Carvacrol suppresses the expression of inflammatory marker genes in D-galactosamine–hepatotoxic rats

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\textbf{ABSTRACT}

\textbf{Objective:} To unravel the mechanism of anti-inflammatory activity of carvacrol in D-galactosamine (D-GalN)–induced hepatotoxic rats. \textbf{Methods:} The mRNA and protein expression levels of tumor necrosis factor–alpha (TNF–\(\alpha\)), interleukin–6 (IL–6), inducible nitric oxide synthase (iNOS), cyclooxygenase–2 (COX–2) and nuclear factor kappa–B (NF–\(\kappa\)B) were assayed by semi–quantitative reverse transcriptase polymerase chain reaction (RTPCR) and western blot analysis. \textbf{Results:} We found that the mRNA and protein expressions of TNF–\(\alpha\), IL–6, iNOS, COX–2 and NF–\(\kappa\)B were significantly up–regulated in D-galactosamine induced hepatotoxic rats and treatment with carvacrol significantly down–regulated the expressions of these genes showing the mechanism behind the anti-inflammatory activity of carvacrol. \textbf{Conclusions:} All above results reveal that the carvacrol well known anti–inflammatory activities in D-galactosamine induced hepatotoxic rats.

1. Introduction

Endotoxemia and sepsis occurs frequently in the patients leading to fulminant hepatic failure, which remains an extremely poor prognosis and high mortality due to the lack of effective preventives and therapies\cite{1,2}. D–galactosamine (GalN)–induced acute liver injury in rats is a widely used as experimental animal model to investigate the underlying mechanisms of clinical fulminate hepatic failure and develop an effective therapeutic strategies to endotoxin challenge\cite{3,4}. In this model, the liver injury critically depends on macrophage–derived pro–inflammatory cytokines, including TNF–\(\alpha\), cyclooxygenase–2(COX–2), iNOS, interleukin–6 (IL–6) and NF–\(\kappa\)B. The macrophages of body tissues, especially Kupffer cells of liver, are known to largely contribute to the production of these cytotoxic and inflammatory mediators\cite{5,6}.

TNF–\(\alpha\) is the primary mediator of the systemic toxicity of endotoxin and secretion of cytokines such as IL–1, IL–6 and IL–10 and activates in T–cells and other inflammatory cells\cite{7}. The activation of the immune system has also been shown to play an important role in the development of liver diseases\cite{8,9}. In this regard, recent reports of abnormal presence of a variety of cytokines, in particular, TNF–\(\alpha\), released from inflammatory cells are thought to be important for understanding in detail the mechanism of liver injury\cite{10,11}. Nitric oxides (NOs) are well known pro–inflammatory mediators in the pathogenesis of inflammation\cite{12}. NO produced by iNOS has been reported to have beneficial microbiocidal, anti–viral, anti–parasitic, and anti–tumoral effects\cite{13}. However, sustained NO production can be deleterious to the host, and has been implicated in the pathogenesis of many inflammatory diseases\cite{14}. Prostaglandin (PG), which is synthesized by COX, is another important mediator of inflammation\cite{15}. Two distinct COX isoforms are well known. COX–1 is constitutively expressed in nearly all tissues, and provides PGs to maintain physiological functions, such as, cytoprotection in the stomach and the regulation of renal blood flow. In contrast, COX–2 is induced by several proinflammatory stimuli, such as, growth factors, cytokines...
and endotoxin. In particular, PGE2 is a major COX–2 product at inflammatory sites, where it contributes to local blood flow increase, edema formation, and pain sensitization[16,17].

NF-κB is one of the most important transcription factors in terms of inflammatory responses, and controls the expressions of numerous genes, such as, iNOS, COX–2, TNF–α, IL–6, and IL–1β[18,19]. NF–κB is predominantly a heterodimer composed of p65 and p50 subunits. In an unstimulated cell, NF–κB is located in the cytoplasm due to binding with inhibitors of κB (IκB). However, pro-inflammatory stimuli cause IκB to be rapidly phosphorylated and degraded via IκB kinase complex, and liberated NF–κB is then translocated to the nucleus, where it binds to its target sites and induces the transcriptions of pro-inflammatory mediators[20]. In addition, several studies have reported that the activation of NF–κB is triggered by mitogen–activated protein kinases, such as, extracellular signal–regulated kinase, p38 mitogen–activated protein kinase, and c-Jun N-terminal kinase[21–23]. A major role of MAPKs is the regulation of cell growth and differentiation, as well as the control of cellular responses to cytokines and stresses. In particular, in stimulated macrophages, MAPK phosphorylation is known to be a prerequisite for the productions of NO and proinflammatory cytokines[21,24].

Carvacrol, [2-methyl-5-(1-methylethyl)-phenol] is a predominant monoterpenic phenol which occurs in many essential oils[25]. Carvacrol was reported to produce a wide variety of pharmacological properties including anti-inflammatory[26], antioxidant[27], antitumor[28,29]. In this study, we have evaluated the anti-inflammatory action of carvacrol in D-GalN hepatotoxic rats and no study has been carried out on these aspects so far.

2. Materials and methods

2.1. Chemicals

D–galactosamine, carvacrol, primary and secondary antibodies for TNF–α, IL–6, iNOS, COX–2 and NF–κB were purchased from Sigma–Aldrich Co. (St. Louis, Missouri, USA). All other chemicals used were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

2.2. Animals

Male albino Wistar rats (weighing 160–180 g) were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University and maintained in an air-conditioned room (25±1°C) with a 12 h light/12 h dark cycle. Feed and water were provided ad libitum to all the animals. The study was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg no.160/1999/CPCSEA, Proposal number: 427), Annamalai University, Annamalainagar.

2.3. Experimental induction of hepatotoxicity

Hepatotoxicity was induced in animals by an intraperitoneal injection of D–galactosamine [400 mg/kg body weight(BW)] in a freshly prepared physiological saline as a single dose on the first day.

2.4. Experimental design

The animals were divided into five groups of six animals each as given below. Carvacrol and silymarin were administered orally once in a day in the morning for 6 d.

- Group I: Normal rats received 0.5% DMSO;
- Group II: Normal + carvacrol (20 mg/kg BW);
- Group III: D-GalN control (400 mg/kg BW) in saline;
- Group IV: D-GalN + carvacrol (20 mg/kg BW);
- Group V: D-GalN + silymarin (25 mg/kg BW).

On 8th day morning the animals were anesthetized by an intramuscular injection of ketamine (25 mg/kg BW) and sacrificed by cervical dislocation. Liver was removed, cleared off blood and immediately transferred to ice–cold containers containing saline and used for the determination of various inflammatory markers.

2.5. Reverse transcription polymerase chain reaction (RT–PCR)

For total RNA, the liver tissue samples were minced and homogenized (100 mg/mL) in RNA isolation buffer. The homogenate was transferred to a 15 mL polypropylene tube and added in order: 0.1 mL of 2 M sodium acetate (pH 4.0), 1 mL of saturated phenol (80%) and 0.2 mL CHCl₃: isoamyl alcohol (49:1). Mixed thoroughly by inversion, following the addition of each reagent. After incubation on ice for 15 min, the samples were centrifuged at 10 000 rpm for 15 min at 4°C. To the aqueous phase equal volume of ice–cold isopropanol was added and kept at −20°C for 1 h. The RNA was precipitated at 12 000 rpm for 15 min and washed with 80% ethanol. The pellet was dried briefly in vacuum and dissolved in minimal volume of sterile DEPC treated water. The amount of RNA was quantified by UV–absorbance spectrophotometry. Total RNA (2 μg) was reverse transcribed and 4 μL cDNA obtained was used for polymerase chain reaction (PCR) amplification to estimate the expression of TNF–α, IL–6, iNOS, COX–2 and NF–κB. GAPDH was used as an internal standard. Primer sequences and the resultant PCR products (Gene expressed) are listed in Table 1. After amplification, the PCR samples were
electrophoreses in 1.2% agarose gel stained with ethidium-bromide. The bands were compared by a densitometer using 'Image J' analysis software.

2.6. Primers used for SQRT–PCR studies in rats

| Gene | Primer sequence |
|------|----------------|
| TNF-α | Forward: 5′−GTAGCCCACGTGAGCAGGAC−3′  
Reverse: 5′−CCCTTCCACCTGGGAGAC−3′ |
| IL−6 | Forward: 5′−TGGCTGGAATCTTGGC−3′  
Reverse: 5′−CCGAATGTCCTGGCGGCTGA−3′ |
| iNOS | Forward: 5′−GAGCCTTACGACGGGTG−3′  
Reverse: 5′−GCGAATGGTCCTGGCGGCTGA−3′ |
| COX−2 | Forward: 5′−GGGGCGTCTCCCTGGG−3′  
Reverse: 5′−GGGGAGTACCGAGGAAAG−3′ |
| NF−κB | Forward: 5′−CCAGGGACCTGGCTTCCTTG−3′  
Reverse: 5′−TGGGGCGTCTCCCTGGG−3′ |
| GAPDH | Forward: 5′−TCGAGTCTACTGGCGTCTT−3′  
Reverse: 5′−ATGAGCCCTTCCACGAT−3′ |

2.7. Western blot analysis

Cells were collected by centrifugation and washed once with phosphate buffered saline. The washed cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% NP−40, 1 mM PMSF, 0.5 mM DTT, 5 mM sodium fluoride (NaF), 0.5 mM sodium orthovanadate containing 5 μg/mL each of leupeptin and aprotinin and incubated for 30 min at 4 °C. Cell debris was removed by centrifugation followed by quick freezing of the supernatants. The total protein concentration was determined by Bradford method. 60 μg of protein from control and carvacrol treated proteins were electroblotted on to a nitrocellulose membrane after separation by 10% SDS–polyacrylamide gel electrophoresis. The blot was incubated for 1 h with blocking solution (5% skim milk) at room temperature after incubation for 4 h with a 1:10000 dilution of monoclonal anti-TNF-α, IL−6, iNOS, COX−2, NF−κB and β−actin antibodies (Santa Cruz Biotechnology Inc.). The data were expressed as ratio of TNF−α or IL−6/GAPDH. *P<0.05 compared with normal rats. Lanes: Normal rats-1; Normal + carvacrol-2; D-GalN control-3; D-GalN + carvacrol-4; D-GalN + silymarin-5. GAPDH-glyceraldehyde 3−phosphate dehydrogenase (internal standard).

2.8. Statistical analysis

Statistical evaluation was performed using one−way ANOVA followed by Duncan’s multiple range test using statistical package of social science (SPSS Inc., Chicago, IL, USA) 10.0 for Windows. Significance level was set at P<0.05.

3. Results

3.1. TNF−α, IL−6, iNOS, COX−2 and NF−κB mRNA expression

Figures 1, 2, 3, 4 and 5 show the effect of carvacrol on TNF−α, IL−6, iNOS, COX−2 and NF−κB mRNA expression in D−galactosamine induced hepatotoxic rats, respectively. The mRNA expressions of TNF−α, IL−6, iNOS, COX−2 and NF−κB were up−regulated significantly in D−galactosamine induced hepatotoxic rats while treatment with carvacrol significantly down−regulated the mRNA expressions.
Figure 3. Agarose electropherogram of mRNA (a) and effect of carvacrol on iNOS and COX-2 mRNA level in the liver of D-GalN hepatotoxic rats (b). The data were expressed as ratio of iNOS and COX-2/GAPDH. *P<0.05 compared with normal rats. Lanes: Normal rats-1; Normal + carvacrol-2; D-GalN control-3; D-GalN + carvacrol-4; D-GalN + silymarin-5. GAPDH—glyceraldehyde 3-phosphate dehydrogenase (internal standard).

Figure 4. Band intensity scanned by densitometer (a) and effect of carvacrol on iNOS and COX-2 mRNA level in the liver of D-GalN hepatotoxic rats (b).

Figure 5. Effect of carvacrol on NFκB mRNA level in the liver of D-GalN hepatotoxic rats. a: Agarose electropherogram of mRNA. b: Band intensity scanned by densitometer. The data were expressed as ratio of NFκB/RGAPDH. *P<0.05 compared with normal rats. Lanes: Normal rats-1; Normal + carvacrol-2; D-GalN control-3; D-GalN + carvacrol-4; D-GalN + silymarin-5. GAPDH—glyceraldehyde 3-phosphate dehydrogenase (internal standard).

Figure 6. Effect of carvacrol on TNF-α, IL-6, iNOS, COX-2 and NFκB protein expression in the liver of D-GalN hepatotoxic rats. a). TNF-α, IL-6, iNOS, COX-2 and NFκB protein expression by western blot. b). Band intensity scanned by densitometer. Histograms from densitometric analysis expressed as arbitrary units. *P<0.05 compared with normal rats. Lanes: Normal rats-1; Normal + carvacrol-2; D-GalN control-3; D-GalN + carvacrol-4; D-GalN + silymarin-5.

3.2. TNF-α, IL-6, iNOS, COX-2 and NFκB protein expressions

Figure 6 shows the effect of carvacrol on TNF-α, IL-6, iNOS, COX-2 and NFκB protein expressions in D-galactosamine induced hepatotoxic rats. The protein expressions were up-regulated significantly in D-galactosamine induced hepatotoxic rats and treatment with carvacrol significantly inhibited the protein expression of these genes.

4. Discussion

D-GalN is thought to alter hepatic function by inhibiting hepatic mRNA synthesis through depleting uridine phosphates and uridine diphosphate sugars[30]. This results in an inability of the cells to produce important components of the cell membrane, leading to cell damage. The Kupffer
cells are thought to play a major role in response to inflammatory agents, mainly to those entering the circulation from the gut, which, in turn, promotes the expression of TNF-α [31]. TNF-α is known to be a pleiotropic cytokine that contributes to the triggering of an inflammatory cascade involving the induction of cytokines including IL-1, IL-6, IFN-γ, nitric oxide and cell adhesion molecules, etc [32,33]. In respect of apoptosis, TNF-α combined with TNF-α receptor on the hepatocyte membrane activates caspase-3 and eventually induces apoptosis at an early stage through a series of signal transmission. It has been reported that TNF-α-induced neutrophil transmigration at the later stages of liver injury has been shown to be a critical step in hepatocyte necrosis [34,35]. Therefore, TNF-α is in association with a wide range of inflammatory or auto-immune diseases including various liver lesions. IL-6 acts as a pro-inflammatory cytokine in the context of endotoxemia and acute liver failure. It is well known that IL-6 serum levels correlate with the severity of sepsis [36,37]. IL-6 can also enhance TNF-α-induced apoptosis of hepatocytes sensitized by D-galactosamine [38]. In our study, D-GalN toxicity significantly upregulated the mRNA and protein expressions of hepatic TNF-α and IL-6 and treatment with carvacrol significantly reduced the mRNA and protein expression. The results implicated that carvacrol could attenuate inflammatory processes by suppressing the expression of TNF-α and IL-6 and thereby alleviating liver injury.

Recently, there has been much interest in the role of improper activation or up-regulation of iNOS or COX-2 in the pathogenesis of inflammatory disorders, including toxin-induced liver damage [39,40]. The iNOS-catalyzed oxidative deamination of L-arginine to produce NO following exposure to pro-inflammatory cytokines (e.g., TNF) or endotoxins (e.g., lipopolysaccharide), could trigger disadvantageous cellular responses and may result in inflammation and sepsis [31]. COX-2 is another important inflammatory mediator through its rate-limiting synthesis of the precursors of prostaglandins and thromboxanes [42]. The consequence of high levels of iNOS and COX-2 causes the production of high concentrations of NO and eicosanoids through the initiation of the COX–prostanoid pathway, respectively [43,44], which cause cellular inflammation and necrosis. Treatment with carvacrol leads to a reduction in the expression level of both iNOS and COX-2. This may in part be the result of the regulatory activity and expression of NF-κB. We propose that anti-inflammatory effect of carvacrol may in part be regulated by TNF-α and NF-κB which could attenuate the downstream inflammatory process. In our recent study, we have shown that carvacrol treatment effectively diminished D-GalN-induced liver toxicity by reducing the oxidative stress [45]. In the present study showed that iNOS and COX-2 mediates acute D-GalN-induced liver injury and their inhibition by carvacrol exerts beneficial effects in the prevention of acute hepatic damage.

In addition to inducing direct cellular damage, oxidative stress could activate transcription factors including NF-κB which regulate the expression of various inflammatory genes implicated in hepatotoxicity [46]. NF-κB plays a key role in the process of inflammation. The expression of iNOS and COX-2 has been shown to be dependant on NF-κB activation [47]. Carvacrol significantly suppressed the expression of iNOS, COX-2, IL-6 and TNF-α genes. This was most likely due to suppression of NF-κB. This is consistent with the reports that NF-κB response elements are present on the promoter for the IL-6, iNOS, COX-2 and TNF-α genes [48–50]. In unstimulated cells, NF-κB is found in cytoplasm and is bound to IκBα and IκBβ, which prevents it from entering the nucleus [51]. The release of NF-κB from IκB results in the passage of NF-κB into the nucleus, where it binds to specific sequence in the promoter region of target genes. In the present study, carvacrol significantly suppressed the expression of NF-κB probably through the inhibition of degradation of IκB, which, in turn, suppressed the expression of TNF-α, IL-6, COX-2 and iNOS genes.

Thus, the administration of carvacrol suppressed hepatic injury in rats by inhibiting the TNF-α, IL-6, COX-2, iNOS and NF-κB mRNA and protein expressions in liver. Our results thus provide a mechanistic basis for the anti-inflammatory activity of carvacrol.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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