3*H*-1,2-Dithiole-3-thione targets nuclear factor κB to block expression of inducible nitric oxide synthase, prevents hypotension, and improves survival in endotoxemic rats

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Running Title: Dithiolethione protection during endotoxemia

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Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BP, aterial blood pressure; D3T, 3*H*-1,2-dithiolethione; EMSA, electrophoretic mobility shift assay; eNOS, endothelial nitric oxide synthase; IκB, inhibitor of NFκB; NFκB, nuclear factor kappa beta; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide.

Gastrointestinal, Hepatic, Pulmonary, Renal

Abstract

Septicemia is a major cause of death associated with non-coronary intensive care. Systemic production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is a major cause of hypotension and poor organ perfusion seen in septic shock. Here we show that pretreatment of F344 rats with the cancer chemoprotective agent, 3*H*-1,2-dithiole-3-thione (D3T), blocks lipopolysaccharide (LPS)-mediated induction of hepatic iNOS, and significantly reduces the associated serum levels of NO metabolites and enzyme markers of toxicity provoked by treatment with lipopolysaccharide (LPS). Immunohistochemical analysis shows that this protective effect is largely due to suppression of iNOS expression in hepatocytes. Importantly, pretreatment of animals with D3T blunts LPS-mediated hypotension and dramatically increases their survival. Inasmuch as iNOS expression can be regulated by nuclear factor kappa beta (NFκB), mechanistic studies show that D3T blocks NFκB nuclear translocation and DNA binding, and that these effects are accompanied by changes in the levels of phospho-IκBα. In conclusion, this study identifies new drug classes and targets that may improve the prevention and treatment of septic shock, as well as chronic diseases associated with the NFκB and iNOS pathways.

Introduction

Systemic production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is a major cause of the hypotension and poor organ perfusion that is seen in septic shock (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990). Studies of iNOS *null* mutant mice have shown such mice to be resistant to the hypotension and death caused by lipopolysaccharide (LPS) (MacMicking et al., 1995; Wei et al., 1995). Inhibition of nitric oxide synthase activity with selective inhibitors of iNOS have been shown to have beneficial effects in rodent models of septic shock, whereas inhibition of endothelial nitric oxide synthase (eNOS) may lead to adverse effects including enhanced organ damage and excessive vasoconstriction (Thiemermann, 1997; Vos et al., 1997). Treatments such as dexamethasone that act to block the expression of iNOS protein decrease circulatory and organ failure, although such agents must be administered prior to endotoxin in order to achieve these beneficial outcomes (reviewed in Thiemermann, 1997).

Dithiolethiones such as 3*H*-1,2-dithiolethione (D3T) are potent chemopreventive agents that induce phase 2 and antioxidative enzymes to enhance the detoxication of chemical carcinogens. Moreover, dithiolethiones have been shown in animals to protect against neoplasia in multiple target organs (Kensler et al., 1999). Use of these agents for the prevention of cancer in humans is currently under clinical investigation (Wang et al., 1999; Lam et al., 2002). Both the cancer chemopreventive and gene expression effects of D3T have been shown to be mediated by the Keap1-Nrf2 pathway (Kwak et al., 2001; Kwak et al., 2003). Keap1 is a sulfhydryl-rich protein that sequesters Nrf2 in the

cytoplasm. Administration of sulfhydryl reactive compounds such as sulforaphane (SFN) or D3T abolishes the Keap1-Nrf2 interaction, facilitating nuclear translocation of Nrf2 (Venugopal and Jaiswal, 1996; Itoh et al., 1997; Dinkova-Kostova et al., 2002; Sekhar et al., 2002; Zipper and Mulcahy, 2002). Nrf2, a "cap n collar" basic region leucine zipper transcription factor, forms heterodimers with small Maf proteins to regulate genes containing antioxidant response elements in their upstream regulatory regions (Venugopal and Jaiswal, 1996; Itoh et al., 1997). Of particular relevance to this study, Vos and coworkers reported that depletion of glutathione by pretreatment of rats with diethylmaleate prevented the induction of hepatic iNOS RNA and protein 6 hr post-treatment with LPS (Vos et al., 1999). While these authors related this effect only to intracellular glutathione status, earlier studies on the regulation of phase 2 enzyme induction showed that agents that lowered glutathione levels were effective activators of reporter gene transcription driven by an antioxidant response element (Bergelson et al., 1994). In 2001, Heiss *et al*. showed that co-treatment of RAW cells with SFN blocked the LPS-mediated induction of iNOS mRNA and protein. SFN did not directly interact with NO or inhibit iNOS activity. Rather, SFN was shown to inhibit DNA binding of NFκB, without affecting the degradation of the inhibitor of NFκB, IκB (Heiss et al., 2001). Earlier this year, the link between the Keap1-Nrf2 phase 2 regulatory pathway and protection against oxidant and inflammatory stress was established *in vitro* (Dinkova-Kostova et al., 2005). Potent anti-inflammatory triterpenoids (Honda et al., 2000) were shown to block the induction of iNOS and cycloxygenase-2 by γ-interferon (IFN-γ) in RAW cells. A strong correlation of the potency of these triterpenoids for this anti-inflammatory effect and induction of a phase 2 response was demonstrated over a range

of several log concentrations. Using mouse embryo fibroblast from wild-type and Nrf 2-deficient mice, these authors established that the effect of triterpenoids to block NO production in response to treatment of these cells with IFN-γ and tumor necrosis factor-α was Nrf2-dependent (Dinkova-Kostova et al., 2005).

Collectively, these reports support the hypothesis that agents that activate Nrf2 signaling could effectively suppress iNOS elevation, NO production, and lessen the severity of endotoxin-induced toxicity *in vitro*. Herein we report the results of a study of an *in vivo* model of LPS-induced toxicity. Pretreatment with a potent activator of Nrf2 blocked induction of iNOS, lessened hypotension and other indices of systemic toxicity, and dramatically improved survival of LPS-treated rats.

Methods

Animals and Treatments. The experiments were carried out on 6-8 week old male Fischer F344 rats (Harlan, Indianapolis, IN) weighing 116-160 g. The animals received humane care in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animlas.* Semi-purified diet (AIN-76A formulation, Harlan Teklad, Madison, WI) and water were available *ad libitum*. All experimental protocols were approved by the Animal Welfare Committee of the University of Memphis. LPS, *E. coli* serotype 0127:B8, was from Sigma (St. Louis, MO); D3T was from LKT (St. Paul, MN). Rats were divided randomly into four groups, and acclimated to the diet for 1 week. Animals in Groups 1 (V) and 3 (VL) were given vehicle, saturated sucrose, by gavage on three alternate days; Groups 2 (D) and 4 (DL) were given D3T suspended in saturated sucrose at a dose of 0.3 mmol/kg body weight by gavage on three alternate days, as per (Roebuck et al., 2003). Twenty-four hr after the last treatment with either vehicle or D3T, animals in Groups 1 (V) and 3 (D) were injected i.p. with PBS (pH 7.4); Groups 2 (VL) and 4 (DL) were injected i.p. with LPS in PBS (pH7.4) at a dose of 5 mg/kg body weight, as per (Vos et al., 1999). For biochemical endpoints, 4 animals/group were treated for 6 hr with PBS or LPS. The animals were euthanized with pentobarbital (60 mg/kg, i.p.), and the livers were removed, washed in PBS, cut into small pieces, and either snap frozen in liquid nitrogen or used directly for nuclear protein isolation. Heparinized blood samples were drawn by cardiac puncture for determination of serum aspartate transaminase (AST), alanine transaminase (ALT) and the NO metabolites, nitrite plus nitrate. To determine the impact of D3T pretreatment on the LPS-induced hypotension, blood pressure (BP) was measured on the ventral artery of tails by

simultaneously detecting the pulse of the artery and the pressure of an inflatable tail cuff (Krege et al., 1995; Johns et al., 1996). The rats were held in a restrainor of appropriate size to immobilize the tail and trained 15 min per session for three days to familiarize the animals with the restraint and to reduce the variation of the BP measurement. The cuff was fitted around the tail and inflated/deflated using a manual rubber-bulb. The pulse sensor SS4LA and pressure sensor SS19L were connected to the monitor of a Biopac MP35 system with multiple channels (Biopac Systems, Inc, Goleta, CA). The environment temperature around the rat tail was controlled at 32^{o}C throughout the BP measurement. After catching the pulse, the cuff was inflated to block the blood flow on the tail artery. The cuff was then slowly deflated until recovery of the pulse. The pressure change and pulse were recorded by computer in order to determine the systolic pressure, which was used to represent BP in this study. For survival analysis, animals were individually housed and monitored for a period of 18 hr, following the last measurement of BP. Surviving animals were euthanized by asphyxiation with CO_2.

Enzymes, NO Metabolite, and GSH Assays. The activities of ALT and AST in plasma were measured using kits from Biotron Diagnostics Inc (Hemet, CA) (Vos et al., 1999). In order to measure total nitrite plus nitrate, plasma samples were thawed and deproteinized by incubating them with 95% ethanol at 4^{o}C for 30 min, followed by centrifugation for 10 min at 13000 x g. NO metabolites were measured according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim Germany). Levels of intracellular GSH equivalents were determined using a colorimetric detection assay (BioVision Research Products Mountain View CA) based on the method of (Akerboom and Sies, 1981). Tissue samples were homogenized in buffer, mixed with 5%

sulfosalicylic acid, and centrifuged at 8000 x g for 10 minutes. Glutathione content in the supernatant was determined as the conversion of DTNB [5.5'-dithiobis-(2-nitrobenzoic acid)] to colored 2-nitro-5-thiobenzoic acid by measuring the spectrophotometric absorbance at 415 nm in a Benchmark Microplate Reader (Bio-Rad Hercules CA). A standard curve was made using GSH.

Protein Isolation and Western Blot Analysis. Total protein was isolated from the frozen tissue and used for iNOS analysis by western blot (Vos et al., 1999). Sixty µg of each sample was separated by SDS/PAGE under reducing conditions and transferred onto 0.2 µm nitrocellulose membranes (Schleicher & Schuell, Keene, NH). iNOS was identified using an affinity-purified rabbit polyclonal antibody (50 µg/ml) (BD Biosciences, San Jose, CA). Nuclear and cytosolic proteins were extracted with NE-PER reagents (Pierce Laboratories, Rockford, IL) following the company's protocol and stored at -80^{o}C in aliquots. Nuclear (2 µg) and cytosolic (50 µg) proteins of each sample were used for the assay of p65 by western blot, using rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). IkB-α (Santa Cruz Biotechnology), phospho-IkB-α (Cell Signaling, Beverly, MA), and IkB-β (Santa Cruz Biotechnology) were used for other western blot analyses. For detection of eNOS, protein-containing particulate fractions were prepared from frozen liver samples according to (Pollock et al., 1991), with some modifications. Tissues were suspended in 10 volumes of ice-cold buffer A (50 mM Tris-HCl (pH-7.4), 0.1 mM EDTA, 0.1% 2-mercaptoethanol), containing 1 mM phenylmethylsulfonyl fluoride and 2 µM protease inhibitor cocktail (Sigma, P8340), and homogenized in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 600 x g for 10 min and the supernatant was again centrifuged at

100,000 x g for 60 min. The pellet was resuspended in Buffer A with 10 % (v/v) glycerol. Electrophoresis was carried out in the Bio-Rad mini gel apparatus using 7.5% SDS-PAGE gels under reducing conditions. Levels of eNOS protein were detected using specific antibodies against eNOS (BD Transduction, Cat. # 610296, San Jose, CA) at a 1:1000 dilution. The immunocomplexes were detected by enhanced chemiluminescence (Super–Signal System, Pierce Laboratories, Rockport, IL). The density of bands was quantified by using a scanner (Expression 836XL, EPSON, Long Beach, CA) and measurement software (Image J, W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/, 1997-2005).

Immunohistochemistry. Immunohistochemistry was carried out as previously described (Vos et al., 1999). Briefly, samples were prepared by mounting 4-μm slices of formalin-fixed/paraffin-embedded liver sections. Slides were deparaffinized with xylyne and stained with hematoxylin and eosin for routine histology. For immunohistochemistry, polyclonal iNOS antibody (2 μg/ml) (Santa Cruz Biotechnology) or normal rabbit serum, as a negative control, were used. After incubation with the secondary antibody, the slides were washed with PBS and labeled with a biotin-horseradish peroxidase conjugate, and developed by incubation with 3, 3'-diaminobenzidine and hydrogen peroxide (ABC kit, Vector Laboratories, Burlingame, CA). The slides were dehydrated and counter-stained with hematoxylin.

Electrophoretic Mobility Shift Assay (EMSA). DNA binding conditions for NFκB and EMSA were as described (Lenardo and Baltimore, 1989). Oligonucleotides from the consensus binding site for NFκB were used: (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and reverse complement) (Integrated DNA Technologies, Coralville, IA). Each

NFκB binding oligonucleotide was end-labeled with biotin using a biotin 3'-end labeling kit (Pierce Laboratories, Rockford, IL), and annealed by incubating equal concentrations and heating to 75^{o}C, followed by gradual cooling to room temperature. Binding reactions were performed at room temperature for 25 min using 1 μg of nuclear protein and 20 fmol of biotin-labeled NFκB. DNA-protein complexes were separated on native 6% polyacrylamide gels, and electroblotted onto positively charged Biodyne B nylon membranes. Detection of biotin-labeled DNA by chemiluminescence was performed according to protocol provided in the Lightshift Chemiluminescent EMSA kit (Pierce Laboratories). Cold competition was performed using consensus or mutant NFκB oligonucleotides. Supershift experiments were carried out by adding anti-p65 or -p50 antibodies (Santa Cruz Biotechnologies) to the binding mixture prior to PAGE.

Statistical Analysis. All data were expressed as mean ± SD. Pairwise comparisons were performed by Student t-test. Survival following LPS challenge was assessed by the chi-square test. Differences were considered statistically significant at values of $p \leq 0.05$.

Results

Effect of D3T on iNOS Expression, NO Metabolites and Serum Enzymes. Hepatic iNOS protein is not detected by western blotting in vehicle treated rats (Fig. 1A, upper, lanes 1-2, **V**). Six hr after administration of LPS, hepatic iNOS protein is markedly induced in vehicle-pretreated rats (Fig. 1A, lanes 5-6, **VL**). This effect of LPS on iNOS expression is blocked by pretreatment with D3T (Fig.1A, lanes 7-8, **DL**). As a control for determining response to D3T pretreatment, aflatoxin aldehyde reductase (Afar), a prototype for inducible phase 2 enzyme response, is markedly elevated in response to pretreatment on three alternate days with D3T (Fig. 1A, lower, lanes 3-4; lanes 7-8, **D** and **DL**), whereas levels of iNOS protein are not affected (Fig. 1A, lanes 3-4, **D**). Replication of the effect of D3T pretreatment on the induction of iNOS in response to LPS is shown in Fig. 1B, upper panel. As shown, the action of D3T to block iNOS induction in response to LPS is not complete, and the magnitude of this effect varies between animals (Fig. 1B, lanes 5-8). However, on average, levels of iNOS protein are reduced 80% and this effect is statistically significant (Fig. 1B, lower panel, $p<0.01$). Levels of NO metabolites, total nitrite plus nitrate, measured in the serum were markedly increased by treatment with LPS (Fig. 1C, **VL**). These levels were significantly reduced by pretreatment with D3T ($p< 0.05$), although they remained significantly elevated relative to either vehicle or D3T pretreated animals not receiving LPS (Fig. 1C). Similarly, the level of serum ALT, a liver enzyme released into the serum upon hepatic injury, was significantly lower in D3T plus LPS-treated animals compared to animals treated with LPS alone (Fig. 1D, **VL**-**DL**, $p< 0.05$); yet levels of this enzyme in the D3T-LPS treated animals were about 2-fold greater than the levels observed in serum from

either vehicle- or D3T-treated animals (Fig. 1D). For AST, another serum marker, pretreatment with D3T afforded significant protection against the toxic effects of LPS (Fig. 1E, **VL**-**DL**, $p < 0.05$), with no difference observed between vehicle-, D3T-, or D3T plus LPS-treated animals (Fig. 1E).

Histology and iNOS Immunohistochemistry. Hepatic tissue of vehicle- or D3T-treated animals appeared normal in structure, with none or few inflammatory cells in the portal tracts or parenchymas. There were no major differences in the histology of D3T plus LPS-treated animals and LPS-treated animals, 6 hr post LPS treatment. Both sets of samples showed occasional areas of perivascular lymphocytes and scattered areas of cell necrosis. In the D3T plus LPS-treated animals, one animal showed two large areas of necrosis, whereas the other animals showed only occasional foci of necrosis and occasional neutrophils. In the LPS-treated animals, two animals showed more than 10 regions of necrosis involving 10-30 cells and frequent neutrophil infiltration, whereas the other two LPS-treated animals showed focal necrosis and occasional neutrophils (data not shown). At 6 hr after treatment with LPS, iNOS staining was pronounced in hepatocytes (Fig. 2C). A marked decrease of iNOS observed in the hepatic tissue of D3T plus LPS-treated animals was a result of diminished iNOS expression in hepatocytes (Fig. 2D).

Effects of D3T and LPS on eNOS Expression. Because studies of inhibitors of nitric oxide synthases have shown that non-selective inhibitors that inhibit both iNOS and eNOS activities increase tissue damage and circulatory failure (Thiemermann, 1997), we determined the effect of the treatments on the expression of eNOS protein. The level of expression of hepatic eNOS expression was not altered by any of the treatments as determined by western blot analysis (Fig. 3).

Effects of treatment on GSH levels. Previously, it had been reported (Vos et al., 1999) that depletion of glutathione prevented the induction of hepatic iNOS protein 6hr post-treatment with LPS. To rule out similar effects of D3T, we determined hepatic levels of GSH (Fig. 4). As expected, treatment of animals with D3T resulted in about a 3-fold increase in hepatic GSH levels (Fig. 4, **V, D**). While LPS treatment slightly decreased GSH content, this effect was not statistically significant. However, in animals pretreated with D3T and then challenged with LPS, the levels of GSH were lower than those observed for animals treated only with D3T. Moreover, animals pretreated with D3T and then challenged with LPS maintained significantly higher levels of GSH (Fig. 4, **VL, DL**).

D3T Targets NFκB Regulation of iNOS. Based on the published results of *in vitro* studies of SFN in RAW cells (Heiss et al., 2001) and diethylmaleate in primary cultures of rat hepatocytes (Vos et al., 1999), we focused our initial investigation of the mechanism of the D3T effect to block LPS-mediated induction of iNOS on NFκB, an established regulator of iNOS expression. By EMSA, no NFκB DNA binding was observed in nuclear extracts prepared from liver tissue of either vehicle- or D3T-treated rats (Fig. 5A, lanes 1-2). NFκB DNA binding was observed in samples prepared from LPS-treated animals (Fig. 5A, lane 3). This binding complex was shown by supershift assay to contain both p65 and p50 proteins (Fig. 5A, lanes 6-7). Pretreatment of animals with D3T blocked the LPS-stimulated increase in NFκB DNA binding (Fig. 5A, lane 4). Consistent with these results, western blot analysis detected p65 protein only in nuclear extracts prepared from liver tissue of animals treated with LPS alone (Fig. 5B, upper panel, lanes 5-6). Levels of p65 protein in cytosolic fractions did not vary greatly

between the treatment groups (Fig. 5B, lower panel), consistent with the knowledge that nuclear localization of NFκB is regulated through the degradation of its cytosolic protein partner/inhibitor, IκB (Baeuerle and Baltimore, 1988; Zandi et al., 1997). Canonical cytokine-mediated activation of the NFκB pathway involves phosphorylation of IκBα by IκB kinase, followed by IκBα ubiquitination and proteosome degradation (Courtois et al., 2001; Ghosh and Karin, 2002). Western blot analysis of cytosolic proteins showed no treatment-related differences in the levels of IκBα or IκBβ proteins (Fig. 5C). However, levels of phospho-IκBα were shown to be lower in samples from animals treated for 6hr with LPS than in other treatment groups (Fig. 5C, P-IκBα). Relative densitometric data for this observation is shown in the lower panel of Fig. 5C, expressed as the ratio of P-IκBα to IκBβ.

Effect of D3T on Physiologic Endpoints of Endotoxic Shock. As induction of iNOS in many organs contributes to hypotension, vascular hyporeactivity to vasoconstrictors and organ injury (Millar and Thiemermann, 1997; Nathan, 1997; Rosselet et al., 1998), we expanded our study to include endpoints of systemic effects of LPS. We chose a non-invasive tail cuff procedure to determine atrial blood pressure. Doing so eliminated the need for survival surgery and allowed us to determine BP measurements at baseline, after pretreatment regimens and following LPS challenges, all on the same animals. The decision to measure BP 6 hr post treatment with LPS was based on other studies showing that significant differences in BP occur at this time with LPS (Millar and Thiemermann, 1997; Rosselet et al., 1998). The baseline atrial BP of animals treated with either vehicle or D3T was 140.3 ± 6.1 and 135.5 ± 9.5 mm Hg, respectively (Fig. 6A). There were no significant differences in these values. As expected, the BP was decreased in vehicle plus

LPS group. An average reduction of 30.8 mm Hg (21.9%) was observed in theses animals 2 hr after LPS injection and this value was significantly different (p= 0.003) from its baseline value. The BP was further decreased 6 hr after LPS injection, with an average reduction of 57.5 mm Hg (40.1%) from baseline. Again, this value was statistically different (p=0.001) from its baseline value. Interestingly, the BP value was not reduced after LPS injection in D3T pretreated animals. Actually, there was a slight increase of 9 mm Hg (12.5%) from the baseline value 2 hr after injection of LPS (p=0.049). The reason for this increase is not known, but may be related to the procedure. It is important to note that this effect is transient, whereby the BP returns to baseline by 6 hr after injection of LPS in the D3T pretreated animals. Survival during the period of 24 hr post LPS injection was dramatically improved in animals pretreated with D3T. One hundred percent of the animals pretreated with D3T survived, while only 37.5 percent (3/8) of the animals given vehicle plus LPS survived (Fig. 6B).

Discussion

We have shown that in an acute *in vivo* model of endotoxic shock, pretreatment with D3T inhibited the induction of hepatic iNOS protein by nearly 80 percent. This effect was predominantly the result of decreased iNOS expression in hepatocytes. Accompanying this effect, and presumably a consequence of reduced iNOS expression, were significant reductions in the serum levels of NO metabolites and the toxicity markers ALT and AST. No changes in the hepatic levels of eNOS protein were observed.

As others have reported that depletion of glutathione by pretreatment of rats with diethylmaleate prevented the induction of hepatic iNOS protein (Vos et al., 1997), we studied these effects of D3T. As expected from the pioneering work of Bueding on diothiolethiones (De Long et al., 1986), hepatic levels of GSH were increased 3-fold by treatment with D3T. Of note, challenge with LPS of animals pretreated with D3T decreased by 50 percent the levels of GSH relative to the D3T-treated only group. Thus it appears that one action of D3T is to protect against the oxidative stress component of LPS-mediated toxicity.

A fundamental aspect of the LPS-mediated induction of iNOS is the activation of the transcription factor NFκB. EMSA and western blot analysis of nuclear protein extracts established that pretreatment of animals with D3T blocked LPS-mediated nuclear translocation and DNA binding of NFκB. This action of D3T was distinct from what has been reported for SFN in RAW cells. While SFN also inhibited the DNA binding of NFκB, it did not prevent nuclear translocation of p65. Thus, unlike the *in vitro* effect of SFN (Heiss et al., 2001), and other vicinal dithiol-binding agents such as phenylarsine

oxide that modify the structure of NFκB and inhibit its binding to DNA (Oda et al., 2000), the *in vivo* effect of D3T appears to act upstream of nuclear translocation of NFκB. Analysis of the IκB proteins showed that pretreatment with D3T resulted in higher levels of phosph-IκBα relative to samples from untreated animals. Furthermore, D3T pretreatment appeared to alter the LPS-directed decrease in phospho-IκBα protein levels. The observed change in phospho-IκBα and not total IκBα in rat liver is consistent with current knowledge that most of the cellular IκBα protein exists in the non-phosphorylated form (Menon et al., 1995), and that phosphorylation of this protein results in its degradation and activation of NFκB (Courtois et al., 2001; Ghosh and Karin, 2002). Since our analysis was performed at only a single time point, and the kinetics of IκBα synthesis, phosphorylation, and turnover are complex, we are cautious not to over-interpret this data. However, our results are consistent with several other studies of structurally diverse antioxidants that have been shown to alter IκBα degradation [Reviewed in (Surh et al., 2001)]. In the case of (-)-eppigallocatechin gallate, phosphorylation of IκBα was shown to be the target (Nomura et al., 2000), and further studies of IκBα and its associated IK kinase are warranted for D3T.

What then is the mechanism of action of D3T? Our current study design employed an intermittent dose schedule of D3T, followed 24 hr after the last dose with LPS challenge. This model is the same design we have used in cancer chemoprevention (Kensler et al., 1999; Roebuck et al., 2003), where D3T has been shown to induce gene expression through Nrf2 (Kwak et al., 2001; Kwak et al., 2003; Dinkova-Kostova et al., 2005). A recent report from the laboratories of Sporn and Talalay showed a strong correlation of the potencies of triterpenoids for anti-inflammatory effects, including

blocking iNOS expression, and phase 2 enzyme responses. Using mouse embryo fibroblasts from wild-type and Nrf2-disrupted mice, these authors established that the effect of triterpenoids to block NO production in response to treatment of these cells with IFN-γ and tumor necrosis factor-α was Nrf2-dependent (Dinkova-Kostova et al., 2005). Therefore, we believe that it is likely that D3T acts through Nrf2 to inhibit iNOS expression. Ongoing studies in Nrf 2 *null* mice should clarify this point.

While our studies focused on expression of hepatic iNOS, it is known that several organs are sites of LPS-mediated toxicity including lung, intestine, kidney and liver. Two important physiologic endpoints of the systemic effect of LPS include hypotension and death (Mailman et al., 2001). That iNOS is an important mediator of these endpoints has been established in studies of iNOS *null* mice (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990). Furthermore, iNOS is one of three nitric oxide synthase enzymes. The other two forms are constitutively expressed in either neuronal (nNOS) or endothelial (eNOS) cells. Studies using NOS inhibitors either specific to iNOS (S-methyl-isothiourea) or those that are better inhibitors of eNOS (N^6-nitro-L-arginine methyl ester) have shown that inhibitors of iNOS afforded protection against endotoxemia, whereas the eNOS inhibitors augmented toxicity (Vos et al., 1997). Thus, iNOS induction has been causally linked with the systemic toxicity of LPS, yet the relative contribution of different organ systems is still under investigation. By measuring the systemic endpoints of hypotension and survival during endotoxic shock, we demonstrated that the protective effect of D3T pretreatment extends beyond effects on hepatic function. One hundred percent of animals pretreated with D3T and then challenged with LPS survived and maintained normal blood pressure throughout the

measured period. Only 37.5 percent of the animals treated with LPS alone survived the 24 hr follow-up period. All animals in the vehicle-pretreated group exhibited marked hypotension.

To our knowledge, this is the first report showing that D3T or other inducers of phase 2 enzymes protect against endotoxic shock *in vivo*. While the current model of acute endotoxic shock may not adequately simulate human septic shock (Rosselet et al., 1998), the results of this study strongly support the need for further studies on this class of compounds for the prevention and treatment of sepsis and septic shock, as well as other chronic diseases associated with the NFκB and iNOS pathways.

Acknowledgments

We thank Jennifer Tzefakes and Mariallan Shadle, UM and Dr. Jian Jia Su, Dartmouth for technical assistance with the histology.

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Footnotes

This study was supported by the W. Harry Feinstone Center for Genomic Research and National Cancer Institute Grant CA 39416.

[1] A.R.K. and Y.H. contributed equally to this work.

Legends for Figures

Fig 1. Effects of D3T on iNOS expression, NO metabolites and serum enzymes. F344 rats were treated with either vehicle (V) or D3T, 0.3mmol/kg body weight, every other day for 3 days (D). Twenty-four hr after the last dose of vehicle or D3T, animals were treated with either PBS (V or D) or 5 mg/kg body weight LPS (VL or DL) for 6hr. (A)Western blot analyses of crude cell lysate. (*Upper*) iNOS expression in 60 μg/lane. (*Lower*) Aflatoxin aldehyde reductase (Afar) expression, a control for response to D3T (26) in 30 μg/lane. (B)Western blot of 40 μg of cytosolic protein from liver samples of 4 independent animals treated with vehicle plus LPS (VL) or D3T plus LPS (DL). (*Upper*) iNOS expression. (*Middle*) stained gel demonstrating the loading of samples analyzed in upper panel. (L*ower*) densitometric analysis of data shown in B, *upper*. (C-D), Levels of serum endpoints for indicated treatments, n=4 per group, (C) NO metabolites, NOx, (D) ALT, and (E) AST.

Fig 2. Immunohistochemistry of hepatic iNOS (400x). (A) vehicle treated rat liver. (B) D3T treated rat liver. (C) vehicle plus LPS treated rat liver. (D) D3T plus LPS treated rat liver.

Fig. 3. Effect of treatments on eNOS expression. F344 rats, four per group, were treated with either vehicle (V) or D3T, 0.3mmol/kg body weight, every other day for 3 days (D). Twenty-four hr after the last dose of vehicle or D3T, animals were treated with either PBS (V or D) or 5 mg/kg body weight LPS (VL or DL) for 6hr. Western blot of 40 μg of

particulate fraction protein prepared from liver samples: *Upper*) eNOS expression in a representative gel of 8 of 16 samples; (*Middle*) stained gel demonstrating the loading of samples analyzed in upper panel; (L*ower*) densitometric analysis of the complete set of data showing the mean and standard deviation for each set of four samples.

Fig. 4. Effect of treatments on levels of reduced glutathione. F344 rats, four per group, were treated with either vehicle (V) or D3T, 0.3mmol/kg body weight, every other day for 3 days (D). Twenty-four hr after the last dose of vehicle or D3T, animals were treated with either PBS (V or D) or 5 mg/kg body weight LPS (VL or DL) for 6hr. For each treatment, levels of GSH are expressed in units of nmol/mg wet weight of tissue. Shown is the mean and standard deviation for each set of four samples.

Fig 5. Mechanistic studies of the effect of D3T on LPS-meidated iNOS induction. (A) EMSA assay of NFκB DNA binding in nuclear extracts of animals treated with vehicle (V), D3T (D), vehicle plus LPS (VL), or D3T plus LPS (DL). Nuclear extracts were added at 1 μg per sample. Lanes 6-7, supershift assays using antibodies against p65 and p50 protein subunits, respectively. (B) Western blot of p65 subunit of NFκB. (*Upper*) nuclear extracts (n p65, 2 μg/lane). (*Lower*) cytosolic extracts (c p65, 50μg/lane). (*C*)Western blot of inhibitory proteins of NFκB in samples of cytosolic protein (100 μg/lane). (*Upper*) IκBα. (*Middle*) phospho- IκBα. (*Lower*) IκBβ. Shown below the last blot is a bar graph of the densitometric analysis of the middle and lower western blots shown in (C) expressed as the ratio of p-IκBα/ IκBβ. Samples are vehicle alone (V), D3T alone (D), vehicle plus LPS (VL), and D3T plus LPS (VD).

Fig 6. Protective role of D3T on blood pressure and survival in LPS treated rats. (A) Effect of treatment of vehicle plus LPS or D3T plus LPS on ventral tail arterial pressure in conscious animals, n=6 per group. The BP of each animal was measured at 1 hr before LPS injection (-1, baseline), and 2 hr and 6 hr after LPS injection. The *p* value was generated by paired comparison with the baseline. (B) Twenty-four hour survival after injection of vehicle plus LPS (VL) or D3T plus LPS (DL), n=8 per group.

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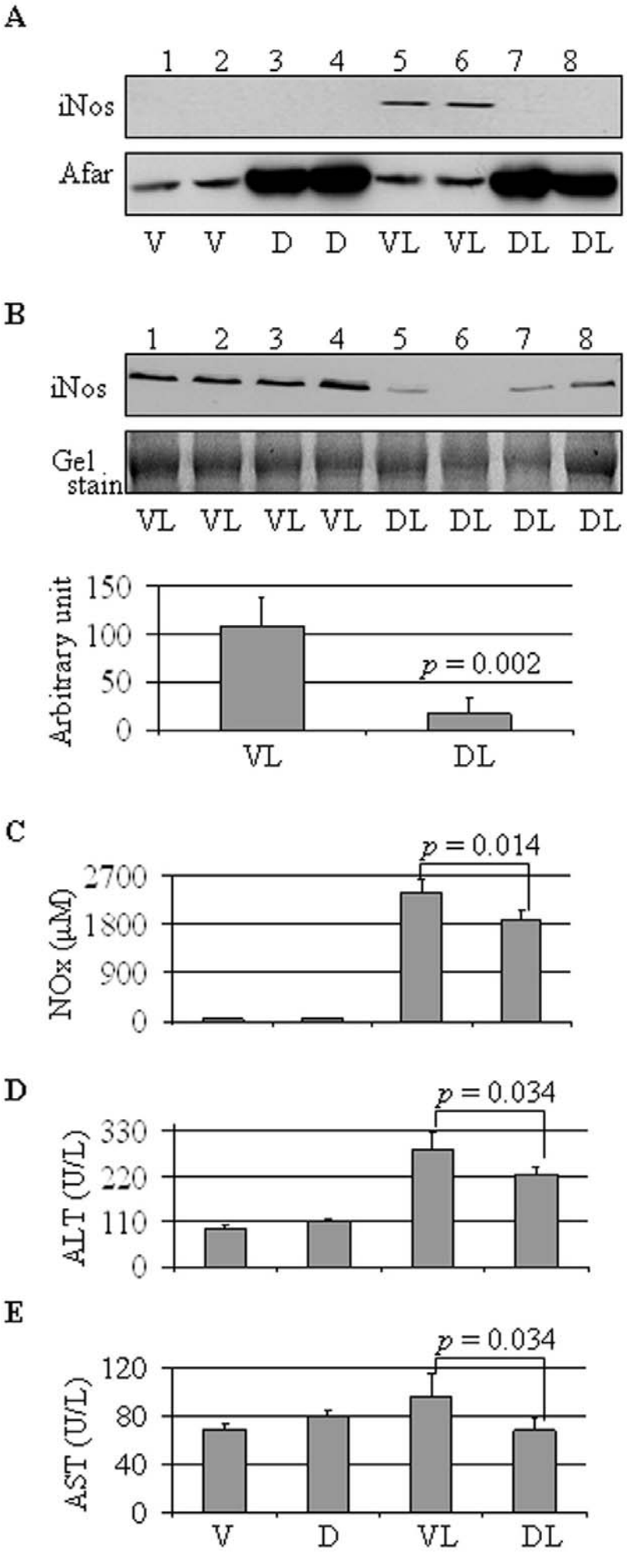

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Fig 2

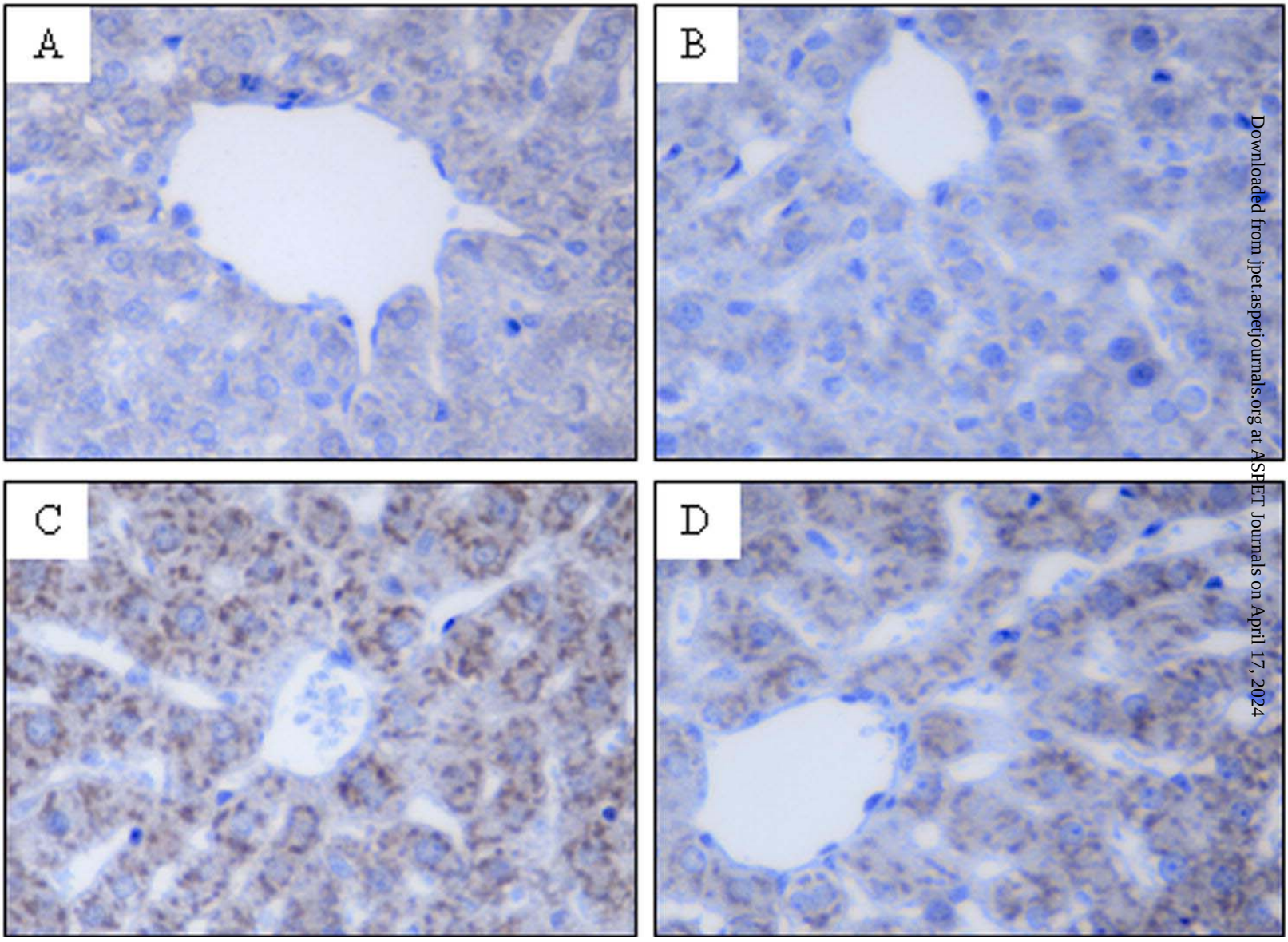

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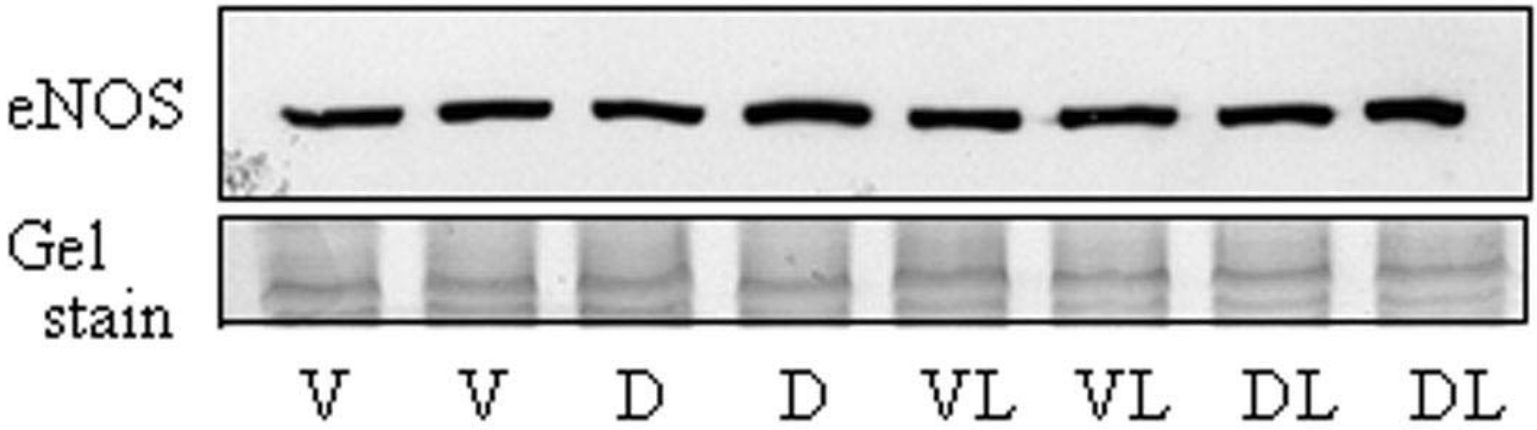
Fig 3



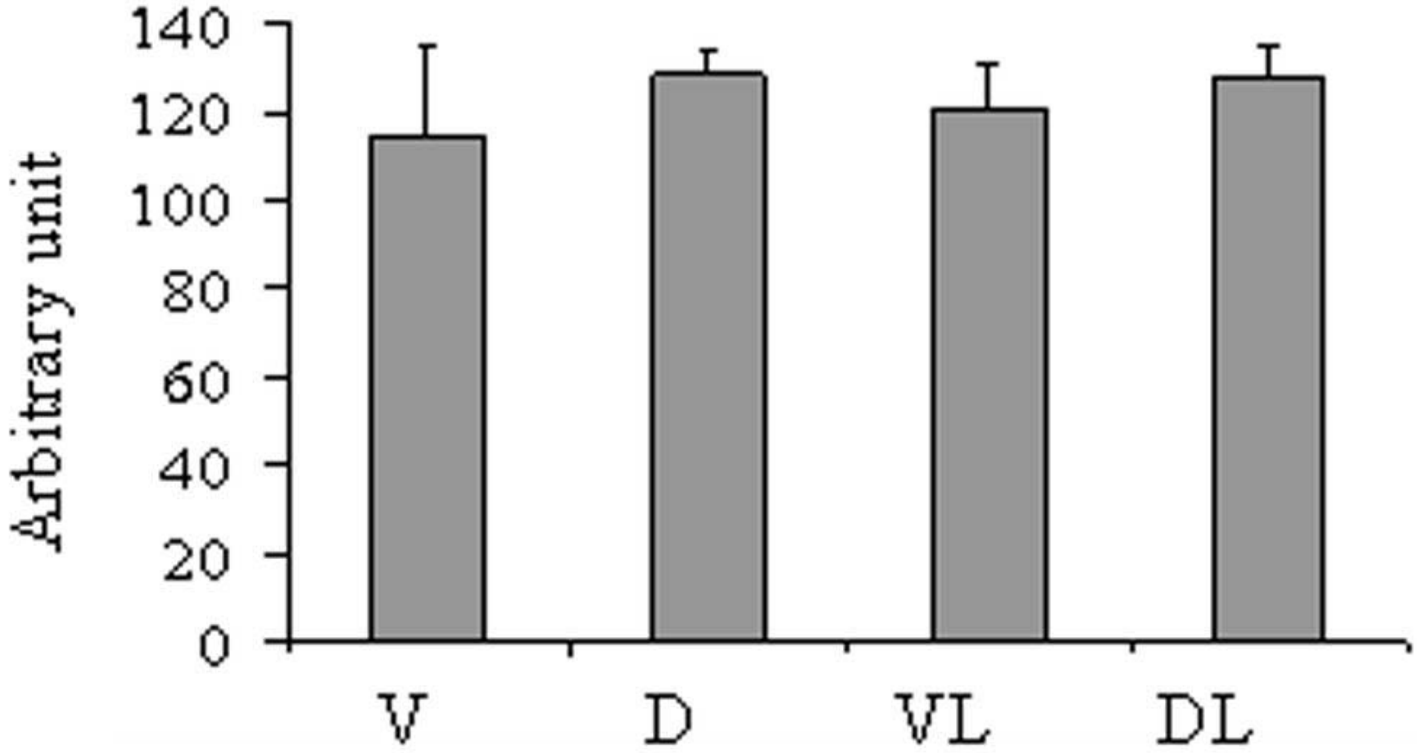


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Fig 4

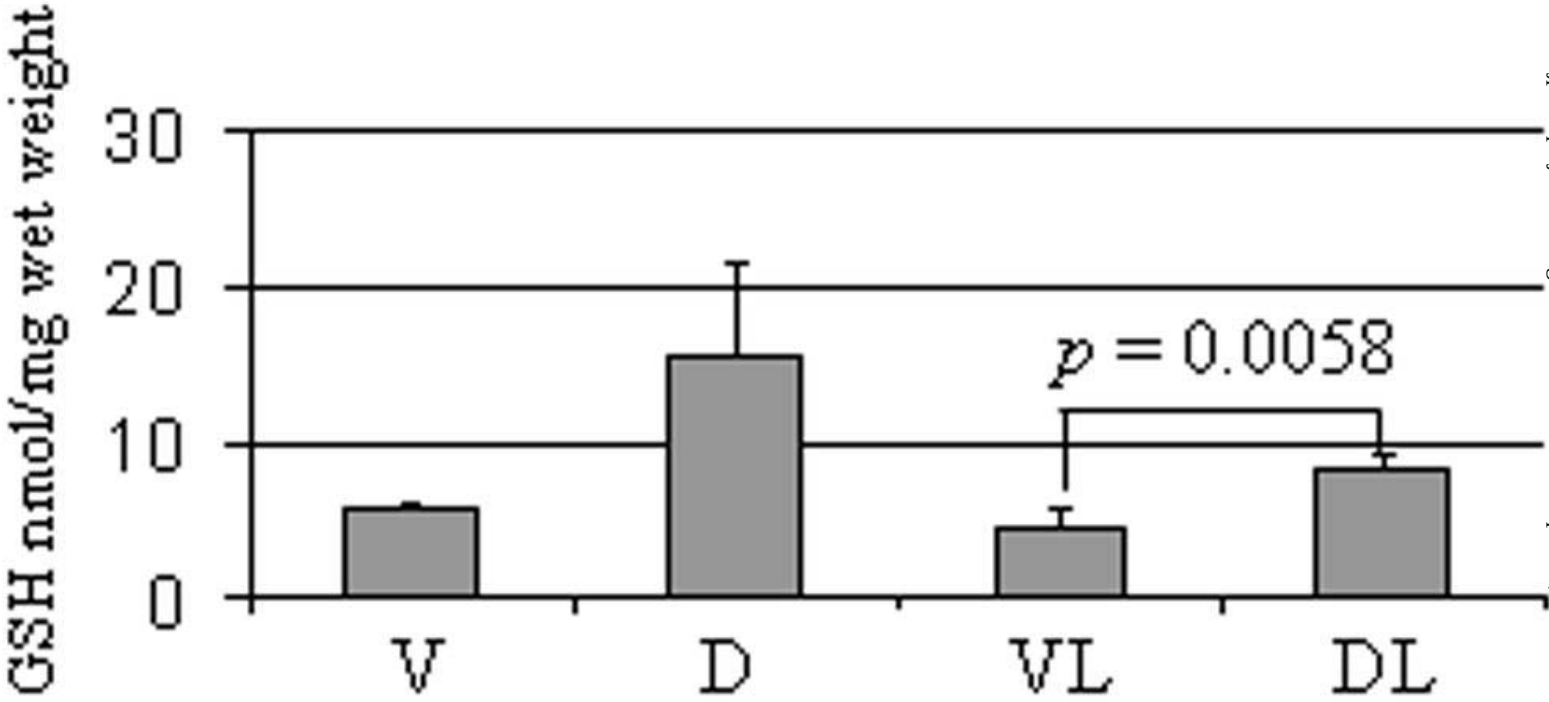

Fig 5

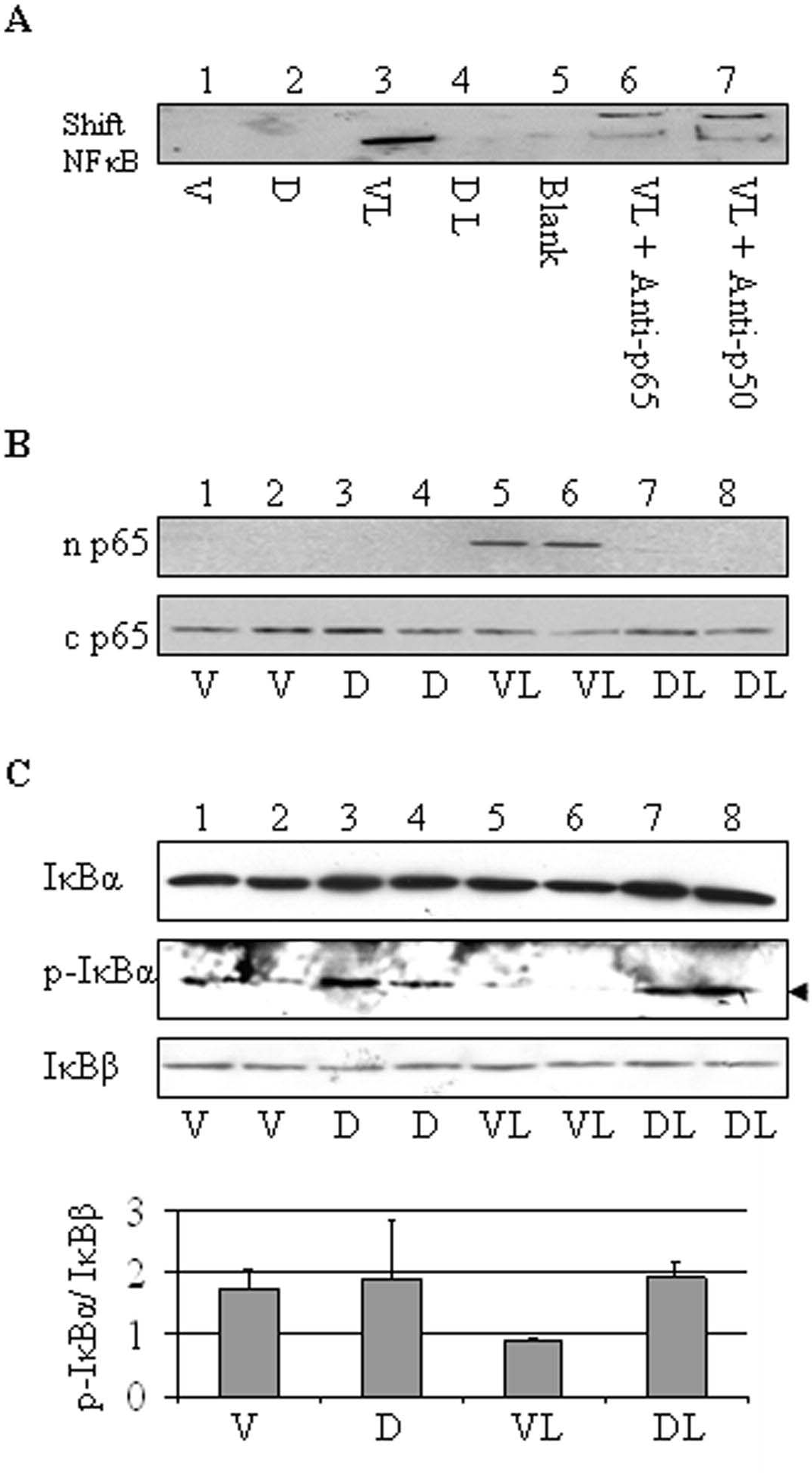

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Fig 6