Inhibitors of inducible nitric oxide (NO) synthase are more effective than an NO donor in reducing carbon-tetrachloride induced acute liver injury

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Summary. The exact functional role of nitric oxide (NO) in liver injury is currently a source of controversy. NO is enzymatically synthesized by nitric oxide synthase (NOS). In this study, we assessed the role of inducible NOS (iNOS) in carbon tetrachloride (CCl₄)-induced acute liver injury using inhibitors of iNOS, and an NO donor. Adult ICR mice were injected with CCl₄ with or without the iNOS inhibitors (5-methylisothiourea hemisulfate [SMT] and L-N⁶-(1-iminoethyl)-lysine [L-NIL]) and an NO donor (Sodium Nitroprusside [SNP]). Blood and liver tissues were collected for analysis. Immunohistochemistry (IHC), serum alanine aminotransferase (ALT), serum total 8-isoprostane analysis, RT-PCR, Western Blotting (WB) and EMSA were done. Our results showed increased levels of ALT, necrosis, total 8-isoprostane and nitrotyrosine after CCl₄ administration. iNOS inhibitors and SNP abrogated these effects but the effect was more pronounced with SMT and L-NIL. RT-PCR, WB and IHC in CCl₄-treated mice demonstrated upregulation of TNF-α, iNOS, and COX-2. The administration of iNOS inhibitors with CCl₄ diminished the expression of these proinflammatory mediators. NF-κB was also upregulated in CCl₄-treated mice and was reversed in mice pretreated with iNOS inhibitors. SNP pretreated mice also showed a lower expression of COX-2 when compared with CCl₄ treated mice but TNF-α, iNOS and NF-κB activity were unaffected. We propose that a high level of nitric oxide is associated with CCl₄-induced acute liver injury and the liver injury can be ameliorated by decreasing the NO level with iNOS inhibitors and an NO donor with the former more effective in reducing CCl₄-induced liver injury.

Key words: Nitric oxide, Liver injury, Oxidative stress; Lipid peroxidation, Inflammation

Introduction

Nitric oxide (NO) is a pluripotent short-lived free radical molecule that influences a variety of physiological functions in most organs and tissues (Marshall et al., 2000). NO has a wide spectrum of functions that includes neurotransmission, immune function, and regulation of blood flow. NO is enzymatically synthesized from the catabolism of L-arginine to L-citrulline by nitric oxide synthases (NOS). At least three different isoforms of NOS are known; all isoforms are found in the liver. eNOS and iNOS are the most abundant isoforms in the liver, the neuronal isoform is present only in the nerve endings of the large blood vessels. In the liver, a low level of NO produced by eNOS regulates hepatic perfusion and is sufficient for cellular signaling (Tanaka et al., 1999). iNOS, in contrast, is readily upregulated in the liver in conditions such as endotoxemia, sepsis and hepatitis (Kane et al., 1997; Wray et al., 1998). iNOS is found principally in hepatocytes (Geller et al., 1993) and Kupffer cells (Harbrecht et al., 1995). However, the precise role of nitric oxide in acute liver injury remains controversial. Appropriate stimulation by a number of agents upregulate iNOS in many cells including those in the liver. These agents include endotoxin and...
inflammatory cytokines. There are conflicting conclusions regarding the nature of the contribution of NO to the pathogenesis of acute toxin-induced liver injury. A beneficial role for NO is based on observations that NO inhibits migration of neutrophils into the liver (Pinzani et al., 1994; Zhang et al., 1994). On the other hand, there is evidence that induction of iNOS plays a harmful role by inducing tissue damage through the formation of peroxynitrite (McKim et al., 2003). The controversy regarding the role of iNOS in acute liver injury could be attributed to the lack of isoform-specific inhibitors. In addition to inhibiting iNOS, inhibitors of NOS are likely to inhibit other enzymes, such as cyclooxygenase-2 and lipid peroxidation.

In this study, we assessed the role of iNOS in CCl₄-induced acute liver injury using inhibitors of iNOS, and an NO donor. We evaluated the differences in the degrees of histological and biochemical indices of CCl₄-induced acute liver injury in mice treated with iNOS inhibitors and an NO donor.

**Materials and methods**

**Animal Experiments**

Eight-week-old male ICR mice, weighing 30-40 g, were maintained in our animal facilities on standard laboratory chow and received care in compliance with the requirements of The University of Hong Kong and the National Institute of Health guidelines. The Laboratory Animal Unit of The University of Hong Kong is fully accredited by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC International). Eight groups of animals (n = 6-8) were studied to determine the effects of NO in acute liver injury by injecting CCl₄ (Merck, Darmstadt, Germany) with or without iNOS inhibitors or NO donor in the ICR mice. Drugs were given as a single dose and the mice were killed 6 hours after injection. iNOS inhibitors and NO donor were given subcutaneously 30 minutes before the intraperitoneal administration of CCl₄. Based on our previous studies where we determined that a dose of 50 µl/kg of CCl₄ and killing after 6 hours were the optimum duration and amount of CCl₄ that would induce necrosis and nitric oxide production, we carried out the study in CCl₄ and saline injected mice which were matched for age, sex and weight (Chen et al., 2004). Based on our pilot experiment, we tried three doses (0.5 mg/kg, 1 mg/kg and 3 mg/kg) of sodium nitroprusside (SNP) and found that 1 mg/kg was the most optimum and effective dose. Similarly, we also tried three doses (10 mg/kg, 30 mg/kg and 50 mg/kg) of 5-methylisothiourea hemisulfate (SMT) and l-N⁵-(1-iminoethyl)-lysine (L-NIL) and the most optimum and effective dose was 30 mg/kg for both drugs. Olive oil was used as a vehicle for CCl₄ and sterile saline for the iNOS inhibitors and NO donor. Blood and liver tissue samples were collected after the mice were killed. The following were the experimental groups: 1) Control + vehicle (saline/olive oil); 2) CCl₄ (50 µl/kg; i.p.); 3) SNP (1mg/kg, s.c., NO donor; Calbiochem La Jolla, CA, USA); 4) SMT (30 mg/kg, s.c., selective iNOS inhibitor; Sigma, St. Louis, MO, USA); 5) L-NIL (30mg/kg, s.c., selective iNOS inhibitor; Alexis Corp., Lausanne, Switzerland); 6) SNP + CCl₄; 7) SMT + CCl₄; 8) L-NIL + CCl₄.

**Tissue collection and histopathological analysis**

Fresh liver blocks were cut and immediately fixed in 10% phosphate buffered formalin, then dehydrated in graded alcohols and embedded in paraffin. Paraffin sections of 5 µm thickness were rehydrated and stained with hematoxylin and eosin. Stained sections were observed under light microscopy and later subjected to image analysis (Leica QWIN, Cambridge, United Kingdom). The percentage area of necrosis was determined by dividing the sum area of necrosis by the sum of the reference area of ten low power fields.

**Determination of alanine aminotransferase (ALT) activity in serum**

Alanine aminotransferase activity in serum was used as a biochemical indicator of hepatic injury. A reaction mixture containing L-alanine (80 mM), NADH (0.2 mM) and lactate dehydrogenase (2 units) in potassium phosphate buffer (0.2 mM, pH 7.4) and serum (0.35 ml) was incubated at 37°C for 3 min to determine the basal rate of NADH consumption at 340 nm. α-Ketoglutarate (10 mM) was subsequently added to measure the rate of NADH utilization by alanine aminotransferase present in serum. The activity of the enzyme was expressed in units per liter of serum. All chemicals used in this assay were obtained from Sigma, St. Louis, MO, USA.

**Enzyme immunoassay (EIA) for total 8-isoprostane**

A competitive immunoassay was used to quantify the 8-isoprostane level of the serum sample (Cayman Chemical, Ann Arbor, MI, USA). The assay is based on the competition between 8-isoprostane and 8-isoprostane-acetylcholinesterase (AchE) conjugate (tracer) for a limited number of 8-isoprostane-specific rabbit anti-serum binding sites. Briefly, 50 μl of purified serum sample in EIA buffer and 50 μl tracer were added to the wells that had been previously coated with antisera. They were then incubated for 18 hours at room temperature and subsequently washed with washing buffer to remove all unbound reagents. Addition of Ellman’s reagent resulted in the development of a yellow color whose optical density was read at 412 nm. The intensity of the color, determined spectrophotometrically, was inversely proportional to the amount of total 8-isoprostane present in the well. The value for the total 8-isoprostane was adjusted by recovery factors for each separate sample after hydrolysis and purification. The amount of total 8-
isoprostane was expressed in picogram per milliliter of plasma.

RNA extraction and analysis of mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

To examine the expression of tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and iNOS in liver tissue, total RNA was extracted using the RNeasy mini kit (QIAGEN Inc., Valencia, CA, USA). The sequences of primer pairs, 5’ and 3’ and the predicted size of the amplified polymerase chain fragments have been previously published (Nanji et al., 1995, 1997). Reverse transcription and amplification (Invitrogen; Life Technologies, CA, USA) were done as described previously (Nanji et al., 1997). After subjecting the PCR products to electrophoresis and ethidium bromide staining, the gels were analyzed by densitometry (Nanji et al., 1997). To normalize signals from different RNA samples, 1 µl of the same reverse transcriptase reaction was amplified with GAPDH specific primers.

Immunohistochemical and Western Blot analysis

Sections were immunostained with antiserum to iNOS using the biotin-avidin-peroxidase method. Briefly, endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 5 minutes at room temperature. The sections were permeabilized in 0.1% trypsin and 0.1% CaCl2, in phosphate buffered saline (PBS, 0.01M, pH 7.5). The sections were pre-incubated with 10% normal serum to reduce non-specific binding of the antiserum, then incubated overnight, at 4°C, with rabbit polyclonal iNOS antibody (Transduction Laboratories, San Diego, CA, USA) diluted at 1:100 in PBS containing 2% normal serum. Control sections were incubated with normal rabbit IgG. Sections were washed three times in PBS, then incubated with biotinylated goat anti-rabbit IgG at a dilution of 1:200 for 30 minutes at room temperature. The sections were further washed and incubated with an avidin and biotinylated peroxidase complex (1:50) for 30 minutes at room temperature. Finally, the sections were washed and the peroxidase was visualized by immersing in 0.05% diaminobenzidine containing 0.03% hydrogen peroxide in Tris-HCL buffer (pH 7.5) for 2 minutes. The sections were rinsed in water and counterstained with hematoxylin. Positive staining was indicated by a brown color.

The specificity and the relative differences of iNOS protein expression were confirmed by Western blot analysis. Briefly, liver tissue was rapidly homogenized and lysed in 5 volumes of cold RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF) on ice for 30 minutes. The cell debris was removed by centrifuging at 1,300 rpm for 30 minutes at 4°C. The protein concentration in the supernatant was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Polyacrylamide gel electrophoresis using 20 µg of total protein was performed using the Mighty Small II run gel system (SE 250/260, Hoefer, Pharmacia Biotech Inc., San Francisco, CA, U.S.A.). The protein was transferred onto a polyvinylidene fluoride blotting membrane using a TE series transfer electrophoresis unit (Hoefer, Pharmacia Biotech Inc., San Francisco, CA, USA). The membrane was incubated in a blocking buffer for 1 hour and then incubated with polyclonal iNOS antibody (1:1000 dilution; Transduction Laboratories, San Diego CA, USA) overnight at 4°C. The membrane was washed and then incubated with a 1:2000 dilution of secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech Inc., Buckinghamshire, England) at room temperature for 2 hour. The iNOS protein was detected using an ECL Western Blotting detection kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, England).

The immunohistochemical staining and Western blot procedures used for nitrotyrosine and COX-2 were similar to that described for iNOS. Instead of using the antibody against iNOS, the sections and liver homogenates were incubated with the anti-nitrotyrosine antibody (Upstate Biotechnology, Lake, Placid, NY, USA) and COX-2 (Cayman Chemicals, Ann Arbor, MI, USA) at 1:200 dilutions for immunohistochemistry and 1:1000 for Western Blotting.

Evaluation of activation of nuclear factor kappa B (NF-κB)

Nuclear protein fractions from liver homogenates were prepared as described previously (Nanji et al., 1999). Electrophoretic mobility shift assays were performed on the nuclear extracts from livers in the different experimental groups as described previously (Nanji et al., 1999). Densitometric scanning analysis was performed using laser scanning densitometry. The specificity of binding was determined by prior addition of 100-fold excess of unlabeled competitor consensus oligonucleotide. Supershift experiments were performed on 5% non-denaturing gels using antiserum against the p50 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Data from each group were expressed as mean ± SEM. A non-parametric Mann-Whitney U test (two-tailed) was used for group comparisons using the GraphPad Prism (GraphPad Software, Inc., San Diego CA, USA). A p value of <0.05 was considered to be statistically significant.

Results

In this study, we assessed the role of iNOS in CCl4-induced acute liver injury using inhibitors of iNOS and
an NO donor. We evaluated the differences in the degree of histological indices of CCl₄-induced acute liver injury in mice treated with iNOS inhibitors and an NO donor. Our results indicated that inhibition of iNOS showed greater protection against acute liver injury than the NO donor.

**Percentage of necrosis**

The amount of hepatocellular necrosis which was represented by the percentage area of necrosis showed the highest amount of necrosis in the CCl₄ treated mice (Figs. 1A, 2A). The vehicle-treated control group and

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**Fig. 1.** A. Haematoxyline and Eosin staining of the liver section in CCl₄ treated mice with evidence of moderate amount of centrilobular necrosis. B. Haematoxyline and Eosin staining of the liver section in L-NIL pretreated mice followed by CCl₄ showing marked reduction in the amount of centrilobular necrosis. C. Immuno-histochemical staining of liver section with antibody against iNOS in CCl₄ treated mice showing intense iNOS protein expression at the centrilobular region and marked reduction in iNOS protein expression in L-NIL + CCl₄ treated mice (D). E. Immunohistochemical staining of liver section with antibody against nitrotyrosine in CCl₄ treated mice showing intense nitrotyrosine formation at the centrilobular region and marked reduction in nitrotyrosine formation in L-NIL + CCl₄ treated mice (F). G. Immunohistochemical staining of liver section with antibody against COX-2 in CCl₄ treated mice showing intense COX-2 protein expression at the centrilobular region and marked reduction in COX-2 protein expression in L-NIL + CCl₄ treated mice (H). n = 6-8; x 200
mice treated either with the iNOS inhibitors (SMT or L-NIL) or SNP only showed no evidence of necrosis. The mice pretreated with SMT and L-NIL followed by CCl\textsubscript{4} showed a marked reduction (p<0.0001) in the amount of hepatocellular necrosis (Figs. 1B, 2A). A significant difference (p<0.001) was also observed among SNP, SMT and L-NIL followed by CCl\textsubscript{4}.

**Serum Alanine Aminotransferase (ALT)**

Overall the values of the serum ALT showed a trend similar to that seen with the degree of hepatocellular necrosis. The serum ALT levels were below 80 U/L in the vehicle control group and mice treated with SNP or SMT or L-NIL only (Fig. 2B). The CCl\textsubscript{4} treated mice had the highest level of serum ALT, the mean value was about 4,200 U/L. Significant marked reduction (p<0.01) in the levels of serum ALT was observed in the mice pretreated with SNP and iNOS inhibitors followed by CCl\textsubscript{4} (Fig. 2B). A significant reduction (p<0.01) was also observed among SNP, SMT and L-NIL followed by CCl\textsubscript{4}.

**Enzyme immunoassay of total 8-isoprostane**

The serum total 8-isoprostane levels were between 320 to 470 pg/ml in the vehicle control groups and mice treated with the drugs only (Fig. 2C). The highest levels of serum total 8-isoprostane were observed in the CCl\textsubscript{4} treated mice with a mean value of about 750 pg/ml. In mice pretreated with SMT, L-NIL and SNP and then followed by CCl\textsubscript{4}, a significant reduction in the levels of serum total 8-isoprostane was observed with the lowest values in the L-NIL pretreated mice (p<0.01). This observation is consistent with the levels of serum ALT and the percentage of necrosis observed in the L-NIL treated groups (Fig. 2A,B). A significant reduction was also observed between SNP and SMT, SNP and L-NIL, and SMT and L-NIL followed by CCl\textsubscript{4} (p<0.05, p<0.01 and p<0.01, respectively).

**RT-PCR, Immunohistochemistry and Western Blotting**

The mRNA levels of TNF-α and iNOS were approximately two-fold higher in the CCl\textsubscript{4} treated mice when compared with the control groups (p<0.01; Fig. 3A,B). Both of the mRNA levels of TNF-α and iNOS were markedly reduced in the SMT + CCl\textsubscript{4} and L-NIL + CCl\textsubscript{4} treated mice (p<0.01) when compared with CCl\textsubscript{4} treated mice. Of note is that the L-NIL + CCl\textsubscript{4} treatment group had the lowest levels of TNF-α and iNOS mRNA (p<0.001 and p<0.01, respectively). COX-2 mRNA in liver was increased in CCl\textsubscript{4} treated mice and was significantly lower (p<0.01) in the CCl\textsubscript{4} treated mice which were pretreated with SNP, SMT and L-NIL (Fig. 3C). The control groups did not express COX-2 mRNA.

The immunohistochemical analysis showed an increased expression of iNOS as well as the formation of nitrotyrosine in the CCl\textsubscript{4} treated mice and significant

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**Fig. 2.** A. The percentage of area of necrosis in the controls and the different experimental groups. Administration of CCl\textsubscript{4} increased the degree of necrosis (p<0.0001) and was significantly reduced (p<0.0001) by pretreatment with the iNOS inhibitors and NO donor. No necrosis was observed in the control groups. A significant difference (p<0.001) was also observed among SNP, SMT and L-NIL followed by CCl\textsubscript{4}. B. The serum alanine aminotransferase (ALT) levels in the controls and the different experimental groups. The serum ALT levels were increased (p<0.01) in the CCl\textsubscript{4} treated group and were significant reduced (p<0.01) in the groups pretreated with SMT, SNP and L-NIL+ CCl\textsubscript{4}. A significant reduction (p<0.01) was also observed among SNP, SMT and L-NIL followed by CCl\textsubscript{4}. C. The serum total 8-isoprostane levels in the controls and the different experimental groups. Serum total 8-isoprostane levels paralleled the degree of liver injury in the rats receiving the CCl\textsubscript{4} treatment and iNOS inhibitors. Administration of CCl\textsubscript{4} increased the serum total 8-isoprostane levels (p<0.05) and was significantly reduced (p<0.01) by pretreatment with the iNOS inhibitors and NO donor. (n=6-8).
reduction was detected in the CCl4 treated mice pretreated with SMT or L-NIL. These proteins were localized mainly in the hepatocytes in the centrilobular region of the liver (Fig. 1C-F).

To assess the validity of changes in iNOS protein by immunohistochemical analysis, we analyzed iNOS protein expression using Western Blot analysis. The data confirmed the observation made with immunohistochemistry where iNOS protein expression in CCl4 and SNP + CCl4 treatment showed the highest values (p<0.05) when compared with the controls and other treatment groups (Fig. 4A). Significant reductions in the iNOS protein expression were observed with pretreatment with SMT or L-NIL (p<0.05 and p<0.01, respectively) prior to CCl4 when compared with the CCl4 alone or SNP + CCl4 treated mice. The level of the formation of nitrotyrosine showed a trend similar to that seen with iNOS protein expression (Fig. 4B). Basal formation of the nitrotyrosine was detected in the control mice (vehicles, SNP, SMT and L-NIL). The CCl4 treated mice showed the highest formation of nitrotyrosine and a significant reduction (p<0.01) was observed after pretreatment in CCl4 treated mice with SNP, SMT and L-NIL (Fig. 4B). Pretreatment with L-NIL and CCl4 showed concentrations of nitrotyrosine formation similar to those seen in controls.

Immunohistochemical analysis of COX-2 protein showed that COX-2 protein was expressed mainly in the centrilobular hepatocytes of CCl4-treated mice and a significant reduction was observed in the SMT and L-NIL pretreated mice administered with CCl4 (Fig. 1G-H). The lowest level of COX-2 expression was seen in the SMT and L-NIL plus CCl4 treated mice. Western Blotting analysis confirmed this trend which showed that COX-2 protein expression was highest in CCl4-treated mice with a marked reduction (p<0.01) in mice pretreated with SNP, SMT or L-NIL followed by CCl4. Control mice did not show expression of COX-2 protein (Fig. 4A).

Electrophoretic Mobility Shift Assay (EMSA)

To evaluate the DNA binding activity of NF-κB, electrophoretic mobility shift assay (EMSA) of nuclear protein extracts from whole liver was performed. Nuclear localization of NF-κB was increased (p<0.01) in CCl4 treated mice. SNP pretreatment did not significantly decrease the degree of NF-κB activation in CCl4 treated mice. However NF-κB activation was significantly reduced (p<0.001) in mice administered with SMT and L-NIL prior to CCl4 treatment (Fig. 5). The reduction in activation of NF-κB was significantly greater in mice pretreated with L-NIL followed by CCl4 (p<0.001). The protein/DNA complex was further characterized by using competition and supershift assays. A 100-fold excess of non-radioactive NF-κB or STAT 3 oligonucleotide was added to an EMSA binding reaction containing nuclear protein extracts from the CCl4-treated mice. Addition of the NF-κB

**Fig. 3.** Reverse-transcriptase-polymerase chain reaction analysis of messenger RNA (mRNA) concentrations of (A) tumor necrosis factor-alpha (TNF-α), (B) inducible nitric oxide synthase (iNOS), and (C) cyclooxygenase-2 (COX-2) in liver samples obtained from mice in the different experimental groups. Treatment with the iNOS inhibitors SMT and L-NIL led to a significant decrease (p<0.001 and p<0.01 respectively) in TNF-α and iNOS mRNAs. Treatment with SNP, the NO donor, had no effect of both the TNF-α and iNOS mRNAs. However, the two iNOS inhibitors and an NO donor decreased (p<0.01) the COX-2 mRNA levels. (n=6-8).
oligonucleotide completely abrogated complex formation, and addition of STAT 3 oligonucleotide had no effect (data not shown).

**Discussion**

We chose carbon tetrachloride as a hepatotoxin that induces acute liver injury characterized by expression of proinflammatory mediators as well as evidence of hepatocellular necrosis. CCl$_4$ in the liver is converted to trichloromethyl free radicals by its dehalogenation by cytochrome P-450 2E1, which form the trichloromethyl peroxyl radicals (Halliwell and Gutteridge, 1984). This reactive oxygen species (ROS) generates lipid peroxides which in addition to activating Kupffer cells also induce cell membrane dysfunction leading to hepatocellular damage. The time interval of six hours used in the current study is within a time period when proinflammatory mediators such as TNF-α and iNOS are expressed (Morio et al., 2001; Chen et al., 2004). Our data indicated that at a dose of 50 µl/kg of CCl$_4$, a moderate amount of centrilobular necrosis was observed in CCl$_4$ treated mice and the degree of necrosis was significantly ameliorated by pretreatment with the iNOS inhibitors and an NO donor. The reduction in necrosis was greater in the mice pretreated with the iNOS inhibitors, L-NIL and SMT than in SNP pretreated mice. The partial protective effect of SNP can, in part, probably be attributed to its ability to scavenge the lipid peroxyl radical LOO$^-$ and diminish the level of oxidative stress (Zhu and Fung, 2000). Consistent with this
hypothesis is the observation that there was a significant reduction seen in the levels of serum total 8-isoprostane and liver tissue nitrotyrosine in the SNP treated mice.

**Effects of iNOS inhibitors on hepatocellular damage**

The notion that NO is involved in acute liver injury is based on observation that increased NO production is associated with toxin-induced liver injury. However, whether the augmented production of NO serves a protective or deleterious role in the liver remains a controversial issue. The fact that expression of iNOS and the formation of nitrotyrosine, a footprint of peroxynitrite formation, coincided with liver injury supports the contention that iNOS is involved in toxin-induced liver injury. The observation in the current study was that the iNOS inhibitors, SMT and L-NIL were able to significantly reduce the degree of hepatic damage caused by CCl₄ treated mice. In the SMT treated mice but not iNOS inhibitors. In support of the observation that SNP acts mainly by suppressing lipid peroxidation, we showed that levels of TNF-α and iNOS were not affected by SNP pretreatment.

The main findings of our study was that iNOS inhibitors and SNP attenuated the effects of CCl₄ administration and elicited a significant reduction in liver injury as indicated by the decreased in hepatocellular damage, decreased alanine amino-transferase activity and total 8-isoprostane in serum, decreased expression of iNOS (only in iNOS inhibitors) and reduced nitrotyrosine formation. These effects were more pronounced in iNOS inhibitors than NO donor. The iNOS inhibitors with CCl₄ diminished the expression of proinflammatory mediators and NF-κB activity. SNP pretreated mice showed lower expression of COX-2 when compared with CCl₄ treated mice but TNF-α, iNOS and NF-κB activity were unaffected. We propose that a high level of nitric oxide is associated with CCl₄-induced liver injury which can be ameliorated by reducing the nitric oxide level with iNOS inhibitors and an NO donor with the former more effective than the latter. The effect of SNP could be partly attributed to its ability to scavenge lipid peroxyl radical LOO•.

**Effect of the NO donor (SNP) on CCl₄-induced hepatotoxicity**

Although treatment with SNP reduced the degree of hepatic damage assessed by necrosis and ALT levels, a major difference seen between SNP and the iNOS inhibitors was that the levels of TNF-α and iNOS mRNAs were reduced with iNOS inhibitors but unaffected by SNP. The lesser degree of protection provided by SNP versus the iNOS inhibitors probably relates to the fact that SNP was less able to inhibit NF-κB, TNF-α and iNOS than the iNOS inhibitors.

Additionally, nitric oxide interacts with superoxide (O₂•⁻) to form peroxynitrite, which is a very potent oxidant. Peroxynitrite in turn induces oxidative stress, leading to hepatic cellular damage and necrosis. CCl₄ treated mice showed significant increases in the amount of necrosis, serum ALT, serum total 8-isoprostane, nitrotyrosine and proinflammatory mediators such as TNF-α, iNOS and COX-2 mRNAs and proteins. These effects were markedly abrogated by pretreatment with iNOS inhibitors. In support of the observation that SNP acts mainly by suppressing lipid peroxidation, we showed that levels of TNF-α and iNOS were not affected by SNP pretreatment.

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