amelioration of mitochondrial dysfunction. Moreover, we also observed that treatment of diabetic rats with PDTC could reduce blood glucose. This result is consistent with previous reports and may be related to that PDTC can inhibit oxidative stress and NF-κB activation resulting in the suppression of inflammation and apoptosis of pancreatic β cells [38]. Taken together, these results indicate that ADMA stimulates UCP2 expression via enhance of oxidative stress.

ADMA has been shown to be a maker of ROS since it can inhibit NOS activity leading to the uncoupling of NOS, making the electrons divert to molecular oxygen rather than to L-arginine, and resulting in the production of superoxide rather than NO [13]. In this study, suppressed NO production and inhibited NOS activity were observed in both the liver of diabetic rats and ADMA-treated hepatocytes in parallel with enhance of oxidative stress. These results illuminate that ADMA enhances oxidative stress through uncoupling NOS.

In summary, the present study reveals that elevated endogenous ADMA contributes to hepatic mitochondrial dysfunction in diabetic rats and provides the new insight into the mechanisms underlying ADMA-induced mitochondrial dysfunction. Furthermore, chronic treatment with antioxidant PDTC not only attenuated endogenous ADMA accumulation but also improved mitochondrial dysfunction. Therefore, interventions targeted to modulate endogenous ADMA level may present a new approach to the prevention and treatment of mitochondrial dysfunction in diabetes mellitus.

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