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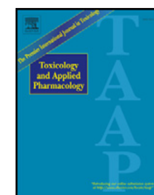

Insights antifibrotic mechanism of methyl palmitate: Impact on nuclear factor kappa B and proinflammatory cytokines

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ABSTRACT

Fibrosis accompanies most chronic liver disorders and is a major factor contributing to hepatic failure. Therefore, the need for an effective treatment is evident. The present study was designed to assess the potential antifibrotic effect of MP and whether MP can attenuate the severity of oxidative stress and inflammatory response in chronic liver injury. Male albino rats were treated with either CCl_4 (1 ml/kg, twice a week) and/or MP (300 mg/kg, three times a week) for six weeks. CCl_4-intoxication significantly increased liver weight, serum aminotransferases, total cholesterol and triglycerides while decreased albumin level and these effects were prevented by co-treatment with MP. As indicators of oxidative stress, CCl_4-intoxication caused significant glutathione depletion and lipid peroxidation while MP co-treatment preserved them within normal values. As markers of fibrosis, hydroxyproline content and α-SMA expression increased markedly in the CCl_4 group and MP prevented these alterations. Histopathological examination by both light and electron microscope further confirmed the protective efficacy of MP. To elucidate the antifibrotic mechanisms of MP, the expression of NF-κB, iNOS and COX-2 and the tissue levels of TNF-α and nitric oxide were assessed; CCl_4 increased the expression of NF-κB and all downstream inflammatory cascade while MP co-treatment inhibited them. Collectively these findings indicate that MP possesses a potent antifibrotic effect which may be partly a consequence of its antioxidant and anti-inflammatory properties.

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Introduction

Progressive hepatic fibrosis is the final common pathway for most chronic liver injuries, leading to cirrhosis with risk of liver failure and hepatocellular carcinoma (HCC). It is now recognized that fibrosis is a dynamic process and may be reversible prior to the establishment of advanced architectural changes to the liver (Thompson and Patel, 2010). Collective evidences indicated that liver fibrosis incorporates uncontrolled inflammation as a part of its etiology. Kupffer cells which act as resident macrophages in the liver represent the primary inflammatory effectors which initiate the inflammatory cascade leading to tissue remodeling and fibrosis (Steib et al., 2007).

Nuclear factor kappaB (NF-κB) comprises a family of inducible transcription factors consisting of p65 and p50 subunits of Rel protein family that serves as important regulators of the host immune and inflammatory response (Cao et al., 2006). Recently, attention has been drawn to the pathophysiological role of NF-κB in the diseased liver. *In vivo* studies using rodent models of liver diseases and cell-targeted perturbation of NF-κB activity have revealed complex and multicellular functions in hepatic inflammation, fibrosis, and the development of hepatocellular carcinoma — a process termed as "inflammation–fibrosis–cancer axis" (Elsharkawy and Mann, 2007). NF-κB as a critical mediator of inflammatory response, regulates the expression of many Kupffer cell-derived proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2) and nitric oxide (NO) which are involved in the process of fibrogenesis (Elsharkawy and Mann, 2007). Furthermore, NF-κB was found to be activated during transdifferentiation of hepatic stellate cells (HSCs) into myofibroblasts like cells which are characterized by increased cell proliferation, *de novo* expression of α-smooth muscle actin (α-SMA) and excessive deposition of extracellular matrix (ECM) such as collagen, proteoglycan and hyaluronate leading to subsequent hepatic fibrosis scar (Cho et al., 2004; Friedman, 2003).

With better understanding of the impact of regulating NF-κB signaling pathway, the potential for preventing NF-κB activation has been regarded as a promising strategy for discovering new therapeutic agents. In the last few years, much attention has been focused on inhibiting NF-κB pathway as a target for prevention or treatment of liver fibrosis and HCC. Several NF-κB inhibitors, like caffeic acid, captopril, silymarin and curcumin have demonstrated antinecrotic, anticholestatic, antifibrotic and anticancer activities in liver (Muriel, 2009). Indeed, much of current knowledge of NF-κB signaling in

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liver diseases is related to the canonical pathway, the inhibitor of kappaB kinase (IKK) complex and the RelA subunit (Robinson and Mann, 2010). In this pathway, IKKα and/or IKKβ, two kinase subunits are responsible for phosphorylation of inhibitory protein known as IκB which sequesters NF-κB in the cytoplasm in an inactive complex. Then IκB is ubiquitinated and degraded by proteasome unmasking the NF-κB nuclear localization signal and leading to its transport from the cytoplasm to the nucleus, where it binds to κB enhancer elements of specific target genes to induce their transcription (Hoffmann et al., 2006).

As a promising inhibitor of IκB phosphorylation, methyl palmitate (MP) was shown to suppress Kupffer cell function as measured by colloidal carbon clearance and latex beads uptake (Cai et al., 2005; Cowper et al., 1990). The inhibition of phagocytic activity was accompanied by differential expression of cytokines, NO, and COX-2 in different types of macrophages. Accordingly, based on these *in-vitro* studies MP could be considered as a universal macrophage inhibitor that could be through inhibition of NF-κB pathway (Cai et al., 2005; El-Demerdash 2011; Sarkar et al., 2006). Recently, MP effectiveness as a promising antifibrotic agent was investigated *in vivo* against carbon tetrachloride (CCl_4)-induced liver fibrosis and bleomycin-induced lung fibrosis (El-Demerdash, 2011; Rodriguez-Rivera et al., 2008). In the present study we elucidated the molecular mechanisms underlying the promising antifibrotic effect of MP by studying its effects on different oxidative stress, inflammatory and fibrosis markers as well as the expression of NF-κB in an experimental model of carbon tetrachloride (CCl_4)-induced liver fibrosis.

Materials and methods

Materials. Methyl palmitate (MP), carbon tetrachloride (CCl_4), reduced glutathione (GSH), Ellman's reagent [5,5-dithio-bis (2-nitrobenzoic acid); DTNB], hydroxyproline, chloramine-T, p-dimethylaminobenzaldehyde, bovine serum albumin and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). N-butanol, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and trichloroacetic acid (TCA) were purchased from El-Nasr Chemical Co (Egypt). All other chemicals and solvents were of highest grade commercially available.

Animals. The study was conducted according to ethical guidelines (Ain Shams University, Egypt). Male albino rats (150–250 g) were obtained from Nile Co. for Pharmaceutical and Chemical industries, Egypt. Rats were housed in an air-conditioned atmosphere, at a temperature of 25 °C with alternatively 12 hour light and dark cycles. Animals were acclimated for 2 weeks before experimentation. They were kept on a standard diet and water *ad libitum*. Standard diet pellets (El-Nasr, Abu Zaabal, Egypt) contained not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture.

Experimental design. Animals were divided randomly into four groups (ten animals per group) and treated for six weeks as follows; the first group was given CCl_4 (1 ml/kg, 1:1 mixture with corn oil, i. p.), twice weekly to induce liver fibrosis. The second group was given both CCl_4 and MP (300 mg/kg, i.p. dissolved in corn oil) three times per week at alternating days with CCl_4. The third group was given MP alone and the last group was considered as control group and given corn oil. At the end of 6 weeks the rats were anesthetized and blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 1000 *g* for 10 min and used for the assessment of liver functions. Then, rats were sacrificed and liver tissues were dissected and washed with ice-cold saline. Livers were homogenized in saline then the homogenate was used for assessment of oxidative stress markers (reduced glutathione (GSH)), and lipid peroxides, inflammatory makers (TNF-α and total NO) as well as a fibrosis marker; hydroxyproline content. In addition, specimens from the three major lobes of each liver were fixed in appropriate buffer for light and electron microscopical examination as well as immunohistochemical detection of NF-κB(p65), inflammatory markers; iNOS and COX2 and fibrosis marker; α-SMA.

Assessment of hepatotoxicity indices. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method of Reitman and Frankel (1957). Serum levels of total cholesterol (TC), triglycerides (TG) and albumin were estimated using available commercial kits (Spectrum diagnostics, Cairo, Egypt). Liver index was calculated according to the formula: (liver weight/body weight) × 100.

Assessment of oxidative stress markers. To determine GSH, 0.5 ml homogenate was added to a tube with 0.5 ml of 10% TCA. The tubes were shaken gently and intermittently for 15 min, followed by centrifugation at 3000 rpm for 10 min. An aliquot of the resulting supernatant (0.2 ml) was added to a tube containing 1.7 ml phosphate buffer and 0.1 ml Ellman's reagent then the absorbance was read at 412 nm within 5 min (Ellman, 1959). The results were expressed as μM of GSH/g of wet tissue. Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Mihara and Uchiyama (1978). Briefly, the reaction mixture (0.5 ml homogenate + 2.5 ml 20% TCA + 1.0 ml 0.6% TBA) was heated for 20 min in a boiling water bath followed by cooling and addition of 4 ml *n*-butanol with shaking. The alcohol layer was separated by centrifugation at 2000 *g* for 10 min and absorbance was measured at 535 nm. The results were expressed as nmol of MDA/g of wet tissue using 1,1,3,3-tetraethoxypropane as standard.

Assessment of inflammatory markers. Both TNF-α and total NO levels in liver homogenate were assessed. In addition, immunohistochemical detection of iNOS and COX-2 was carried out. Determination of TNF-α was performed using commercial ELISA kit (Assaypro Co., USA) according to the manufacturer's instructions. The quantities of rat TNF-α were expressed as ng/mg protein. Protein was determined according to Lowry et al. (1951) using bovine serum albumin as standard. Total NO content was estimated in liver homogenate spectrophotometrically as formed nitrite (NO_2^-) according to the method described by Miranda et al. (2001). Briefly, the homogenate was incubated with absolute ethanol for protein precipitation for 48 h. Following centrifugation, the supernatant was incubated with vanadium trichloride followed by addition of Griess reagent at 37 °C for 30 min. Then, the absorbance was measured at 540 nm. Total NO content was calculated based on a standard curve constructed using sodium nitrate ($NaNO_3$) and the results were expressed as μM/g of wet tissue.

Assessment of liver fibrosis markers. As an index of liver fibrosis, hydroxyproline was estimated in the liver using the method of Woessner (1961). Briefly, 0.5 ml of 20% liver homogenate was digested in 1 ml of 6 M HCl at 120 °C for 8 h. An aliquot of digested homogenate (25 μl) was added to 25 μl citrate–acetate buffer and finally 500 μl of chloramine T solution was added then the mixture was left at room temperature for 20 min. Then, 500 μl Ehrlich's solution was added and the mixture was incubated at 65 °C for 15 min. After cooling for 10 min, the color developed was measured spectrophotometrically at 550 nm. Results were expressed as μg/g of wet tissue. Besides hydroxyproline assay, liver fibrosis was further assessed using Masson's trichome staining for collagen fiber detection and immunohistochemical staining for α-SMA.

Histopathological examination. For light microscopy, liver specimens were taken from the right lobe and fixed in 10% formalin and

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