NAD Blocks High Glucose Induced Mesangial Hypertrophy via Activation of the Sirtuins-AMPK-mTOR Pathway

Li Zhuo, Bo Fu, Xueyuan Bai, Bin Zhang, Lingling Wu, Jing Cui, Shaoyuan Cui, Ribao Wei, Xiangmei Chen and Guangyan Cai

Department of Nephrology, Kidney Center and Key Lab of the People's Liberation Army, General Hospital of People's Liberation Army, Beijing

Key Words

NAD • Sirtuins • AMPK • mTOR • Mesangial hypertrophy

Abstract

Background/aims-Since the discovery of NAD-dependent deacetylases, Sirtuins, it has been recognized that maintaining intracellular levels of NAD is crucial for the management of stress-response of cells. Here we show that high glucose(HG)-induced mesangial hypertrophy is associated with loss of intracellular levels of NAD. This study was designed to investigate the effect of NAD on HG-induced mesangial hypertrophy. **Methods-**The rat glomerular mesangial cells (MCs) were incubated in HG medium with or without NAD. Afterwards, NAD⁺/NADH ratio and enzyme activity of Sirtuins was determined. In addition, the expression analyses of AMPK-mTOR signaling were evaluated by Western blot analysis. **Results-**We showed that HG induced the NAD⁺/NADH ratio and the levels of SIRT1 and SIRT3 activity decreased as well as mesangial hypertrophy, but NAD was capable of maintaining intracellular NAD⁺/NADH ratio and levels of SIRT1 and SIRT3 activity as well as of blocking the HG-induced mesangial hypertrophy *in*

vitro. Activating Sirtuins by NAD blocked the activation of pro-hypertrophic Akt signaling, and augmented the activity of the antihypertrophic AMPK signaling in MCs, which prevented the subsequent induction of mTOR-mediated protein synthesis. By AMPK knockdown, we showed it upregulated phosphorylation of mTOR. In such, the NAD inhibited HG-induced mesangial hypertrophy whereas NAD lost its inhibitory effect in the presence of AMPK siRNA. **Conclusion-**These results reveal a novel role of NAD as an inhibitor of mesangial hypertrophic signaling, and suggest that prevention of NAD depletion may be critical in the treatment of mesangial hypertrophy.

Copyright © 2011 S. Karger AG, Basel

Introduction

The early pathological manifestations of diabetic nephropathy consist of altered glomerular hemodynamics and whole-kidney and, in particular, glomerular hypertrophy [1-3]. Later manifestations include microalbuminuria, frank proteinuria, and progressive fibrosis leading to end-stage renal disease. Increases in fractional volume of the mesangium correlate with mesangial cell hypertrophy. However, high glucose is one

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
1015-8987/11/0276-0681\$38.00/0

Accessible online at:
www.karger.com/cpb

Xiangmei Chen and Guangyan Cai
General Hospital of People's Liberation Army,
Fuxing Road 28, Beijing, 100853 (People's Republic of China)
Tel. +86 1066935462
E-Mail xmchen301@126.com and E-Mail caiguangyan@sina.com

of the leading factors of diabetic nephropathy that introduce mesangial cell hypertrophy, manifesting as increase in overall protein synthesis without concomitant enhancement in DNA synthesis [3]. One novel approach is the activation of endogenous cell signaling pathways that negatively regulate mesangial hypertrophy. Exogenous agents which can facilitate the activity of these pathways are of particular interest as new therapeutic tools for the management of mesangial hypertrophy.

At the cellular level various signaling mechanisms have been described which lead to development of mesangial hypertrophy. Among them, oxidative stress is recognized as a critical common signal to various stimuli, which directs to evolution of pathologic hypertrophy. Severe oxidative stress can result in increased NAD turnover due to increased activity of NAD-consuming enzymes such as PARP1 (poly ADP-ribose polymerase-1) and/or decreased activity of NAD salvage pathways, with a net result of depletion of intracellular NAD levels [4]. Loss of NAD can make a cell unable to carry out its energy-dependent functions and to defend itself against oxidative stress because of loss of activity of certain cell-survival factors which are NAD-dependent, such as Sirtuins.

Sirtuins are class-III HDACs, which are expressed as seven different (SIRT1-SIRT7) isoforms in mammals. They are considered to be key regulators of many cellular functions, including stress-resistance, energy metabolism, apoptosis, and aging [5]. It is reported that SIRT1 regulate intracellular metabolism and attenuate reactive oxidative species (ROS)-induced apoptosis leading to longevity and stress resistance. Recent study showed SIRT1 overexpression in proximal tubules rescues cisplatin-induced kidney injury, concomitant elimination of renal ROS levels [6]. In fact, SIRT1 also can prevent oxidative stress-induced apoptosis through p53 deacetylation in mesangial cells [7]. Another Sirtuin analogue SIRT3 has been shown to be highly expressed in the kidney and heart [8, 9]. However, it is activated during stress of kidney injury and cardiomyocytes. Increased activity of SIRT3 protects cardiomyocytes from oxidative stress mediated cell-death by increased expression of antioxidants, MnSOD and catalase [10]. Previous studies have shown that intracellular NAD⁺/NADH ratio regulates the levels of Sirtuins, including SIRT1 and SIRT3 [11]. While high levels of NAD upregulates the levels of Sirtuins, high levels of NADH and nicotinamide do the opposite. A recent report has indicated that exogenous NAD can enter into neurons and protect them from degeneration and ischemia induced cell death [12]. It is therefore likely

that by maintaining an adequate intracellular level of NAD, mesangial cells could be also protected from hypertrophy and cell-death by increasing the activity of one or more Sirtuin analogues.

In this study we report that high glucose induces mesangial hypertrophy is associated with depletion of cellular NAD levels. Exogenous supplementation of NAD restores the intracellular levels of NAD and blocks the mesangial hypertrophic response. We also demonstrate that anti-hypertrophic effects of NAD are mediated via activation of Sirtuins-AMPK-mTOR pathway. To the best of our knowledge, this report represents the first application of NAD in the treatment of mesangial hypertrophy.

Materials and Methods

The rat mesangial cell (MC) line (the cell bank of the Chinese Academy of Sciences, Beijing) was thawed, cultured in RPMI1640 (Life Technologies BRL Gaithersburg, MD) that was supplemented with 10% FBS (Life Technologies BRL, Gaithersburg, MD), 100 units/ml penicillin, and 100 g/ml streptomycin at 37°C. MCs were cultured for 48h in normal-(5.5 mM) or high (30 mM) D-glucose medium with or without NAD(250 μM). As an osmotic control, MCs were grown in normal-glucose medium that contained 24.5 mM mannitol.

Reactive oxygen species (ROS) were detected using CM-H₂DCFDA (Invitrogen) as per the manufacturer's instructions. Briefly, MCs were cultured for 48h in low- or high glucose medium with or without NAD (250 μM). Cells were stained with CM-H₂DCFDA. CM-H₂DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2',7'-dichlorofluorescein (DCFH) and thereby trapped within the cells (17). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The ROS generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission, 515 to 540 nm). The effect of DCFH photooxidation was minimized by collecting the fluorescence image with a single rapid scan under identical conditions, such as contrast and brightness, for all samples. The cells were then imaged by differential interface contrast microscopy and analyzed with use of Image J (<http://rsb.info.nih.gov/ij/>).

The NAD⁺/NADH Quantification Kit (BioVision) provided a convenient tool for sensitive detection of NAD⁺/NADH ratio. There is no need to purify NAD⁺/NADH from sample mix. The NAD⁺/NADH Quantification Kit were used according to manufacturer's instructions.

Deacetylation was measured using the Fluor de Lys kit (AK-555-SIRT1 and AK-557-SIRT3; Biomol). The acetylated lysine residue was coupled to an aminomethylcoumarin moiety. The peptide was deacetylated by SIRT1 or SIRT3, followed by the addition of a proteolytic developer that released the fluorescent aminomethylcoumarin. Briefly, enzyme preparations

were incubated with 3 mM NAD⁺ for 45 min at 37°C followed by incubation in developer for 15 min at 25°C. Fluorescence was measured by excitation at 360 nm and emission at 460 nm and enzymatic activity was expressed in relative fluorescence units [13]. Assays were performed in triplicate.

The day before transfection, plates were inoculated with an appropriate number of MCs in serumcontaining medium to ensure 40-60% confluence the following day. AMPK $\alpha_{1/2}$ siRNA (Santa Cruz, CA) mixed with jetPRIME™ (Polyplus-transfection, USA) was added to the cells at a concentration of 10 nmol/L. The medium was replaced with culture medium at 24h transfection and the cells were incubated for 48h at 37°C. Luciferase siRNA (Shanghai GenePharma Co., Ltd) was used as a negative control (NC).

Cells (1×10^6 ; each sample) were treated with 0.25% trypsin, washed twice with cold PBS, and fixed with 70% alcohol in PBS for 12 h at 4°C. The cells then were washed twice with PBS and stained for 30 to 60 min at 4°C in 100 μ g/ml propidium iodide (PI) solution (with 100 μ g/ml RNase). Stained cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ). The mean cell size was analyzed by measurement of light scatter parameters: forward scatter (FSC) using flow cytometry [14]. FSC is related to the cell size and the optical refraction index of the outer membrane of the cell.

Because the total protein/cell number ratio is a well-established measurement of cellular hypertrophy, this parameter was used to determine whether the alteration of cell growth was accompanied by cell hypertrophy [15]. MCs were cultured for 48h in low- or high-glucose medium with or without NAD, and at the end of the treatment period, cells were trypsinized and washed twice with ice-cold PBS and counted in a hemocytometer chamber. The cells then were lysed to measure the total protein content by the Bradford method. The total protein/cell number ratio expressed as μ g/ 10^5 cells was used as a hypertrophy index.

Relative Sirtuins expression was quantified by real-time RT-PCR. Total RNA was extracted from MCs by using TRIzol reagent (Invitrogen, USA), and 5 μ g of the total RNA was reverse-transcribed to cDNA. Real-time PCR was performed with cDNA and gene-specific primer pairs using a Taqman kit (Toyobo, Osaka, Japan) and SYBR Green PCR Master Mix (TransGen Biotech, Beijing, China) in an iCycler apparatus (Bio-Rad, Hercules, CA, USA). The primer sets used were: 5'-TTG GCA CCG ATC CTC GAA C-3' and 5'-CCC AGC TCC AGT CAG AAC TAT-3' for SIRT1; 5'-TGC TAC TCA TTC TTG GGA CCT-3' and 5'-CAC CAG CCT TTC CAC ACC-3' for SIRT3; 5'-AAC GAC CCC TTC ATT GAC -3' and 5'-TCC ACG ACA TAC TCA GCA C-3' for GAPDH. PCR amplification was performed using the following conditions: initial denaturation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 30s, annealing at 56°C (SIRT1 and SRIT3) or 60°C (GAPDH) for 30s (with fluorescence measured simultaneously), and extension at 72°C for 30s. Data analysis based on measurements of the threshold cycle was performed used the $2^{-\Delta\Delta Ct}$ method, as described by Livak [16]. The experiments were repeated in triplicate.

The cells were lysed in RIPA buffer that was composed of 50 mM Tris-Cl (pH 7.6), 5mM EDTA, 150mM NaCl, 0.5% NP-40,

Fig. 1. NAD blocks the HG-induced oxidative stress of mesangial hypertrophy. (A) MCs were cultured for 48 hours in normal- or high-glucose medium with or without NAD. Cells were stained with CM-H₂DCFDA. ROS (reactive oxygen species) production was measured by differential interface contrast microscopy (600x). (B) Quantification of mean fluorescence intensity in different groups of cells. * $P < 0.05$ versus Control or Osmotic control. $\Delta P < 0.05$ versus High glucose. Values are mean $\pm$ SE of 3 experiments.

Fig. 2. NAD suppresses the high glucose (HG)-induced down-regulation of NAD⁺/NADH ratio. Mesangial cells (MCs) were cultured for 48 hours in normal-(5.5 mM) or high (30 mM) D-glucose medium with or without NAD(250 μ M). The NAD⁺/NADH ratio was decreased in MCs treated with HG, but NAD suppressed the HG-induced down-regulation of NAD⁺/NADH ratio. * $P < 0.05$ versus Control or Osmotic control. $\Delta P < 0.05$ versus High glucose. Values are mean $\pm$ SE of 3 experiments.

and 0.5% Triton-X-100 and contained 1 μ g/ml leupeptin, aprotinin, and antipain; 1mM sodium orthovanadate; and 0.5mM

Fig. 3. NAD suppressed the HG-induced down-regulation of Sirtuins activity. MCs were cultured for 48h in normal- or high-glucose medium with or without NAD. (A) The relative quantitation of SIRT1 mRNA was expressed as the ratio of SIRT1/GAPDH, which in control cells was set to 1. (B) The relative quantitation of SIRT3 mRNA was expressed as the ratio of SIRT3/GAPDH, which in control cells was set to 1. (C) Western blots of SIRT1 and SIRT3. (D) Quantitative protein level of SIRT1 and SIRT3. Data were expressed as relative percentages versus actin, and groups were compared using ANOVA. (E) The deacetylase activity of SIRT1 was decreased in MCs. (F) The deacetylase activity of SIRT3 was decreased in MCs. SIRT1 and SIRT3 deacetylase activity were measured using the Fluor de Lys kit. The comparison among groups was conducted by ANOVA. Values are means $\pm$ SD (n=3); * P <0.05 versus Control or Osmotic control. ΔP <0.05 versus High glucose. AFU, Arbitrary Fluorescence Units.

phenylmethylsulfonyl fluoride. Protein concentration was measured by the Bradford assay. A total of 50 μ g of total protein was separated by 6-16% SDS-PAGE and then transferred to a membrane, which was blocked with 5% skim milk, probed with a primary antibody overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody. Antibodies were procured from Cell Signaling (mTOR, p-mTOR, p-Raptor), ABcam (AMPK, p-AMPK, Raptor, p70S6k and 4EBP1) and Santa Cruz Biotechnology (Santa Cruz, CA; SIRT1, SIRT3, Akt, p-Akt, and β -Actin). Immunoreactive bands were visualized using enhanced chemiluminescence.

All data analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL); data are expressed as mean $\pm$ SD. Comparison among groups was conducted with ANOVA. P < 0.05 was considered significant.

Results

NAD protects mesangial cell from high glucose-mediated hypertrophy

Mounting evidence has indicated that induction of reactive oxygen species (ROS) is necessary for mesangial hypertrophy of the development of diabetic nephropathy[17, 18]. Therefore, in order to test the mesangial cells protective effects of NAD, we first examined that the ability of NAD to block high glucose-induced ROS

Fig. 4. NAD blocks the HG-induced cell cycle arrested. High glucose inhibited cell cycle progression, while NAD could block the effect. Cell cycle analyses were performed by flow cytometry, and G0/G1, S and G2/M populations were indicated aside each histogram. The data shown are from one representative experiment from a series of three with similar results.

production of mesangial hypertrophy. Cultures of MCs were treated with high glucose (HG), in the presence or absence of NAD. Cells were stained with CM-H₂DCFDA, a non-fluorescent dye which fluoresces by ROS-mediated removal of an acetate group by intracellular esterases. We found that NAD-treatment

	Control	Osmotic control	High glucose	NAD	OS+NAD	HG+NAD
protein/cell number ratio	39.5±1.41	42.9±1.34	49.7±1.13*	29.1±0.71* [▲]	30.3±2.83* [▲]	43.3±1.56* [▲]
FSC	518.55±0.25	521.57±1.77	555.23±2.28*	492.26±4.22* [▲]	493.17±5.6* [▲]	501.82±3.24* [▲]

Table 1. NAD blocks the HG-induced mesangial cell hypertrophy. MCs were cultured for 48h in normal- or high-glucose medium with or without NAD. Total protein content was determined by Bradford assays, and cells were counted manually to determine the total protein/cell number ratio expressed as $\mu\text{g}/10^5$ cells. The mean cell size was analyzed by measurement of light scatter parameters: forward scatter (FSC) using flow cytometry. Values are means $\pm$ SD (n=5); comparison among groups was conducted by ANOVA. * $P < 0.05$ versus Control or Osmotic control. [▲] $P < 0.05$ versus High glucose.

	HG	HG+NC	HG+AMPK siRNA	HG+NAD	HG+NC+NAD	HG+NAD+AMPK siRNA
protein/cell number ratio	52.8±0.42	53.7±0.57	55.9±1.06*	44.6±0.99* [▲]	45.4±1.27* [▲]	51.6±1.20* [▲]
FSC	526.22±4.38	524.60±2.51	549.70±6.34*	479.56±2.37* [▲]	480.51±11.90* [▲]	517.64±2.66* [▲]

Table 2. Down-regulation of AMPK by siRNA reverses the effects of NAD on HG-induced hypertrophy in MCs. MCs were transfected with a mixture of siRNAs targeting AMPK or luciferase (NC), and cultured for 48h in high-glucose medium with or without NAD. Total protein content was determined by Bradford assays, and cells were counted manually to determine the total protein/cell number ratio expressed as $\mu\text{g}/10^5$ cells. The mean cell size was analyzed by measurement of light scatter parameters: forward scatter (FSC) using flow cytometry. Values are means $\pm$ SD (n=5); comparison among groups was conducted by ANOVA. * $P < 0.05$ versus HG or HG+NC group. [▲] $P < 0.05$ versus HG+AMPK siRNA group.

suppressed the HG-induced mean fluorescence intensity of the dye to a level which was comparable to what was observed with control cells, thus suggesting that NAD-treatment was capable of blocking the HG-induced ROS synthesis of mesangial cells (Fig. 1).

These data also indicated that hypertrophy of MCs may result from loss of cellular NAD levels, and if so, then supplementation of NAD should be able to rescue cells from developing hypertrophy. To test this hypothesis, we treated MCs with HG, or HG-treatment was given together with NAD. We found that HG-treatment caused depletion of cellular NAD levels, and it showed that the intracellular NAD^+/NADH ratio decreased (Fig. 2).

However, Sirtuins is the enzyme that catalyzes NAD^+ -dependent protein deacetylation. Previous studies have shown that intracellular NAD^+/NADH ratio regulates the levels of Sirtuins, including SIRT1 and SIRT3 [11]. While high levels of NAD upregulates the levels of Sirtuins, high levels of NADH do the opposite. Our experimental data showed that the NAD^+/NADH ratio and the levels of SIRT1 and SIRT3 activity were decreased in MCs treated with HG (Fig. 2 and Fig. 3), but NAD suppressed the declines (Fig. 2 and Fig. 3).

Hypertrophy could be cell cycle-dependent or cell cycle-independent [19]. Cell cycle independent hypertrophy may be caused by reduced protein degradation due to an inhibition of various proteases [20]. The concept that mesangial cell hypertrophy is rather an active cell cycle-dependent process comes from studies in which murine mesangial cells were exposed to high D-

glucose concentrations. Raising the medium glucose concentrations from 0.56 to 2.5 mM has a biphasic effect on cell proliferation. It stimulates an early limited proliferation after 24 h. However, prolonged exposure to high glucose inhibits mesangial cell proliferation and induces hypertrophy [21]. A similar biphasic growth response of mesangial cells has also been observed in experimentally induced diabetes *in vivo* [22]. Cell cycle analysis revealed that prolonged incubation in high glucose arrests cells in the G1-phase of the cell cycle [19]. In this study, we also confirmed that HG induces cell cycle arrest. However, NAD attenuated cell cycle arrest in response to HG (Fig. 4).

Cell hypertrophy could be defined as cell enlargement due to an increase in protein synthesis without concomitant changes in DNA synthesis [3]. This increase in protein synthesis also correlates well with hypertrophy measured as a ratio of total protein to cell number [23]. Otherwise, the mean cell size was analyzed by measurement of light scatter parameters: forward scatter (FSC) using flow cytometry [14]. FSC is related to the cell size and the optical refraction index of the outer membrane of the cell. We routinely observed that HG induced mesangial cell hypertrophy, manifested as an increase in ratio of total protein to cell number and FSC, meanwhile NAD suppressed the HG-induced cell hypertrophy (Table 1).

So, these data indicated that HG-induced mesangial hypertrophy was associated with depletion of cellular NAD levels, and supplementation of NAD should be able to rescue cells from HG-induced cell hypertrophy.

Fig. 5. Expression of AMPK-mTOR pathway by HG-induced hypertrophy in MCs. MCs were cultured for 48h in normal- or high-glucose medium with or without NAD. (A) Western blot of AMPK-mTOR pathway. (B) Quantitative protein level of AMPK-mTOR pathway. Data were expressed as relative percentages versus actin, and groups were compared using ANOVA. Values are means $\pm$ SD (n=3). * P <0.05 versus Control or Osmotic control. ^ P <0.05 versus High glucose.

Fig. 6. Inhibition of AMPK reverses the effects of NAD on Sirtuins-AMPK-mTOR pathway in MCs. MCs were transfected with a mixture of siRNAs targeting AMPK or luciferase (negative control, NC), and cultured for 48h in high-glucose medium with or without NAD. (A) Western blot of Sirtuins-AMPK-mTOR pathway. (B) Quantitative protein level of Sirtuins-AMPK-mTOR pathway. Data were expressed as relative percentages versus actin, and groups were compared using ANOVA. Values are means $\pm$ SD (n=3). * P <0.05 versus HG group. ^ P <0.05 versus HG+NAD group.

NAD treatment blocks mesangial hypertrophy via activation of the AMPK-mTOR signaling pathway

Glucose is a metabolic fuel that regulates energy status of the cell. States of energy depletion lead to the activation of AMP-activated protein kinase (AMPK), resulting in inhibition of energy-consuming pathways [24] and activation of ATP generating processes [25]. Cell hypertrophy entails an increased rate of protein synthesis, which accounts for a significant part of cell energy

consumption. In addition, it has been shown that glucose deprivation, through use of a nonhydrolyzable glucose analog (2-deoxyglucose), results in decreased mTOR signaling [26]. It is currently suggested that mTOR functions as a sensor of cellular energy state through input from the AMPK pathway [27]. We therefore focused our attention on the involvement of the AMPK-mTOR signaling pathway in NAD-mediated mesangial cells protection. The results showed that AMPK activation (phosphorylation) was reduced substantially in HG-

stimulated cells, but not when HG stimulation was given together with NAD (Fig. 5). AMPK activation is also known to negatively regulate the activity of PKB/Akt [28, 29], an upstream kinase of mTOR. Contrary to the finding for AMPK, we found increased phosphorylation of Akt and of mTOR in HG-stimulated cells, but not in NAD-treated cells (Fig. 5). These results indicated that NAD prevented the development of mesangial hypertrophy via activation of the anti-hypertrophic mechanism by augmenting the activity of the AMPK-mTOR signaling pathway.

Inhibition of AMPK reverses the effects of NAD on HG-induced hypertrophy in mesangial cells

To explore the role of AMPK-mTOR pathway on the effect of NAD on high glucose-induced mesangial hypertrophy, siRNA was used to infect MCs to shut down AMPK expression. As shown in Figure 6, endogenous AMPK expression in mesangial cells infected with AMPK siRNA was remarkably suppressed compared with those infected with control siRNA. Notably, the knockdown of AMPK downregulated the phosphorylation of AMPK and upregulated phosphorylation of mTOR and its downstream targets, nevertheless the expression of SIRT1 and SIRT3 were no change (Fig. 6). After stimulation with HG and treatment with NAD, the expression of p-AMPK was increased (Fig. 6) and mesangial hypertrophy relief (Table 1). It is worthy of note that AMPK knockdown abolished these effect (Fig. 6, Fig. 7 and Table 2). NAD inhibited HG-induced mesangial hypertrophy whereas NAD lost its inhibitory effect in the presence of AMPK siRNA. These data strongly suggest that AMPK pathway is necessary for the inhibiting effect of NAD on HG-induced hypertrophy in mesangial cells.

It has been reported by numerous studies that AMPK activation limits oxidative stress and ROS production. This indicates that AMPK activation could also contribute to NAD effects on ROS levels in response to high glucose. However, We also found ROS production decreased by NAD in the presence of AMPK siRNA (Fig. 8). This experiment has demonstrated that NAD effects partly could be mediated on anti-oxidant properties of Sirtuins.

Discussion

This study was designed to investigate the effects of exogenous supplementation of NAD in the development of mesangial hypertrophy. By using *in vitro*

Fig. 7. Down-regulation of AMPK by siRNA reverses the effects of NAD on relief in cell cycle arrested. HG inhibited cell cycle progression, while NAD could block the effect, whereas NAD lost its inhibitory effect in the presence of AMPK siRNA. Cell cycle analyses were performed by flow cytometry, and G0/G1, S and G2/M populations were indicated aside each histogram. The data shown are from one representative experiment from a series of three with similar results.

Fig. 8. ROS production decreased by NAD in the presence of AMPK siRNA. (A) MCs were transfected with a mixture of siRNAs targeting AMPK or luciferase (negative control, NC), and cultured for 48h in high-glucose medium with or without NAD. Cells were stained with CM-H₂DCFDA. ROS (reactive oxygen species) production was measured by differential interface contrast microscopy (600x). (B) Quantification of mean fluorescence intensity in different groups of cells. *P<0.05 versus HG group. ▲*P*<0.05 versus HG+NAD group. Values are mean ± SE of 3 experiments.

models of hypertrophy, we showed that mesangial hypertrophy is associated with depletion of cellular NAD levels. NAD treatment restored the cellular NAD levels and blocked the HG-mediated mesangial hypertrophic response. We also identified a new substrate of Sirtuins. Sirtuins deacetylase activity is dependent upon NAD, and NAD depletion in agonist-induced cardiac hypertrophy would therefore be expected to decrease Sirtuins activity, however, NAD treatment restored the cellular NAD levels, and maintained the activity of AMPK [30]. AMPK has been implicated in the activation of SIRT1 by enhancing the intracellular NAD⁺/NADH ratio via activation of Nampt [31]. Because increased intracellular NAD⁺/NADH ratio also activates SIRT3 [32], it is likely that stabilization of AMPK influences the activity of SIRT3 as well. The upstream kinase of AMPK is LKB1, which is known to be activated by SIRT1-mediated deacetylation in HEK293 cells [33]. Studies have demonstrated that LKB1 is activated by SIRT3 in the heart [30]. However, mTOR functions as a sensor of cellular energy state through input from the AMPK pathway [27]. Thus, Sirtuins is upstream of AMPK, followed by mTOR. We conclude that increased glucose concentration reduces the level of Sirtuins activity, resulting in decreased AMPK activation and increased Akt activation, consequently increased mTOR activation, which induces hypertrophy of mesangial cells. Although Several studies have demonstrated that Sirtuins were upstream of AMPK [34, 35]. Some publications indicated that AMPK seems to influence SIRT1 activity through an AMPK-induced modulation of NAD⁺ metabolites [31, 36]. For example, pharmacological or physiological activation of AMPK is followed by a robust increase in NAD⁺ within hours, which derives from the metabolic rearrangements promoted by an increase in fatty acid oxidation rates [31]. Otherwise, a recent study has also mentioned that AMPK and SIRT1 both regulate each other and share many common target molecules [37]. It was so complicated, while the relationship between Sirtuins and AMPK need more studies in the future. However, activating Sirtuins by NAD prevented a hypertrophic response by shutting down the major signaling pathways that induce mesangial hypertrophy. Therefore, experiments carried out to deduce that activating Sirtuins by NAD blocks mesangial hypertrophy by maintaining the activity of the anti-hypertrophic AMPK signaling pathway, and by shutting down the pro-hypertrophic mTOR signaling, thereby reducing the cellular protein synthesis.

Protein synthesis consumes a significant amount of cellular energy [27]. Induction of protein synthesis by

glucose, a source of energy, is likely to involve regulation of energy sensors such as AMPK. AMPK is a heterotrimeric protein consisting of a combination of $\alpha_{1/2}$ -, $\beta_{1/2}$ -, and $\gamma_{1/2/3}$ -subunits [38], with the α_1 - rather than α_2 -subunit expressed in the kidney [39]. Changes in the cellular energy state activate AMPK through various mechanisms involving allosteric regulation of AMPK, activation by an upstream AMPK kinase, and diminished activity of phosphatases [40]. AMPK activation increases glucose uptake and fatty acid oxidation [41] as well as up-regulates expression of various metabolic genes (e.g. the glucose transporter, GLUT4, uncoupling protein-3, and cytochrome c) [42-44]. Consequently AMPK serves as a sensor/modulator of intermediary metabolism by directing cellular events to increase energy availability and sustain high energy phosphate levels.

To date, numerous studies have shown that mTOR signaling pathway has a wide range of functions including regulation of protein synthesis, cell proliferation, hypertrophy, apoptosis, autophagy and senescence [45, 46]. Studies demonstrated that increased AMPK activity would diminish translation initiation and attenuate protein synthesis through down-regulated mTOR signaling [47]. Inoki [27] identified that, under low cellular energy conditions, activated AMPK phosphorylates TSC2 on Thr1227 and Ser1345 residues (these residues correspond to Thr1271 and Ser1387, respectively, in human TSC2) and enhances its inhibitory function leading to decreased mTOR activity. This research group has also reported that AMPK-dependent phosphorylation of TSC2 on Ser1387 primes TSC2 for further phosphorylation by GSK-3 on multiple residues. This series of phosphorylation steps leads to subsequent inhibition of mTOR activity under energy-deprived conditions [48]. Additionally, a recent study [49] reported that AMPK can directly phosphorylate the mTOR binding partner Raptor on two well-conserved serine residues (Ser722/Ser792), and Raptor phosphorylation is required to inhibit mTOR activity through cellular energy stress-induced AMPK activation. Consistent with these observations, it was demonstrated that activation of AMPK, by treatment with 5-aminoimidazole-4-carboxamide-1- β -D-ribose nucleoside (AICAR), results in decreased protein synthesis and a repression of mTOR-mediated signaling in high glucose induced renal hypertrophy [50]. Findings in this report extend previous observations in which AMPK-mTOR pathway normally inhibits protein synthesis and cell hypertrophy and that the suppression appears to mediate high-glucose-induced protein synthesis and mesangial hypertrophy. Studies also show that

the NAD inhibited HG-induced mesangial hypertrophy whereas NAD lost its inhibitory effect in the presence of AMPK siRNA. These data are available to indicate the AMPK pathway is necessary for the inhibiting effect of NAD on HG-induced hypertrophy in mesangial cells.

Another pathway which should be also considered involved in the anti-hypertrophic effects of NAD is the ability of NAD to reduce cellular ROS levels. Many previous studies have shown that reduction of cellular ROS levels is necessary for prevention of the mesangial hypertrophic response. Reduced cellular ROS levels after NAD treatment could be also related to activation of Sirtuins. Scientists have recently shown that SIRT3 activation induces the expression of antioxidants, MnSOD and catalase by enhancing the activity of Foxo3a. Increased activity of antioxidants reduces cellular ROS levels and thereby blocking the activity of Ras and Ras-mediated activation of mTOR signaling [10]. We also found ROS production decreased by NAD in the presence of AMPK siRNA. These findings thus indicate that NAD can block cell hypertrophy by at least two ways, first by stabilization

of the activity of AMPK-mTOR pathway, and second by curtailing the cellular ROS levels by NAD-mediated activation of antioxidants.

In summary, we demonstrated that exogenous NAD can block high glucose-induced mesangial hypertrophy via activation of the AMPK-mTOR pathway. Because oxidative stress is a common cause of many diseases, such as diabetic nephropathy, it is likely that diabetic nephropathy is associated with depletion of cellular NAD levels. If so, then as demonstrated in this study, NAD treatment might be a potential target for treating diabetic nephropathy in the future.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program) (2007CB507400), a grant from the National Natural Science Foundation of China (30771014, 81070267), and Postdoctoral Science Foundation of China (20090450200).

References

- Lehmann R, Schleicher ED: Molecular mechanism of diabetic nephropathy. *Clin Chim Acta* 2000;297:135-144.
- Mason RM, Wahab NA: Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol* 2003;14:1358-1373.
- Wolf G, Ziyadeh FN: Molecular mechanisms of diabetic renal hypertrophy. *Kidney Int* 1999;56:393-405.
- Pillai JB, Isbatan A, Imai S, Gupta MP: Poly(adp-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by nad⁺ depletion and reduced sir2alpha deacetylase activity. *J Biol Chem* 2005;280:43121-43130.
- Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X, Tian B, Wagner T, Vatner SE, Sadoshima J: Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007;100:1512-1521.
- Hasegawa K, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, Sueyasu K, Washida N, Tokuyama H, Tzukurman M, Skorecki K, Hayashi K, Itoh H: Kidney-specific overexpression of sirt1 protects against acute kidney injury by retaining peroxisome function. *J Biol Chem* 2010;285:13045-13056.
- Kume S, Haneda M, Kanasaki K, Sugimoto T, Araki S, Isono M, Isshiki K, Uzu T, Kashiwagi A, Koya D: Silent information regulator 2 (sirt1) attenuates oxidative stress-induced mesangial cell apoptosis via p53 deacetylation. *Free Radic Biol Med* 2006;40:2175-2182.
- Bao J, Lu Z, Joseph JJ, Carabenciov D, Dimond CC, Pang L, Samsel L, McCoy JP Jr, Leclerc J, Nguyen P, Gius D, Sack MN: Characterization of the murine sirt3 mitochondrial localization sequence and comparison of mitochondrial enrichment and deacetylase activity of long and short sirt3 isoforms. *J Cell Biochem* 2010;110:238-247.
- Shi T, Wang F, Stieren E, Tong Q: Sirt3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem* 2005;280:13560-13567.
- Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP: Sirt3 blocks the cardiac hypertrophic response by augmenting foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009;119:2758-2771.
- Lin SJ, Ford E, Haigis M, Liszt G, Guarente L: Calorie restriction extends yeast life span by lowering the level of nadh. *Genes Dev* 2004;18:12-16.
- Ying W, Wei G, Wang D, Wang Q, Tang X, Shi J, Zhang P, Lu H: Intranasal administration with nad⁺ profoundly decreases brain injury in a rat model of transient focal ischemia. *Front Biosci* 2007;12:2728-2734.
- Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS, Huber LJ: Inhibition of sirt1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* 2006;26:28-38.
- Yitzhaki S, Barnea A, Keysary A, Zahavy E: New approach for serological testing for leptospirosis by using detection of leptospira agglutination by flow cytometry light scatter analysis. *J Clin Microbiol* 2004;42:1680-1685.
- Nagai K, Matsubara T, Mima A, Sumi E, Kanamori H, Iehara N, Fukatsu A, Yanagita M, Nakano T, Ishimoto Y, Kita T, Doi T, Arai H: Gas6 induces akt/mtor-mediated mesangial hypertrophy in diabetic nephropathy. *Kidney Int* 2005;68:552-561.

- 16 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 2001;25:402-408.
- 17 Ohtake K, Ishiyama Y, Uchida H, Muraki E, Kobayashi J: Dietary nitrite inhibits early glomerular injury in streptozotocin-induced diabetic nephropathy in rats. *Nitric Oxide* 2007;17:75-81.
- 18 Jaimes EA, Tian RX, Pearce D, Raij L: Up-regulation of glomerular cox-2 by angiotensin ii: Role of reactive oxygen species. *Kidney Int* 2005;68:2143-2153.
- 19 Shankland SJ, Wolf G: Cell cycle regulatory proteins in renal disease: Role in hypertrophy, proliferation, and apoptosis. *Am J Physiol Renal Physiol* 2000;278:F515-529.
- 20 Franch HA: Pathways of proteolysis affecting renal cell growth. *Curr Opin Nephrol Hypertens* 2002;11:445-450.
- 21 Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadeh FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine tgf-beta. *Kidney Int* 1992;42:647-656.
- 22 Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J, Couser WG, Seidel K: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 1995;47:935-944.
- 23 Mahaimanathan L, Das F, Venkatesan B, Choudhury GG: Mesangial cell hypertrophy by high glucose is mediated by downregulation of the tumor suppressor pten. *Diabetes* 2006;55:2115-2125.
- 24 Viollet B, Andreelli F, Jorgensen SB, Perrin C, Flamez D, Mu J, Wojtaszewski JF, Schuit FC, Birnbaum M, Richter E, Burcelin R, Vaulont S: Physiological role of amp-activated protein kinase (ampk): Insights from knockout mouse models. *Biochem Soc Trans* 2003;31:216-219.
- 25 Hardie DG: The amp-activated protein kinase pathway-new players upstream and downstream. *J Cell Sci* 2004;117:5479-5487.
- 26 Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G: Mammalian tor: A homeostatic atp sensor. *Science* 2001;294:1102-1105.
- 27 Inoki K, Zhu T, Guan KL: Tsc2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577-590.
- 28 Chan AY, Dolinsky VW, Soltys CL, Viollet B, Baksh S, Light PE, Dyck JR: Resveratrol inhibits cardiac hypertrophy via amp-activated protein kinase and akt. *J Biol Chem* 2008;283:24194-24201.
- 29 Cheng SW, Fryer LG, Carling D, Shepherd PR: Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *J Biol Chem* 2004;279:15719-15722.
- 30 Pillai VB, Sundaresan NR, Kim G, Gupta M, Rajamohan SB, Pillai JB, Samant S, Ravindra PV, Isbatan A, Gupta MP: Exogenous nad blocks cardiac hypertrophic response via activation of the sirt3-lkb1-amp-activated kinase pathway. *J Biol Chem* 2010;285:3133-3144.
- 31 Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J: Ampk regulates energy expenditure by modulating nad⁺ metabolism and sirt1 activity. *Nature* 2009;458:1056-1060.
- 32 Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A, de Cabo R, Sauve AA, Sinclair DA: Nutrient-sensitive mitochondrial nad⁺ levels dictate cell survival. *Cell* 2007;130:1095-1107.
- 33 Lan F, Cacicedo JM, Ruderman N, Ido Y: Sirt1 modulation of the acetylation status, cytosolic localization, and activity of lkb1. Possible role in amp-activated protein kinase activation. *J Biol Chem* 2008;283:27628-27635.
- 34 Lin JN, Lin VC, Rau KM, Shieh PC, Kuo DH, Shieh JC, Chen WJ, Tsai SC, Way TD: Resveratrol modulates tumor cell proliferation and protein translation via sirt1-dependent ampk activation. *J Agric Food Chem* 2010;58:1584-1592.
- 35 Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M, Verbeuren TJ, Cohen RA, Zang M: Sirt1 regulates hepatocyte lipid metabolism through activating amp-activated protein kinase. *J Biol Chem* 2008;283:20015-20026.
- 36 Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, Sartorelli V: Glucose restriction inhibits skeletal myoblast differentiation by activating sirt1 through ampk-mediated regulation of nampt. *Dev Cell* 2008;14:661-673.
- 37 Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y: Ampk and sirt1: A long-standing partnership? *Am J Physiol Endocrinol Metab* 2010;298:E751-760.
- 38 Carling D: The amp-activated protein kinase cascade-a unifying system for energy control. *Trends Biochem Sci* 2004;29:18-24.
- 39 Fraser S, Mount P, Hill R, Levidiotis V, Katsis F, Stapleton D, Kemp BE, Power DA: Regulation of the energy sensor amp-activated protein kinase in the kidney by dietary salt intake and osmolality. *Am J Physiol Renal Physiol* 2005;288:F578-586.
- 40 Hardie DG, Carling D, Carlson M: The amp-activated/snfl protein kinase subfamily: Metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 1998;67:821-855.
- 41 Merrill GF, Kurth EJ, Hardie DG, Winder WW: Aica riboside increases amp-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* 1997;273:E1107-1112.
- 42 Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL: Regulation of muscle glut-4 transcription by amp-activated protein kinase. *J Appl Physiol* 2001;91:1073-1083.
- 43 Zhou M, Lin BZ, Coughlin S, Vallega G, Pilch PF: Ucp-3 expression in skeletal muscle: Effects of exercise, hypoxia, and amp-activated protein kinase. *Am J Physiol Endocrinol Metab* 2000;279:E622-629.
- 44 Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI: Chronic activation of amp kinase results in nrf-1 activation and mitochondrial biogenesis. *Am J Physiol Endocrinol Metab* 2001;281:E1340-1346.
- 45 Wullschlegel S, Loewith R, Hall MN: Tor signaling in growth and metabolism. *Cell* 2006;124:471-484.
- 46 Zhuo L, Cai G, Liu F, Fu B, Liu W, Hong Q, Ma Q, Peng Y, Wang J, Chen X: Expression and mechanism of mammalian target of rapamycin in age-related renal cell senescence and organ aging. *Mech Ageing Dev* 2009;130:700-708.
- 47 Bolster DR, Crozier SJ, Kimball SR, Jefferson LS: Amp-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem* 2002;277:23977-23980.
- 48 Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL: Tsc2 integrates wnt and energy signals via a coordinated phosphorylation by ampk and gsk3 to regulate cell growth. *Cell* 2006;126:955-968.
- 49 Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ: Ampk phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* 2008;30:214-226.
- 50 Lee MJ, Feliars D, Mariappan MM, Sataranatarajan K, Mahaimanathan L, Musi N, Foretz M, Viollet B, Weinberg JM, Choudhury GG, Kasinath BS: A role for amp-activated protein kinase in diabetes-induced renal hypertrophy. *Am J Physiol Renal Physiol* 2007;292:F617-627.