Amelioration of Liver Injury by Curcumin in Streptozotocin-Induced Diabetic Rats

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Abstract—We investigated the effect of curcumin on liver injury in diabetic rats induced by streptozotocin (STZ) through modulation of 5′-AMP-activated protein kinase (AMPK)/protein kinase C (PKC)-α/nuclear factor-κB (NF-κB) pathway. Experimental diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg) and curcumin was given at 100 mg/kg by gavage, for 56 days. We observed that curcumin improved the morphological and histopathological changes, decreased lipid peroxidation and increased the activity of antioxidant in diabetic liver. Moreover, treatment with curcumin significantly increased phosphorylation of AMPK, inhibit membrane translocation of NADPH oxidase and PKC-α, NF-κB activation, degradation of IκBα, inducible nitric oxide synthases, nitrotyrosine, transforming growth factor-1β in liver tissues of diabetic rats, as well as inhibit increased mRNA expression of TNF-α and IL-1β. Thus, the results of the present study suggested that curcumin may have a significant role in alleviating liver damage in the STZ-diabetic rats.

Keywords—AMPK, Curcumin, Diabetes, Protein Kinase C

I. INTRODUCTION

DIABETES mellitus is one of the most common endocrine metabolic disorders. Studies have shown that hepatobiliary disorders, such as the inflammation and necrosis or fibrosis of non-alcoholic fatty liver disease can follow diabetes [1]-[2]. Previous study has shown that hepatic fat accumulation and oxidative stress play a critical role in the development of diabetic liver injury [3] and a number of reports have shown that antioxidants could attenuate the complications of diabetes including fatty changes in patients and in experimental models [4]-[5]. Recently, the control of diabetes through the modulation of 5′-AMP-activated protein kinase (AMPK) has received considerable attention since it has been shown that the major metabolic responses to exercise are mediated through AMPK [6]. The activation of AMPK has been shown could prevents hyperactivity of NAD(P)H oxidase induced by high glucose through protein kinase C (PKC) inhibition [7].

PKC is a family of serine/threonine protein kinases involved in the regulations of various aspects of cell functions [8]. Tang et al. (1993) have demonstrated that diabetes invoked selective alterations in the expression of PKC isoforms in hepatocytes which may lead to altered cellular functioning [9].

Natural polyphenol curcumin is a potent antioxidants agent [10]. Curcumin has been shown to modulate the activity of protein kinases [11], membrane ATPases [12], and transcription factors [13]-[14]. Previous study has pointed to the protective effect of curcumin on acute liver injury by inhibiting NF-κB and oxidative stress [15]. Recently, it has been demonstrated that curcumin also activate AMPK [16]-[17]. Although many aspects of curcumin-induced cytoprotection are studied, the molecular mechanism by which curcumin protects liver tissues against streptozotocin (STZ)-induced liver injury is not clear. We hypothesized that curcumin may protect liver tissues against STZ-induced oxidative stress and resulting liver injury through modulation of AMPK, PKC-α and NF-κB pathway as well as oxidative stress.

II. MATERIALS AND METHODS

Animals and Experimental design

All animal studies were treated in accordance with the guidelines for animal experimentation of our institute [18]. Male Sprague-Dawley rats (weight 250-300 g) were obtained from Charles River Japan Inc. (Kanagawa, Japan). Animals were housed at a temperature of 22 ± 1 °C and humidity of 65 – 70%, and were submitted to a 12 hour light/dark cycle, and allowed free access to standard laboratory chow and tap water. Diabetic rats were induced by a single intraperitoneal (i.p.) injection of STZ (Sigma-Aldrich, Inc.; Saint Louis, MO, USA) at a dose of 55 mg/kg, diluted in citrate buffer 20 mM (pH 4.5). Forty eight hours later, blood glucose was measured by tail-vein sampling using Medi-safe chips (Terumo Inc., Tokyo, Japan). Diabetes was defined as a morning blood glucose reading of ≥ 300 mg/dL. Twenty four rats were randomly divided into three groups (n = 8/group): non-diabetic normal control group (N), diabetic rats treated with vehicle 1% gum Arabic (D) and diabetic rats treated with curcumin 100 mg/kg/day [19] diluted in vehicle 1% gum Arabic (Cur). Curcumin was started at 3 weeks after STZ injection and was administered via oral gavage for 8 weeks. All rats were sacrificed at 11 weeks after the induction of diabetes for analysis of liver tissues.
Measurement of malondialdehyde (MDA) content
Liver tissues were rinsed, weighed, resuspended at 50 mg/mL in normal saline and homogenized. After centrifugation at 5000 rpm for 10 min at 4 °C, the supernatants were collected and analyzed with corresponding assay kits (OxiItk, ZeptoMetrix Corporation, New York) in accordance with the manufacturer’s instruction.

Measurement of glutathione peroxidase (GPx) activity
Liver tissues were homogenized in six volumes (per weight of tissues) of cold GPx Assay Buffer and the mixture was centrifuged at 8000 rpm for 15 min at 4 °C in accordance with the total GPx assay kit instruction (OxiItk, ZeptoMetrix Corporation, New York). GPx activity in liver tissues was measured using a kinetic ultraviolet-visible spectrophotometer (Ultraspex 3100, Amersham Biosciences). The oxidation of NADPH to NADP+ was measured by the decrease in absorbance at 340 nm.

Histopathological analysis
Formalin-fixed liver sections (4 μm) were stained with hematoxylin and eosin (H&E) and Azan-Mallory. Morphological analysis was assessed by computerized image analysis system on ten microscopic fields per section examined at a 100-fold magnification (CIA-102; Olympus), with the observer blind to the study group. The histopathological findings were scored as 0 (none), 1 (weak), 2 (moderate), or 3 (severe) and were assessed in a blinded manner [20].

Western blotting analysis
Liver tissue was fractionated into membranous and cytosol fractions using methods similar to those described by Inoguchi et al. (1992) [21]. Western blot analysis was performed on cytosol and membrane fraction using PKC-α antibody and activation was inferred from the ratio of membrane to cytosol fraction. Western blot analysis was also performed to determine p67phox, p22phox, NF-κB, IκBα, AMPK-α, p-AMPK-α, nitrotyrosine, inducible nitric oxide synthase, and transforming growth factor (TGF)-β1.

Determination of tumor necrosis factor (TNF)-α and interleukin (IL)-1β expression by quantitative real-time PCR
Gene expression analysis was performed by real-time reverse transcription polymerase chain reaction (RT-PCR) (Smart cycler; Cepheid, Sunnyvale, CA) using cDNA synthesized from the diabetic specimen. Primer sequences were as follows: IL-1β(forward), CTTCATCTCAGCAGCACATCTCG,(reverse),TCCACGGCAAGACATAGGTAGC;TNFa(forward),CCCCAAAGGATGAGAGGTT,(reverse),CACCTGGTGGTTTGTACGA;GAPDH(forward),GCTCATTTCCTGGTATGACAACG,(reverse),AGGGGTCTACATGGCAACTG. Results were normalized to GAPDH mRNA as an internal control and are thus shown as relative mRNA levels.

Statistical analysis
Data are shown as mean ± S.E.M. and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s methods for post-hoc analysis and two-tailed t-test when appropriate. A value of $p < 0.05$ was considered statistically significant. For statistical analysis, GraphPad Prism 5 software (San Diego, CA, USA) was used.

III. Results

Biochemical parameters in experimental animals
As shown in Table 1, body weight was significantly decreased in diabetic rats, but curcumin treatment could not prevent the decline in body weight. The liver weight corrected for body weight of untreated diabetic rats was significantly increased. Compared with normal group, diabetic rats developed elevated mean plasma glucose. Levels of MDA and the activity of GPX were higher and lower, respectively, in the liver of diabetic rats than those in normal rats. All of these abnormalities were significantly attenuated by curcumin treatment ($P < 0.05$).

Effect of curcumin on liver AMPK activity
Since phosphorylation of the catalytic subunit AMPK-α at Thr172 position is essential for AMPK activation, AMPK activation was monitored by using a specific antibody that recognizes the phosphorylated AMPK-α at Thr172. As shown in Figure 1A, AMPK phosphorylation was reduced in the untreated-diabetic rats by nearly 60% ($P < 0.05$). Curcumin prevented the reduction in AMPK phosphorylation in diabetic rats.

Effect of Curcumin on PKC-α and NF-κB activity and degradation of Ikβ
Diabetic rats demonstrated evidence of PKC-α activation, with a significant increased by 4.1-fold in the ratio of membrane to cytosolic PKC-α that was reduced by curcumin ($P < 0.05$) as shown in Figures 1B. Next, we performed Western blot with nucleic lysate for phosphorylated NF-κB and with the cytosolic protein of liver tissue for NF-κB. The ratio of phosphorylated NF-κB in the nucleus to NF-κB in the cytosol was decreased to 1.4-fold in the curcumin-treated diabetic rats compared with that of untreated diabetic rats (Fig. 1C). In addition, we observed that cytosolic Ikβ in the liver of diabetic was significantly lower than in the normal ($P < 0.05$) and curcumin treatment significantly reduced Ikβ degradation ($P < 0.05$) (Fig. 1D).

Effect of curcumin on TNF-α and IL-1β mRNAs in liver
Liver TNF-α and IL-1β mRNA expression assessed by real-time PCR were significantly higher in diabetic rats compared with those in normal rats ($P < 0.05$), and curcumin treatment significantly attenuated the increased liver TNF-α and IL-1β mRNA expression ($P < 0.05$). The TNF-α/GAPDH mRNA and IL-1β/GAPDH mRNA ratios was 2.4- and 3.1-fold higher in diabetic rats compared with normal rats, respectively, and
curcumin treatment attenuated these increases by 1.8- and 2.4-fold, respectively (Fig. 2A and 2B).

**Effect of curcumin on oxidative stress parameter**

We assessed changes in protein expression of nitrotyrosine and iNOS levels in the liver. As shown in Fig. 2C and 2D, there were increased protein expression of nitrotyrosine and iNOS by about 3.7- and 2.2-fold, respectively, compared to the values obtained from normal rats. Curcumin treatment prevented increase in liver iNOS levels by 1.6-fold (Fig. 2C) and nitrotyrosine levels by 2.7-fold ($P < 0.05$, Fig. 2D). In addition, the protein expressions of p22$^{	ext{phox}}$ and membrane translocation of p67$^{	ext{phox}}$ in liver of diabetic rats were increased. These increases were significantly reduced by curcumin treatment (Fig. 3A and 3B).

**Effect of curcumin on liver expression of TGF-β1**

TGF-β is a major fibrogenic cytokine that play an important role in the progression of liver injury. Using Western blot analysis we found that liver TGF-β protein expression was increased by 2.8-fold in diabetic rats compared with that in the normal rats ($P < 0.05$). This increase in liver TGF-β protein expression was markedly suppressed by curcumin treatment in the diabetic rats ($P < 0.05$) (Fig. 3C).

**Histopathological findings**

The histopathological findings are summarized in Table 2. Figure 4 shows the structural features of hepatocytes in the experimental animals. Fatty liver was shown by H&E staining as an unstained area in liver parenchymal cells (Fig. 4B). In the untreated diabetic rats, microvacuolar vacuolization, focal necrosis and inflammation in the portal area were significantly apparent compared with the normal rats (Fig. 4B1 & 4B2) and curcumin treated diabetic rats (Fig. 4C) improved these findings. Intersitial matrix deposition was studied in Azan-Mallory-stained liver sections as an index of interstitial fibrosis. Diabetic rats showed higher interstitial fibrosis than normal rats (Fig. 4E), which was improved in the curcumin treated diabetic rats (Fig. 4F).

**IV. DISCUSSION**

We found that diabetes-induced liver injury was associated with increased amounts of lipid peroxidation and decreased antioxidant enzyme, indicating oxidative stress, and the dephosphorylation of AMPK as well as translocation of PKC-α to the membrane in the liver of rats with STZ-induced diabetes. Moreover, in STZ-induced diabetic rats, there were excessive amounts of lipid deposits in the liver sections, as shown by H&E staining; all of these abnormalities were ameliorated by curcumin treatment. Curcumin treatment also significantly suppressed NF-κB activity as well as reduced the degradation of cytosolic IκBα: as a consequence, the level of proinflammatory cytokines, TNF-α and IL-1β were further significantly decreased. Curcumin treatment markedly inhibited diabetes-induced increased expression of NADPH oxidase subunits (p67$^{	ext{phox}}$ and p22$^{	ext{phox}}$), nitrotyrosine and iNOS which is an essential mechanism responsible for increased ROS production.

AMPK is a heterotrimeric protein consisting of a combination of $\alpha_{1/2^*}$, $\beta_{1/2^*}$, and $\gamma_{1/2^3}$-subunits [22]. The enzymatic activity of AMPK is dependent on phosphorylation of Thr172 of the $\alpha$-subunit [23]. Several lines of evidence have demonstrated that AMPK serves as a key metabolic sensor in both insulin-sensitive and other tissues that is capable of responding to metabolic stresses by shutting down the synthesis of fatty acids and cholesterol, two major energy-consuming pathways [24]-[25]. Previous studies have demonstrated that PKC was implicated as downstream mediators of the effects of AMPK on glucose transport [26]. Na et al. (2011) have shown that curcumin improved muscular insulin resistance through the upregulation of phosphorylated AMPK [27]. In the present study, we show for the first time that curcumin activates AMPK and inhibit PKC-α activity in STZ-induced liver injury, a model in which oxidative stress plays a major role in determining tissue damage. Furthermore, in this study, we observed accumulation of lipid droplets in the cytoplasm of hepatocytes (Fig. 4B). Our findings of fatty liver are in agreement with the findings of previous studies [28]-[29]. We also found scant fibrosis in the liver of diabetic rats which were attenuated by curcumin treatment (Fig. 4B1, 4B2, 4E).

It has been reported that activated PKC could facilitate increased superoxide anion production through PKC-dependent activation of NADPH oxidase in vascular cells [21]. Activated PKC also induces a number of pathogenic consequences by activating NF-κB [30]. In the present study, Western blot analysis revealed that diabetes-induced translocation of PKC-α as well as NADPH oxidase subunits (p67$^{	ext{phox}}$ and p22$^{	ext{phox}}$) to the membrane fractions were increased markedly in 11-week diabetic liver. All such abnormalities were completely normalized by curcumin treatment. This finding is in agreement with the previous report by Balasubramanyam et al. (2003) which determined that ROS inhibitory effect of curcumin interfered mechanistically with PKC activity [31].

Curcumin is a representative polyphenolic compound found in the dietary spice turmeric. Among a wide range of biological and biochemical activities, curcumin therapeutic effects are being investigated on well-defined models of disease, such as inflammatory renal, pancreatic, and pulmonary diseases [32]-[33]. Nanji et al. (2003) have demonstrated the protective effect of curcumin in rat liver injury induced by alcohol, where curcumin administration prevented ALT increases, blocked the activation of NF-κB, and the expression of proinflammatory cytokines (TNF-α) and iNOS [34]. Other study has also reported that curcumin is a nitric oxide synthase inhibitor and these effects may be attributed to inhibition of nitric oxide synthase rather than nitric oxide scavenging capacity of curcumin [35]. In the present study, we showed that activation of NF-κB as well as proinflammatory cytokines expression, TNF-α and IL-1β were
increased in diabetic rats compared with the levels in normal rats. Curcumin treatment prevented all of these alterations. We also showed that curcumin administration inhibited the expression of iNOS and nitrotyrosine in liver tissues of STZ-induced diabetic rats. It was also found that curcumin elevated the activity of GPx, which is an antioxidant defense system, while decreasing the MDA levels, which is a marker of ROS-induced lipid peroxidation, in the liver of diabetic rats. Previous study has proved that curcumin increases glutathione S-transferase activity [32] as well as inhibited lipid peroxidation [34]. We also showed that the extracellular matrix accumulation, which was confirmed by upregulation of TGF-β1, was reduced by curcumin treatment.

In summary, the results presented here show that administration of curcumin inhibits oxidative stress, NF-κB and PKC-α activation, and increase AMPK phosphorylation in liver of STZ-diabetic induced rats. Given these promising preclinical findings, we believe that the curcumin, which is a potentially safe and inexpensive therapy for clinical use, might be considered as potential adjuvant entity for preventing diabetic liver injury.

REFERENCES


TABLE 1 CHANGES IN BIOCHEMICAL PARAMETERS AFTER 8 WEEKS OF TREATMENT WITH CURCUMIN IN DIABETIC RATS INDUCED BY STREPTOZOTOCIN

<table>
<thead>
<tr>
<th></th>
<th>Group N (n=10)</th>
<th>Group D (n=10)</th>
<th>Group Cur (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW) (g)</td>
<td>536 ± 19.8</td>
<td>277.4 ± 20.7</td>
<td>321.6 ± 19.9</td>
</tr>
<tr>
<td>Liver weight (LW)/BW</td>
<td>28.7 ± 0.8</td>
<td>51.3 ± 7.1</td>
<td>37.9 ± 2.3*</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>118.6 ± 6</td>
<td>744.4 ± 9.3*</td>
<td>597.1 ± 9.9*</td>
</tr>
<tr>
<td>MDA (nmol/mg tissue)</td>
<td>0.2 ± 0.01</td>
<td>0.5 ± 0.02*</td>
<td>0.3 ± 0.02*</td>
</tr>
<tr>
<td>GPx (U/mg protein in tissue)</td>
<td>0.03 ± 0.01</td>
<td>0.01 ± 0.03*</td>
<td>0.02 ±0.02*</td>
</tr>
</tbody>
</table>

TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase; MDA: malondialdehyde; GPx: glutathione peroxidase. Values are expressed as means ± SEM. *p < 0.01 vs group N, †p < 0.05 vs group D.

Table 2 Effects of curcumin on histopathological findings in diabetic rats induced by STZ

<table>
<thead>
<tr>
<th></th>
<th>Group N (n=8)</th>
<th>Group D (n=8)</th>
<th>Group Cur (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvascular vacuole</td>
<td>0</td>
<td>0.45 ± 0.05**</td>
<td>0**</td>
</tr>
<tr>
<td>Focal necrosis</td>
<td>0</td>
<td>2 ± 0.5**</td>
<td>0.7 ± 0.05**</td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>0</td>
<td>1 ± 0.05**</td>
<td>0.5 ± 0.01**</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0</td>
<td>3 ± 0.6**</td>
<td>1.7 ± 0.05**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. **P < 0.05 vs group N, ***P < 0.05 vs group D.

Fig. 1 Representative Western blots of total AMPK and phospho-AMPK, together with quantification of AMPK activation are shown (A). Representative Western blots and densitometric data of protein analysis of cytosolic and membranous fractions of PKC-α is presented (B). Representative Western blots and densitometric data of NF-κB is shown (C). Representative Western blots and densitometric data of protein analysis of IκBα (D). Each bar represents mean ± SE. The blots are representatives of five independent experiments. N, age-matched normal rats; D, untreated diabetic rats; Cur, diabetic rats treated with curcumin 100 mg/kg/day. **P < 0.05 vs N, ***P < 0.05 vs D.

Fig. 2 Real-time PCR analysis shows diabetes-induced upregulation of TNF-α mRNA (A) and IL-1β mRNA (B), which was downregulated by curcumin treatment. Representative Western blots and densitometric data of protein analysis for iNOS (C). Representative Western blots and densitometric data of protein analysis for nitrotyrosine (D). Each bar represents mean ± SE. The blots are representatives of five independent experiments. N, age-matched normal rats; D, untreated diabetic rats; Cur, diabetic rats treated with curcumin 100 mg/kg/day. **P < 0.05 vs N, ***P < 0.05 vs D.

Fig. 3 Representative Western blots and densitometric data of protein analysis for p67phox (A). Representative Western blots and densitometric data of protein analysis for p22phox (B). Representative Western blots and group data depicted protein abundance of TGF-β (C) in the liver tissues of diabetic rats, curcumin-treated diabetic rats and normal control rats. Each bar represents mean ± SE. The blots are representatives of five independent experiments. N, age-matched normal rats; D, untreated diabetic rats; Cur, diabetic rats treated with curcumin 100 mg/kg/day. **P < 0.05 vs N, ***P < 0.05 vs D.

Fig. 4 Light microscopic photographs of the livers of experimental animals showed (A) the liver of normal control group, (B) lipid accumulation indicated by the unstained area in liver tissues, (B1) microvascular fattening and focal necrosis (arrow), (B2) portal inflammation (*) in the untreated diabetic group, (C) in curcumin-treated diabetic group, the severity of these changes was less than those in the untreated diabetic group. H&E (X 400). Azan-Mallory staining for fibrosis of the cross-sectional tissue slices of liver. Fibrosis is indicated by the blue area (X 100).