RESEARCH PAPER

Change in post-translational modifications of histone H3, heat-shock protein-27 and MAP kinase p38 expression by curcumin in streptozotocin-induced type I diabetic nephropathy

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Background and purpose: Curcumin has been used to treat cancer, diabetes and other pathologies. However, little is known regarding its role in altering post-translational modifications of histone H3. A recent report suggests that acute hyperglycaemia induces a global down-regulation of gene expression in human tissues and epigenetic regulation of gene expression could be a novel mechanism underlying the pathological processes of hyperglycaemia. The present study was undertaken to examine changes in histone modification by curcumin treatment which prevents development of type I diabetic nephropathy.

Experimental approach: Male Sprague-Dawley rats were rendered diabetic using a single dose of streptozotocin (55 mg kg^{-1}, i.p.). Diabetic nephropathy was assessed by measurements of blood urea nitrogen, albumin and creatinine levels. Post-translational modifications of histone H3, heat shock protein-27 (HSP-27) and mitogen-activated protein (MAP) kinase p38 expression were examined by western blotting.

Key results: Treatment of diabetic rats with curcumin significantly decreased blood urea nitrogen and creatinine and increased albumin; variables associated with the development of diabetic nephropathy. There were also increased levels of HSP-27 and MAP kinase (p38) in diabetic kidney. However, curcumin treatment prevented this increase in HSP-27 and p38 expression. Moreover, at nuclear level curcumin prevented the decrease in dephosphorylation and increases acetylation of histone H3.

Conclusions and implications: Our results suggested that protection against development of diabetic nephropathy by curcumin treatment involved changes in post-translational modifications of histone H3, expression of HSP-27 and MAP kinase p38 in diabetic kidney.

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Introduction

Diabetic nephropathy is one of the most serious complications of diabetes and the most common cause of end-stage renal failure. At present, diabetic kidney disease affects about 15–25% of type I diabetes patients (Hovind *et al.*, 2003) and 30–40% of patients with type II diabetes (Yokoyama *et al.*, 2000). Diabetic nephropathy is characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix proteins such as collagen, fibronectin and laminin. Diabetic nephropathy is characterized by proteinuria, a decline in renal function, glomerulosclerosis and interstitial fibrosis. Diabetic nephropathy is diagnosed on the basis of the extent of albumin protein excretion in urine (proteinuria). The usual daily limits of proteinuria are as follows: normal <30 mg, microalbuminuria 30–300 mg and macro-albuminuria >300 mg.

Persistent hyperglycaemia generates intracellular reactive oxygen species (ROS) in mesangial and tubular epithelial cells via protein kinase C, nicotinamide adenine dinucleotide phosphate oxidase and mitochondrial metabolism.

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Moreover, it also upregulates transforming growth factor-β1 (Oh *et al.*, 1998) and extracellular matrix expression (Ayo *et al.*, 1990; Ziyadeh *et al.*, 1994; Oh *et al.*, 1998) in the glomerular mesangial cells. Transforming growth factor-β1, advanced glycation end products and angiotensin II can also induce ROS and these further activate stress-activated signalling pathways in diabetic kidney. Stress-activated signalling pathways such as those of nuclear factor-κB, p38 mitogen-activated protein (MAP) kinase and Jun kinases underlie the development of diabetic complications (Evans *et al.*, 2002). It has also been reported that high glucose levels can activate the p38 MAP kinase pathway in many cell types, including renal cells (Adhikary *et al.*, 2004; Susztak *et al.*, 2006). There is significantly increased apoptosis in the tubular and interstitial cells during the course of progression of diabetic nephropathy (Zhang *et al.*, 1997; Kumar *et al.*, 2004).

Antioxidants effectively inhibit high-glucose- and H_2O_2-induced transforming growth factor-β1 and fibronectin upregulation, thus providing evidence that ROS play an important role in high glucose -induced renal injury (Ha and Lee, 2000; Iglesias-De La Cruz *et al.*, 2001). Among the spices, turmeric is used as flavouring and colouring agent in the Indian diet every day and is known to possess antioxidant properties (Sharma *et al.*, 2006b). Dietary supplementation with curcumin has been reported to have beneficial effects on diabetic nephropathy, and these have been attributed to its ability to lower blood cholesterol levels (Suresh Babu and Srinivasan, 1998). In addition, curcumin is also reported to prevent the acute renal failure and related oxidative stress caused by chronic administration of cyclosporine in rats (Tirkey *et al.*, 2005). Moreover, curcumin also has beneficial effects in retarding glomerulosclerosis caused by adriamycin (Venkatesan *et al.*, 2000).

On a molecular level, curcumin has been reported to cause histone hypoacetylation in human hepatoma cells, but its *in vivo* target molecules remain to be clarified. Recently, Meugnier *et al.* (2007) have shown that glucose toxicity induces a global downregulation of gene expression in humans. Moreover, a recent report suggests a role for histone deacetylase inhibitors in diabetic nephropathy (Lee *et al.*, 2007). In addition, several reports suggest the role of post-translational modification of histones in general and particularly histone deacetylases in regulating gene expression. However, little is known regarding the role of curcumin in modulating post-translational modifications of histone H3 in kidneys during type I diabetes. Hence, the present study was designed to elucidate the role of curcumin on histones and related molecules in streptozotocin (STZ)-induced type I diabetic nephropathy.

Materials and methods

Animal treatment
All the experiments were approved by the Institutional Animal Ethics Committee and complied with the NIH guidelines on handling of experimental animals. Experiments were performed on male Sprague–Dawley rats in the weight range of 240 to 260 g, which were procured from the central animal facility of the institute, kept under controlled environmental conditions at room temperature $22 \pm 2\,^\circ C$ and 12 h light/dark cycles. After 1 week of acclimatization, animals were randomly divided into two groups at the start of the experiment. In the first group, type I diabetes was induced as described previously (Tikoo *et al.*, 2007b). Briefly, diabetes was induced by injecting a single dose of STZ ($55\,mg\,kg^{-1}$, intraperitoneally, dissolved in ice-cold sodium citrate buffer (0.01 M, pH 4.4). Age-matched control rats received only sodium citrate buffer. Animals included in the study as diabetic animals had plasma glucose levels $>16.7\,mmol\,l^{-1}$ after 48 h post induction of diabetes. After 2 weeks, diabetic animals were divided into two groups, namely diabetic/control ($n=6$) and diabetic/treated with curcumin ($50\,mg\,kg^{-1}\,day^{-1}$, postoperatively, for 6 weeks, $n=6$). Along with these groups, there was one age-matched normal/control group ($n=6$) and one normal/curcumin-treated group ($n=6$). Treatment of curcumin was started from the third week and continued till the end of the eighth week (6-week treatment). Each animal in the control group received vehicle (1% sodium carboxymethyl cellulose) ($2\,ml\,kg^{-1}\,day^{-1}$, per oral).

Estimation of plasma glucose, albumin, blood urea nitrogen and creatinine
Blood samples were collected from the retro-orbital plexus of rats under mild ether anaesthesia, in heparinized centrifuge tubes and immediately centrifuged at $2300\,g$ for separation of plasma. Plasma was stored at $-80\,^\circ C$ until assays were performed. The plasma was used for the estimation of glucose, albumin, blood urea nitrogen (BUN) and creatinine as described previously (Tikoo *et al.*, 2007a, b).

Assessment of renal oxidative stress markers
Oxidative stress markers were measured as described previously (Tikoo *et al.*, 2007b). The lipid peroxide level in animal tissues was measured according to the method described by Ohkawa *et al.* (1979). Superoxide dismutase (SOD) activity was estimated according to the method described by Paoletti and Mocali (1990).

Protein isolation and western blotting
Nuclei, histone isolation and western blotting were performed as described previously (Tikoo *et al.*, 1997, 2007a, b). Immunoblot analysis was performed using anti-acetylated histone H3 (rabbit, 1:5000; Upstate, Lake Placid, NY, USA), anti-phosphorylated histone H3 Ser10 (rabbit, 1:2000), anti-histone H3 (rabbit, 1:5000, Upstate), anti-p38 (rabbit, 1:500), anti-heat-shock protein-27 (HSP-27) (rabbit, 1:500), anti-actin (rabbit, 1:2500; Sigma, St Louis, MO, USA) and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit) from Santa Cruz Biotechnology, Inc., Delaware Avenue, CA, USA. Proteins were detected with the enhanced chemiluminescence system and ECL Hyperfilm (Amersham Pharmacia Biotech, UK Ltd, Little Chalfont, Buckinghamshire, England).

Statistical analysis
Experimental values are expressed as mean ± s.e.mean. Comparison of mean values between various groups was performed by one-way analysis of variance followed by multiple comparisons by Tukey's test. P-value < 0.05 was considered significant.

Chemicals
All the chemicals were purchased from Sigma, unless otherwise mentioned.

Results

In this study, the STZ-treated diabetic rats developed uncontrolled type 1 insulin-dependent diabetes mellitus. All the rats had well-developed signs of diabetes after 2 weeks of STZ administration, that is, hyperglycaemia, glycosuria, polyuria, increased water consumption and weight loss.

Change in body weight and kidney weight/body weight ratio by curcumin treatment
Streptozotocin-induced diabetic animals showed significant decrease in body weight and increase in kidney weight. However, kidney weight/body weight ratio was doubled as compared with normal rats. Treatment of curcumin prevented body weight loss in diabetic rats as compared with untreated diabetic rats. In addition, it also reduced the increase in kidney weights in diabetic rats. Diabetic rats had increased kidney weight/body weight ratio, a marker for the development of diabetic nephropathy, and this ratio was significantly decreased by treatment with curcumin (Table 1).

Effect of curcumin treatment on plasma glucose, BUN, plasma creatinine and plasma albumin
After 8 weeks of STZ treatment, plasma glucose level of diabetic rats was significantly higher than in the normal control group. Treatment with curcumin did not show any significant effect on plasma glucose level in diabetic rats (Table 2). The diabetes-induced increase in plasma creatinine/body weight ratio of diabetic animals was reduced by curcumin treatment. In addition, curcumin significantly reduced the elevated BUN of diabetic rats (Table 2). Increased plasma creatinine level and BUN are indications of the development of diabetic nephropathy in rats (Makino et al., 2002; Breyer et al., 2005). Plasma albumin level was significantly decreased in diabetic animals as compared with age-matched control rats, and this decrease was prevented by curcumin treatment. Maintenance of these biochemical variables closer to those in control rats by curcumin treatment suggests that curcumin plays a role, either directly or indirectly, in providing protection against diabetic nephropathy or delay in its development.

Curcumin prevents changes in oxidative stress markers in diabetic rats
Curcumin treatment showed significant change in the levels of thiobarbituric acid-reacting substances and SOD in diabetic rat kidney. Diabetic rats show higher levels of thiobarbituric acid-reacting substances as compared with control (Figure 1a), and curcumin significantly reduced the levels of thiobarbituric acid-reacting substances in diabetic rat kidney. SOD activity in the kidneys was also significantly lower in the diabetic animals as compared with the control animals. However, treatment with curcumin prevented this decrease in SOD activity in diabetic animals (Figure 1b).

Table 1 Effect of curcumin on body weight (g), kidney weight (g) and kidney weight/body weight ratio in diabetic animals

Group	Body weight (BW) (g)	Kidney weight (KW) (g)	KW/BW ratio *1000
Normal/control	411 ± 13	0.67 ± 0.02	1.7 ± 0.03
Normal/curcumin treated	394 ± 15	0.69 ± 0.06	1.7 ± 0.04
Diabetic/control	186 ± 8***	1.13 ± 0.11*	6.0 ± 0.05***
Diabetic/curcumin treated	215 ± 8	0.89 ± 0.04†	3.9 ± 0.15†††

Body weight and kidney weight were measured after 8 weeks.
All values represent means ± s.e.mean ($n = 6$). ***$P < 0.001$; *$P < 0.05$, significantly different from normal/control. †††$P < 0.001$; †$P < 0.05$, significantly different from diabetic/control.

Table 2 Effect of curcumin on plasma glucose (PGL), BUN, plasma creatinine (PCR)/body weight (BW) ratio and plasma albumin (PAL) in diabetic rats

Group	PGL (mmol l⁻¹)	BUN (mmol l⁻¹)	PCR/BW ratio *1000	PAL (g l⁻¹)
Normal/control	7 ± 0.3	3 ± 0.08	2.4 ± 0.2	27 ± 0.4
Normal/curcumin treated	7 ± 0.2	3 ± 0.09	2.6 ± 0.1	28 ± 0.7
Diabetic/control	31 ± 0.8***	7 ± 0.79***	9.7 ± 1.1***	20 ± 1.2***
Diabetic/curcumin treated	29 ± 0.7	4 ± 0.21††	5.9 ± 0.5††	23 ± 0.4††

Abbreviation: BUN, blood urea nitrogen.
Biochemical variables were estimated after 8 weeks.
All the values represent mean ± s.e.mean ($n = 6$). ***$P < 0.001$, significantly different from normal/control. ††$P < 0.01$, significantly different from diabetic/control.

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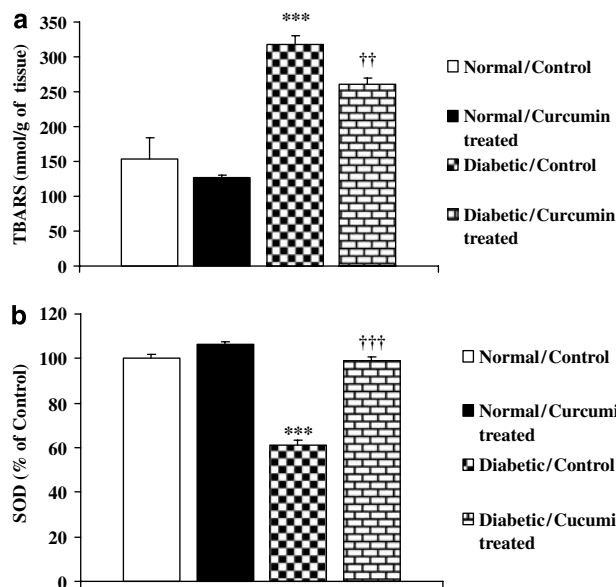
Figure 1 Effect of curcumin on the level of thiobarbituric acid-reacting substances (**a**) and SOD (**b**) in diabetic rats. All the values represent mean ± s.e.mean ($n = 6$). ***$P < 0.001$, significantly different from normal/control; †††$P < 0.001$; ††$P < 0.01$, significantly different from diabetic/control. SOD, superoxide dismutase; TBARS, thiobarbituric acid-reacting substances.

Curcumin decreases the expression of HSP-27 and p38 in STZ-induced diabetic rat kidney

Persistent hyperglycaemia has been reported to upregulate the endogenous stress protein HSP-27 (Chen *et al.*, 2005), and this protein plays a cytoprotective role through its antioxidant, antiapoptotic and actin-stabilizing properties during cell stress (Djamali *et al.*, 2005). Moreover, it has also been reported that hyperglycaemic conditions stimulate the activation of MAP kinase p38 (Igarashi *et al.*, 1999). This kinase is a proapoptotic protein and plays a pathological role in diabetic condition (Susztak *et al.*, 2006). Our data also show increased expression of HSP-27 and MAP kinase p38 in diabetic kidney. However, treatment with curcumin prevented this increase in the expression of HSP-27 and p38 (Figure 2, lanes b and c).

Change in histone H3 acetylation by curcumin treatment in diabetic rats

Histone acetylation plays an important role in eukaryotic gene transcription. Histone acetyltransferase and histone deacetylase are the enzymes controlling the state of histone acetylation *in vivo*. *In vitro*, curcumin at high concentrations (100 μM) causes loss of cell viability, which involves histone hypoacetylation (Kang *et al.*, 2005). However, our *in vivo* results show that treatment with curcumin leads to hyperacetylation of histone H3 in diabetic rats (Figure 3, upper graph, bars b and c). Similarly, curcumin alone in control rats induces hyperacetylation of histone H3 as compared with control untreated rats (Figure 3, upper graph, bars a and d).
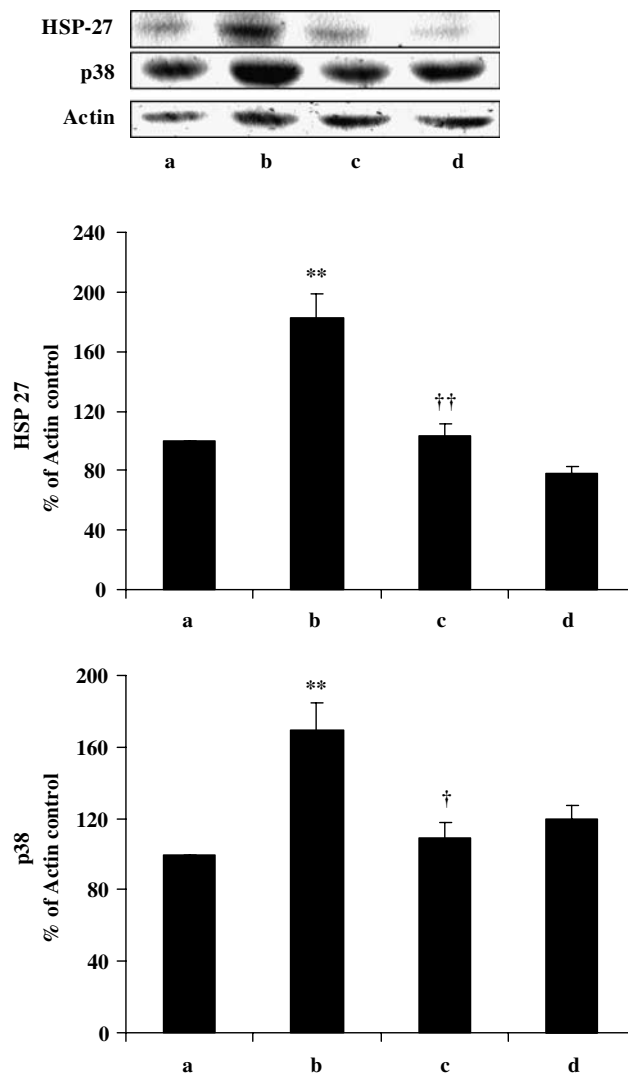
Figure 2 Western blot of HSP-27 and MAP kinase p38 in kidney after treatment with curcumin in diabetic rats. Lane a, normal/control; lane b, diabetic/control; lane c, diabetic/curcumin treated and lane d, control/curcumin treated. Results were normalized with respect to actin in respective controls. Similar results were obtained in three independent sets of experiments. All the values represent mean ± s.e.mean ($n = 3$). **$P < 0.01$, significantly different from normal/control; ††$P < 0.01$; †$P < 0.05$, significantly different from diabetic/control. HSP-27, heat-shock protein-27; MAP, mitogen-activated protein.

Treatment with curcumin changes the phosphorylation of histone H3 in diabetic rats

Activation of p38 is known to be involved in the phosphorylation of histone H3, which leads to modified chromatin structure (Dong *et al.*, 2004). Phosphorylation of histone H3 at Ser10 occurs usually when cells enter into mitosis. Several toxicants have also been shown to induce histone H3 phosphorylation, which results in premature chromatin condensation and cell death, suggesting that cells are undergoing growth arrest (Li *et al.*, 2002). The lower graph in Figure 3 shows a significant reduction of phosphorylated histone H3, that is, increased dephosphorylation of this protein, in kidneys from diabetic rats. However, treatment with curcumin of diabetic animals prevented this
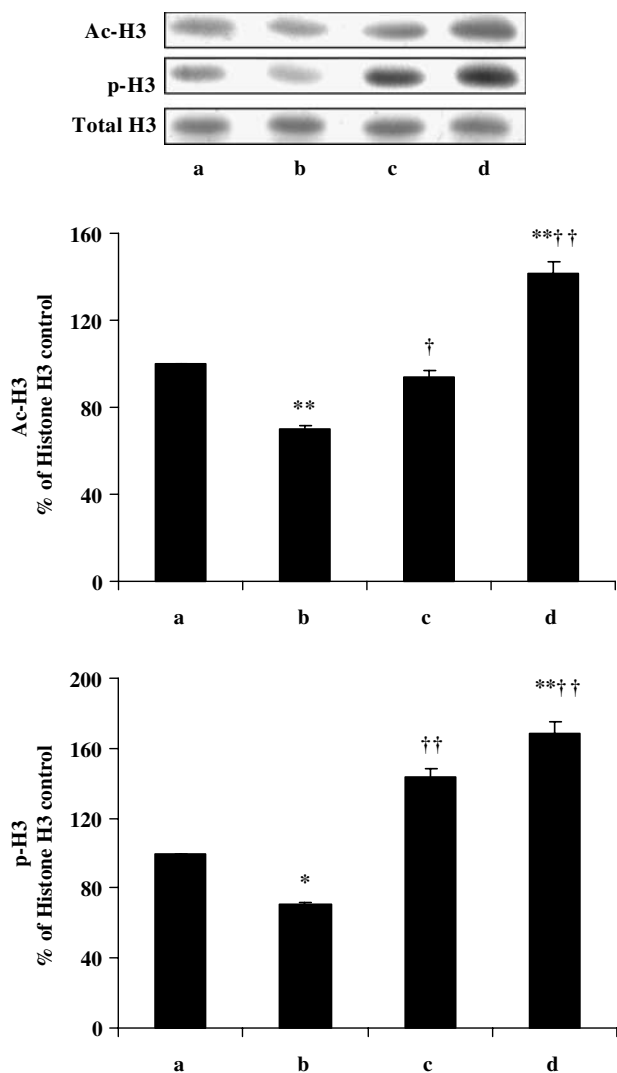
Figure 3 Western blot of acetylation and phosphorylation of histone H3 in kidney after treatment with curcumin in diabetic rats. Lane a, normal/control; lane b, diabetic/control; lane c, diabetic/curcumin treated and lane d, control/curcumin treated. Results were normalized with respect to total histone H3. Similar results were obtained in three independent set of experiments. All the values represent mean ± s.e.mean ($n = 3$). **$P < 0.01$; *$P < 0.05$, significantly different from normal/control; ††$P < 0.01$; †$P < 0.05$, significantly different from diabetic/control. Ac-H3, acetylated histone-H3; p-H3, phosphorylated histone-H3.

dephosphorylation of histone H3. In addition, curcumin on its own increases the phosphorylation of histone H3 in control rat kidney. This change in histone H3 phosphorylation can only be explained if we assume that curcumin either directly or indirectly prevented cells undergoing mitotic arrest under diabetic conditions.

Discussion

We here provide evidence that protection against the development of diabetic nephropathy by curcumin treatment involves changes in the expression of HSP-27 and MAP kinase p38. Our data show that curcumin treatment

prevented the development of diabetic nephropathy by significantly lowering BUN and plasma creatinine/body weight ratio in diabetic animals. This could be explained if there was increased clearance of blood urea and creatinine by the kidney, or if there was decreased protein degradation. Moreover, curcumin also prevented the decrease in plasma albumin concentration in diabetic rats.

Various biological actions of curcumin are mediated by inhibiting cell proliferation (Sikora *et al.*, 1997), oxidative stress (Sharma *et al.*, 2006b) and inflammation (Sharma *et al.*, 2006a). Curcumin has been reported to show its cytoprotection against oxidative stress by increasing heme oxygenase activity in vascular endothelial cells (Motterlini *et al.*, 2000). In addition, it is also reported to inhibit lipid peroxidation, resulting in protection against the cytotoxic action of hydrogen peroxide in renal epithelial cells (Cohly *et al.*, 1998). Moreover, curcumin has been reported to mediate its effects by modulating several important molecular targets, including transcription factors (nuclear factor-κB and activating protein-1) (Brennan and O'neill, 1998; Bengmark, 2006), enzymes (cyclooxygenase-2, 5-lipooxygenase and inducible nitric oxide synthase inhibitor) (Plummer *et al.*, 1999) and cytokines (tumour necrosis factor-α, interleukin-1, interleukin-6 and chemokines) (Bengmark, 2006).

Small heat-stress proteins, such as HSP-27, are molecular chaperones that modulate the ability of cells to respond to several types of injuries (Arrigo, 2001). Protection generated by the expression of HSP-27 against cell death induced by oxidative stress has already been described (Arrigo *et al.*, 2005). Accumulation of abnormal proteins in the kidney and other tissues takes place due to constitutive alterations of intracellular protein recognition, assembly, and turnover in type 1 diabetes mellitus with diabetic nephropathy. In this regard, increased levels of HSP-27 in skin fibroblasts of patients with type I diabetes and diabetic nephropathy have recently been reported (Tessari *et al.*, 2007), suggesting these changes in HSP-27 present in fibroblasts might reflect those in the kidney and be pathophysiologically related to the development of nephropathy in type 1 diabetes mellitus. Our results also indicate that there was an increase in expression of HSP-27 in diabetic kidney. However, treatment of curcumin prevented this increase in HSP-27 expression, either directly or indirectly decreasing the oxidative stress.

High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells (Allen *et al.*, 2003, 2005) and in mesangial cells (Mishra and Simonson, 2005), which is mediated by multiple caspases that are activated by ROS, such as peroxynitrite ($ONOO^-$). Moreover, glucose-induced ROS production initiates podocyte apoptosis and podocyte depletion *in vitro* and *in vivo*, and suggest that podocyte apoptosis/depletion represents a novel early pathomechanism(s) leading to diabetic nephropathy in murine type 1 and type 2 diabetic models (Susztak *et al.*, 2006). Increased phosphorylation of MAP kinase p38 plays an important role in human and experimental diabetic nephropathy (Adhikary *et al.*, 2004). In the present study, we also found an increase in MAP kinase p38 expression in diabetic kidney. These results are consistent with our previous report (Tikoo *et al.*, 2007b). Moreover, treatment with curcumin significantly reduced the expression of this kinase in diabetic kidney,

indicating its antiapoptotic effect. Changes in the expression of HSP-27 and MAP kinase p38 clearly suggested that curcumin was preventing the cells from undergoing apoptosis, which results in the development of diabetic nephropathy.

Recently, global gene expression analysis in healthy men during a hyperglycaemic–euglycaemic clamp, suggested that modifications of gene expression could be an additional effect of glucose toxicity *in vivo* (Meugnier *et al.*, 2007). Acetylation of histones and non-histone proteins is an important post-translational modification involved in the regulation of gene expression in mammalian cells. Dysfunction of histone acetyltransferase is often associated with the manifestation of several diseases (Kang *et al.*, 2006). Recent studies showed that oxidative stress could also regulate gene transcription by differentially affecting changes in histone modifications. Oxidative stress and proinflammatory mediators have been suggested to influence histone acetylation and phosphorylation, via a mechanism dependent on the activation of the MAP kinase pathway (Rahman *et al.*, 2004). Recently, we have reported the dephosphorylation of histone H3 and the activation of MAP kinase p38 in diabetic kidney (Tikoo *et al.*, 2007b). Similarly, in the present study, we also observed a decrease in histone H3 phosphorylation (Ser10) in the diabetic rat kidney. Interestingly, this decrease in phosphorylation of histone H3 is prevented by curcumin treatment, suggesting that cells were not undergoing mitotic arrest.

Phosphorylation of histone H3 at Ser10 facilitates the transcription of immediate-early genes (Mahadevan *et al.*, 1991; Thomson *et al.*, 1999; Labrador and Corces, 2003; Soloaga *et al.*, 2003), whereas during mitosis such phosphorylation facilitates chromosome remodelling and condensation (Wei *et al.*, 1998, 1999; Squires *et al.*, 2003). However, phosphorylation of H3 and apoptotic chromosome condensation are unrelated events and chromosome condensation can occur without phosphorylation of Ser10 (Hendzel *et al.*, 1998). Thus, decreased phosphorylation of histone H3 under diabetic conditions does not necessarily imply that cells are protected against apoptotic cell death. However, these results do demonstrate the regulatory role of histone phosphatases and histone kinases in diabetic conditions. The increase in phosphorylation of histone H3 with curcumin treatment might be due to specific inhibition of histone phosphatases or activation of histone kinases. Our data provide indirect evidence that curcumin increases the phosphorylation of histone H3, which is not histone kinase-dependent, but is dependent on histone phosphatase.

Until now, there have been no reports regarding the effect of curcumin on histone H3 acetylation in diabetic kidney. Several reports indicate that oxidative stress induces hypoacetylation of histones (Kang *et al.*, 2005), which may lead to a decrease in eukaryotic gene transcription and cell-cycle arrest. In contrast to the report that curcumin induces hypoacetylation of histone H3 and H4 in neural progenitor cells (Kang *et al.*, 2006), our *in vivo* data show that treatment of curcumin leads to hyperacetylation of histone H3 in diabetic kidney, as well as in control rat kidney. This can be explained because of the differences in the two cell systems. Increase in histone acetylation suggests an increase the

transcription of various genes. Results of the present study suggest that curcumin treatment increased transcription of certain genes, which are repressed under diabetic conditions and may be involved in the pathophysiology of the disease. However, further studies are required to warrant any conclusion.

In conclusion, our results show for the first time that protection observed in the development of diabetic nephropathy by curcumin treatment in STZ-induced diabetic rats involves changes in post-translational modifications of histone H3 and also changes in the expression of HSP-27 and p38.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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