Anti-Inflammatory Effects of Atorvastatin by the Modulation of NF-κB Expression during Hyperglycemia-Induced Nephropathy in Rat

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Abstract

Objective: Atorvastatin has the pleiotropic effects, including anti-inflammation and antioxidant. Therefore, this study considered to examine the effects of atorvastatin on NF-κB expression, as a main transcription factor for expression of inflammatory cytokines, in hyperglycemia-induced nephropathy in rat.

Materials and Methods: Twenty four male Wistar rats were randomly divided into four groups; Normal, Normal treatment, Diabetic, and Diabetic treatment. Rats were made diabetic by an intravenous injection of streptozotocin (40 mg/kg). Treated rats received atorvastatin for 60 days (40 mg/kg/day). At the end of experiment, blood samples were collected for measurement of blood glucose. Moreover, the mRNA expression level of NF-κB in kidney was determined by RT-PCR technique.

Results: Induction of diabetes significantly increased the mean value of blood glucose in diabetic rats (>450 mg/dl) compared with normal rats (P=0.001). Chronic hyperglycemia also increased the mRNA expression level of NF-κB in diabetic kidney. Moreover, the mean value of kidney index was significantly increased in diabetic rats compared to normal group (P=0.001). Treatment with atorvastatin in diabetic rats for 60 days reduced the mRNA expression level of NF-κB and kidney index compared to non-treated diabetic rats (P=0.014).

Conclusion: Our findings revealed that atorvastatin is able to prevent the development of diabetic nephropathy during chronic uncontrolled hyperglycemia possibly by the inhibition of NF-κB expression in the kidneys of diabetic rats.

Keywords: Nephropathy, Atorvastatin, Hyperglycemia, NF-κB.

Introduction

Diabetic nephropathy is one of the most severe microvascular complications of diabetes. Recent studies reported the prevalence of chronic kidney disease (CKD) and end stage renal disease (ESRD) are increasing in diabetic population (1). Diabetic nephropathy stages are characterized as, hyper-filtration and an increase in urinary albumin excretion, morphological abnormalities of kidney with glomerular basement membrane (GBM) thickening, inflammation and mesangial expansion, the third stage is macro-albuminuria, which is considered as developed nephropathy (2,3).

Several studies indicated, inflammation and inflammatory cells could be involved in the progression of diabetic nephropathy (4-6). Chronic hyperglycemia induces cytokines and chemokines overexpression in kidney cells especially epithelial and mesangial cells, which develop kidney injury by glomerular basement membrane thinking, proteinuria and the kidney tissue fibrosis (7). NF-κB is an important transcription factor. NF-κB is involved in development of diabetic nephropathy. Chronic hyperglycemia, reactive oxygen species (ROS), cytokines and various stimuli activate NF-κB. It triggers the inhibitors of NF-κB (IκBs) degradation. Then, NF-κB enters to nucleus and activates NF-κB-dependent genes (4,8). Clinical and experimental studies have suggested that NF-κB activation contributes to intercellular adhesion molecule-1 (ICAM-1) and cytokines overexpression that promote the progression of diabetic nephropathy (6,9).

Recent studies showed 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are helpful in some pathological situations independent of lipid-lowering effects. These drugs are widely used in treatment of dyslipidemia (10). These drugs have anti-inflammation, antioxidant and anti-thrombotic effects (11-13). Anti-inflammation is one of the major pleotropic effects of statins in diabetic nephropathy (14,15). It is reported that rousvastatin has renoprotective effects by reduction of inflammation. It prevents renal morphological alterations during chronic hyperglycemia (16). Moreover, the findings in patients with chronic kidney diseases showed diminished inflammatory markers and improved renal function in patients who were treated with statins (17). Also clinical findings suggest that statins decrease inflammation, which have many beneficial effects on stroke (18). Several findings have indicated that statins can prevent the over expression of pro-inflammatory chemokines and protect the chronic hyperglycemia kidney injuries (15,19,20).

In the present study we aimed to examine the protective effects of atorvastatin on NF-κB expression, as a main transcription factor for expression of the several inflammatory cytokines, in kidney during hyperglycemia-induced nephropathy in rat.

Materials and Methods

Animal

Male healthy Wistar rats (5-6 weeks old, 200-250 g) were obtained from Center Animal House Facility of Pasteur Institute of Iran (Tehran, Iran). All protocols of the study were approved by institutional Animal Ethics Committee of Science and research Azad University (Tehran, Iran). Rats were housed in standard cages with controlled temperature (22-24°C), humidity (40-60%) and light period (07.00-19.00), while having full access to food (rat chow, Parsdam; Tehran, Iran) and water.

Induction of diabetes

After one week diabetes was induced by a single intravenous injection of streptozotocin (STZ; 40 mg/kg dissolved in normal saline, Sigma, USA) through the lateral tail vein. Non-diabetic rats received the same volume of normal saline. Diabetes was confirmed by determination of high blood glucose level in accompany with polydipsia and polyuria on the 5th day after STZ administration. Rats with blood glucose level above 450 mg/dl were selected as diabetic animals, which were housed in the same room but in separate cages (1 per cage).

Experimental protocol

Twenty four male Wistar rats were randomly divided into four groups in equal numbers (n=6). The first group considered as normal (N), which during the study did not receive any treatments. The second group considered as atorvastatin-treated group (treatment with atorvastatin) (NT), which received daily atorvastatin (40 mg/kg, Hakim Co, Iran) by gavage for 60 days. The third group used as control diabetic (received STZ; 40 mg/kg) (D)

and did not receive any treatment. The fourth group served as atorvastatin-treated diabetic (DT) rats that received atorvastatin orally (40 mg/kg/day) after induction of diabetes by STZ (40 mg/kg). Atorvastatin administration started in 5th day of STZ injection and continued for 60 days.

Blood samples (500 µL) were collected from the tip of snipped tail at day 5 after STZ injection. At the end of 60 days, rats were sacrificed and blood samples were prepared for centrifugation (4500 ×g), and then, serums stored in freezer (-80 ˚C) for assessment of plasma glucose. The plasma glucose was measured by commercial Kit (Pars Azmoon, Iran, Tehran). At the end of experiment (60 days), all rats were anesthetized by intraperitoneal injection of 80mg/kg ketamine and 10 mg/kg xylazine. At the end, kidney weight was measured accurately. The left kidneys were removed and snap-frozen in liquid nitrogen and stored in -80˚C for further analysis.

Evaluation of gene expression of NF-κB

Gene expression of NF-κB was determined using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from 50 mg of left kidney tissue using the RNA extraction kit (Topagene Kavosh, Iran) according to the manufacturer's protocol. The quantity and quality of the extracted RNA samples were estimated by spectrophotometry at 260 and 280 nm. Complementary DNA (cDNA) was synthesized from 5 µg total RNA using the Revert Aid First Strand cDNA Synthesis Kit (BIONEER, Korea). Expression of the β-actin housekeeping gene was used as the reference for the level of target gene expression. cDNA (2 µl) was amplified with PCR kit (BIONEER, Korea) according to the manufacturer's protocol. Also, appropriate primers were used for NF-κB (Forward: 5'-CTGCTTTGACTCA CTCCA-3' Reverse: 5'-GACTGCGATAC CTTAATGA-3') and β-Actin (Forward: 5'-CCACACCCGCCACCAGTTCG-3' and Reverse: 5'-CTAGGGCGGCCCACGATGG A-3') genes. The products of PCR-amplified samples were visualized on 1.5% agarose gel using ethidium bromide. The gel images were digitized by using the Gel Doc (Kiagene, Iran), and the images of the stained sections were also taken.

Histological assessment

At the end of the experiment, animals were sacrificed under deep anesthesia. The kidneys were removed and fixed in formalin (10%) for two weeks. After fixation and tissue processing, coronal serial sections (5µm in thickness) were prepared for conventional histological examination. Paraffin embedded sectioning (each 50µm intervals) was processed routinely for hematoxylin and eosin (H&E) staining. After staining procedure, sections were dehydrated with administration of 70, 80, 96, 100, and 100% ethanol, respectively. The samples were placed in xylene solution for two times (each time was 15 min) owing to clearing. Due to mounting, the samples were covered with entelan sticker and then lamels were placed on them. The histological changes were observed through a light microscope (Nikon, Japan) connected to digital camera (CMEX, Holland) for capturing the photograph

Kidney index

After removing the kidneys under deep anesthesia, all kidneys were weighed in accompany with body weight. Then, the kidney weight index was calculated as follows;

[kidney weight (g)/body weight (g)]×100.

Statistical analysis

All Data were presented as mean±standard error of mean (SEM). Descriptive statistics were calculated for all demographical, Kolmogrov Smirnov test was shown normal distribution of data, so paired t-test was used to determine the differences between data at beginning and termination of tests in each group, and one-way variance (ANOVA) and Tukey Post-Hoc test was used to compare the

data between all groups. Statistical significance was set at $P<0.05$. SPSS (V.21) was used for statistical analysis.

Results

Effect of atorvastatin on plasma glucose

Plasma glucose of normal rats was <200 mg/dl. Treatment with atorvastatin did not change the plasma glucose of normal animals ($P=0.996$). Five days after STZ injection, plasma glucose of diabetic (589±41 mg/dl) and diabetic treated (487±19 mg/dl) groups were elevated compared to normal rats ($P=0.001$). Atorvastatin did not change the blood glucose of diabetic treated rats after 60 days treatment compared to diabetic untreated rats ($P=0.357$), (Table 1).

Effects of atorvastatin on the kidney weight index

Table 1 shows the left kidney index of all groups. The value of left kidney index in normal group was 0.3±0.01. The kidney index of normal treated rats with atorvastatin was 0.36±0.02. There was no significant difference in the kidney index of normal and normal treated groups ($P=0.713$). However, untreated diabetic group showed an increase in the value of kidney index (0.56±0.02) compared to normal group ($P=0.001$). Atorvastatin decreased the value of kidney index in diabetic treated rats (0.47±0.01) compared to diabetic non-treated group ($P=0.014$).

Effect of atorvastatin on NF-κB gene expression

Figure 1 indicates the quantitative analysis of NF-κB gene expression based on bands densitometry, which were formed on agarose gel and were analyzed with Image J software. The mRNA level of NF-κB gene significantly increased in diabetic group compared with normal and normal treated groups ($P=<0.05$). However, in diabetic treated group, atorvastatin prevented the enhancement of mRNA level of NF-κB. It means that in diabetic treated group, NF-κB gene expression was significantly decreased compared to non-treated diabetic group ($P=<0.05$).

Effects of atorvastatin on histological changes

As demonstrated in figure 2, the glomeruli are in normal size and states in normal and normal treatment rats. In histopathological assessments, we didn't see any morphological damages in the kidneys of normal and normal treatment groups. The destroyed glomeruli, glomerular sclerosis and atrophy were clearly observed in the kidneys of diabetic rats. However, these morphological changes were not observed in the kidneys of diabetic treated rats with atorvastatin.

Discussion

Chronic hyperglycemia initiates several degenerative cascades that promote the renal damages during diabetic nephropathy. In this study, chronic hyperglycemia increased the NF-κB expression in the kidney of diabetic rats that accompanied with the kidney index increases. Administration of atorvastatin during the experiment in diabetic treated rats could decrease the gene expression of NF-κB concomitant decrease in the kidney index.

Streptozotocin destroys the β-cell of pancreas and decreases insulin secretion, which induces hyperglycemia (21). In the present study, induction of diabetes induced chronic

Table 1. The mean values of blood glucose and kidney index at the end of experiment

Parameters \ Groups	Normal	Normal + Atorvastatin	Diabetes	Diabetes + Atorvastatin
Plasma glucose at day 5(mg/dl)	182±24	127±6	589±41*	487±19*
Plasma glucose at day 60 (mg/dl)	188±14	195±19	559±35*	526±15*
Left kidney index	0.31±0.01	0.36±0.02	0.56±0.02*	0.47±0.01*[#]

All values are presented as Mean±SEM.

*As significant difference compared to normal groups ($P=0.001$)

[#] As significant difference compared to diabetes group ($P=0.014$)

uncontrolled hyperglycemia. However, treatment with atorvastatin for two months could not decrease the blood glucose of diabetic rats (Fig. 1). There are several controversial studies about the actions of statins on blood glucose level. Several in vivo studies demonstrated that statins interfere in glucose metabolism. These drugs decrease the GLUT4 expression and therefore decrease insulin sensitivity (22-24). Furthermore, the findings of one study suggest that statins could inhibit insulin secretion by inhibition of Ca^{2+} signaling in pancreatic β-cells (25). On the other hands, the findings of recent studies showed atorvastatin has protective effects on β-cell, which is related to increased pancreas proliferation in obese mice (26). Also, atorvastatin could improve insulin sensitivity in both lean and fatty rats (27). In the present study, atorvastatin administration did not change the blood glucose of diabetic rats maybe due to the severity of diabetes.

The gene expression of NF-κB increased following the diabetes in our study. NF-κB is the major transcription factor involved in pathophysiology of diabetic nephropathy, which is activated by many stimuli such as inflammatory cytokines and oxidants in renal tubular cells. This transcription factor induces the transcription of many genes including CCL2 (C-C chemokine C-C Motif ligand 2), CCL5 (Chemokine C-C Motif Ligand 5), nitric oxide synthase, and variety of other inflammatory genes that contribute to structural and functional abnormalities in diabetic kidneys. Inflammation and inflammatory pathways have a central role in progression of diabetic nephropathy. They are activated by metabolic and biochemical factors in diabetic kidney (28). Chronic hyperglycemia increases kidney production of chemokines, pro-inflammatory cytokines and adhesion molecules, which are associated with renal injury. These inflammatory cascades induce the activation of signal transduction systems in kidney cells including endothelial, mesangial and tubular cells. These adverse abnormalities promote diabetic nephropathy in diabetic patients and in animal models (4,29,30).

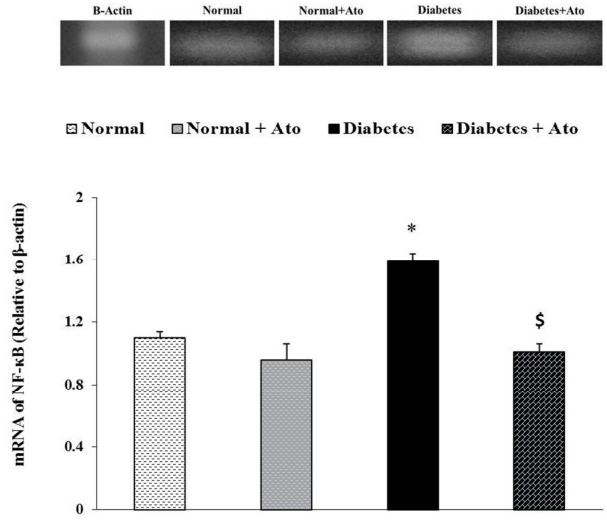

Figure 1. Changes of NF-κB gene expression at the end of experiment
All values are presented as mean±SEM.
***As significant difference compared to normal group (*P*<0.05)**
$ As significant difference compared to diabetic group (*P*<0.05)

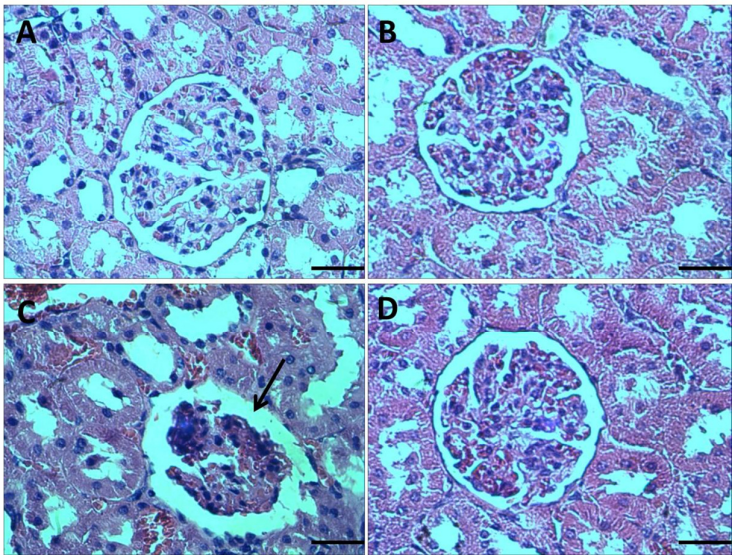

Figure 2. Micrographs (H&E staining) show the histopathological damages of kidney at the end of experiment in diabetic rats (C). Diabetic rats treated with atorvastatin (D) showed less morphological damages such as destroyed glomeruli compared to non-treated diabetic rats. Normal (A) and normal treated rats with atorvastatin (B) did not show morphological damages, (400X, Scal bar; 30 µm).

Several studies showed the beneficial effects of statins (pleiotropic effects) including anti-inflammation, reduction of oxidative stress and enhancing the stability of atherosclerotic plaques by reducing plaque size (31-33). In this study, atorvastatin administration for 60 days decreased the expression of NF-κB gene in renal tissue in diabetic rats. Sironi et al, reported that simvastatin has the anti-inflammatory property by reduction of NF-κB activation in cerebral ischemia (32). Moreover, the values of kidney index in diabetic treated group significantly reduced compared to diabetic group. Moreover, Ozbek et al, reported that atorvastatin prevented the translocation of NF-κB dimmers to nucleus by inhibition of IκB kinase activity in hindered renal damages (31). Additionally, the antioxidant effect of statins is one of the major pleiotropic actions of these drugs. Statins inhibit intracellular oxidative stress pathways by modulation of PKC pathway, which can slow the progression of renal failure. It has been shown that atorvastatin decreases the ROS overproduction by inhibition NF-κB activation. Also, chronic hyperglycemia triggers ROS overproduction, which can develop renal injury by degradation of IkB-alpha inhibitor and free NF-κB dimers translocation to the nucleus. Ultimately, NF-κB activates the target genes such as chemokines and adhesion molecules. It is suggested that atorvastatin inhibit NF-κB activation, directly or indirectly by ROS inhibition, which leads to attenuation of genes expression of chemokines and adhesion molecules (4,31,34,35).

Based on our findings, the kidney weight index significantly increased in diabetic rats. Recent findings have suggested that glomerular hypertrophy takes place in the early stages of diabetic nephropathy (36,37). According to previous findings, overproduction of several growth factors have been reported during diabetic nephropathy, including transforming growth factor (TGF)-beta 1, growth hormone (GH) and insulin like growth factor (IGFs), (36,37). On the other hand in the present study, atorvastatin decreased the kidney weight index during chronic hyperglycemia. Also, our finding revealed the reduction of NF-κB expression

during the hyperglycemia. Moreover, the anti-inflammatory functions of atorvastatin have been reported (38). Therefore, it is concluded that atorvastatin decreases the kidney weight index possibly by inhibition of NF-κB expression.

Conclusions

In conclusion, our study indicates that atorvastatin is able to prevent hyperglycemia-induced renal damages and diabetic nephropathy possibly through attenuation of NF-κB expression in renal tissue. It is suggested that reduction of NF-κB expression by atorvastatin decreases the kidney inflammation and inhibits the progression of diabetic nephropathy independent of plasma cholesterol or glucose alterations.

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