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Vitamin D₃ protects against prednisolone-induced liver injury associated with the impairment of hepatic NF-κB/iNOS/nitric oxide pathway

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Abstract

The study was carried out to define whether prednisolone-induced damage to hepatic cells is accompanied by excessive nitric oxide (NO) levels associated with nuclear factor kappa B (NF-κB)/inducible NO synthase (iNOS) activation and evaluate the efficacy of the treatment with vitamin D$_3$. Histopathological examination, activities of liver transaminases (ALT and AST) and cell death assays consistently showed that prednisolone (5 mg/kg of body weight, 30 days) induces chronic liver injury in female Wistar rats. Specifically, increased hepatocellular necrosis and caspase-3-dependent apoptosis were observed. Prednisolone enhanced iNOS protein expression, NO generation and tyrosine nitration in liver cells. Despite unchanged hepatic level of NF-κB/p65 protein, prednisolone increased inhibitory κB-α (IκB-α) degradation, nuclear translocation and phosphorylation of NF-κB/p65 at Ser311, indicating that NF-κB activation can be involved in the induction of iNOS/NO. All changes were associated with a 2.9-fold decrease in the serum content of 25OHD$_3$ and significant reduction of hepatic VDR expression that points reliably to vitamin D$_3$ deficiency and failures in VDR signalling. Vitamin D$_3$ co-administration (100 IU/rat, 30 days) prevented glucocorticoid-evoked abnormalities in hepatic tissue. In conclusion, prednisolone-induced liver disturbances were associated with the impairment of NF-κB/iNOS/NO responses that can be ameliorated by vitamin D$_3$ treatment through VDR-mediated mechanisms.

Key words: vitamin D$_3$, prednisolone, nuclear factor kappa B, inducible nitric oxide synthase, nitric oxide.
**Introduction**

The high efficiency of synthetic glucocorticoids (GCs) as anti-inflammatory, anti-allergic and immunosuppressive drugs has long been recognized (Vandevyver et al. 2013). However, chronic GC treatment can increase, via genomic and/or non-genomic mechanisms, the risk of serious side effects, in particular osteoporosis, muscle atrophy, adrenocortical insufficiency, hypertension, insulin resistance and impaired glucose tolerance (Ericson-Neilsen and Kaye 2014). Furthermore, GCs were also shown to have extensive adverse effects on hepatic function in several clinical studies. It has been reported recently that the levels of marker liver enzymes were significantly upregulated in blood serum of patients after receiving high doses of methylprednisolone for the treatment of demyelinating disease that is typical of acute hepatitis (Loraschi et al. 2010). Liver damage, due to the development of cholestasis and biliary disease, was also found to be the consequence of GC therapy in persons with abnormal hepatocellular function (Lu et al. 2012).

Despite emerging evidence of deleterious side effects on the liver induced by long-term application of synthetic GCs, the precise mechanisms are still poorly understood. Oxidative stress associated with increased reactive oxygen species (ROS) generation and impaired effectiveness of antioxidant protection have recently received attention as one of the mechanisms of destructive changes in the liver induced by systematical administration of GCs (Aboelwafa and Yousef 2015). Little is known about the possibility of nuclear factor kappa B (NF-
κB)/inducible nitric oxide synthase (iNOS)/nitric oxide (NO) pathway involvement downstream of GC action in liver injury. We hypothesized that simultaneous increase in NO and ROS levels could cause peroxynitrite synthesis and nitrosative protein modifications leading to hepatocellular damage.

The effects of vitamin D₃ (cholecalciferol) in maintaining functional activity of hepatocytes may be useful in correcting liver damage caused by prolonged administration of GCs (Hamden et al. 2009; Sharifi et al. 2014). In addition to the classical role of vitamin D₃ in the regulation of calcium and phosphorus metabolism and bone tissue remodeling, cholecalciferol is now recognized as an important regulator of a plethora of fundamental biological functions in cell differentiation, inhibition of cell growth as well as immune modulation, acting on target cells via its hormonally active metabolite 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, calcitriol) and nuclear vitamin D₃ receptor (VDR) thus modulating gene transcription (Lucas et al. 2014). In recent studies that focus on the mechanisms of cellular protection by vitamin D₃ much attention is paid to the efficacy of cholecalciferol and its hormonally active derivatives in preventing and/or managing various diseases, including osteoporosis, cancer, autoimmune and cardiovascular diseases (Basit 2013). Accumulating evidence also suggests that vitamin D₃ has the ability to correct complications caused by acute or chronic administration of hepatotoxicants, including prednisolone (De Mattia et al. 2013; Han et al. 2013). However, no reliable information is available regarding the vitamin D₃ activity against NO-mediated liver damage associated with prednisolone treatment.
In this study our aim was to investigate the effects of long-term administration of synthetic glucocorticoid prednisolone on NF-κB/iNOS/NO responses and cell injury in rat liver and to evaluate whether vitamin D₃ is capable of attenuating prednisolone-induced abnormalities.

**Materials and methods**

**Animals**

Female Wistar rats (100±5 g) were fed a standard rodent diet. The rats were randomly divided into 3 groups, each including 15 animals: 1 – the control group; 2 – the group of animals that received prednisolone at dose 5 mg per kg of body weight (per os, daily for 30 days); 3 – the group of animals that received prednisolone at dose 5 mg per kg of body weight and 100 IU of vitamin D₃ (per os, daily for 30 days). The study was performed accordingly with national and international guidelines and laws concerning animal welfare.

**Isolation of primary rat hepatocytes**

Primary hepatocytes were isolated as described Shiota et al. (1995). Briefly, the portal vein and inferior vena cava of anesthetized animals were cannulated and oxygenated perfusion solution was slowly infused. After its discoloration the liver was excised and perfused for 1 hour at 37°C with liver digest medium containing 0.05% collagenase (Sigma, USA), 0.146 M NaCl, 5.4 mM KCl, 0.8 mM MgSO₄·7H₂O, 2.0 mM CaCl₂, 0.7 mM Na₂HPO₄·12H₂O, 0.7 mM KH₂PO₄, 1%
albumin (pH 7.4). Thereafter the cells were dispersed, washed 3 times, centrifuged and resuspended.

**Histopathological analysis**

After fixing liver samples, taken for histological studies, in 10% buffered formalin, they were thoroughly washed under running water and dehydrated in ascending grades of ethanol, embedded with soft paraffin, and then cut into 5 µm sections on a microtome (Thermo Scientific, USA). Tissue sections were stained by hematoxylin and eosin (H&E) and examined under light microscope (Carl Zeiss Axioskop 40, Germany) for histopathological assessment.

**Assay of ALT and AST activities**

Blood was obtained via cardiac puncture into sample tubes and plasma was separated by centrifugation. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using the ALT Activity Assay Kit (Sigma, USA) and AST Activity Assay Kit (Sigma, USA) respectively according to the manufacturer’s instruction.

**Propidium iodide staining assay**

Propidium iodide (PI; Sigma, USA), an intercalating dye, was used to determine cell death (Ning et al. 2011). The cells (approximately 1×10^6 cells/ml) were incubated in Hank’s balanced salt solution with PI (50 µg/ml) in the dark for 10 min at room temperature. Data acquisition was carried out on an EPICS XLTM
flow cytometer (Beckman Coulter, USA) using the excitation/emission wavelengths of 530/645 nm.

**Determination of nitric oxide**

Intracellular nitric oxide production in isolated hepatocytes was analyzed using 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma, USA). Upon entering the hepatocytes, intracellular esterases hydrolyze the diacetate moiety, trapping DAF within the cell. DAF specifically reacts with the oxidized form of NO, producing the fluorescent 4,5-diaminofluorescein triazole (Gumpricht et al. 2002). Immediately after isolation, the cells (approximately 0.5×10^6) were incubated in Hank’s balanced salt solution with dye concentration of 10 µM for 30 min (37°C) in a humidified atmosphere of 5% CO₂. Subsequently, the cells were washed, centrifuged, resuspended and fluorescence was measured with an EPICS XLTM flow cytometer (Beckman Coulter, USA) using the excitation/emission wavelengths of 495/515 nm. Results were expressed as a relative fluorescence of the samples in comparison to control.

**Western blot analysis**

Protein expression levels were assessed by Western blot analysis. Briefly, total liver lysates and nuclear protein extracts were prepared from 100 mg liver tissue using protocols previously described Cheng et al. (2011). Protein concentrations in the lysates were quantified by Lowry’s method. Equal protein samples (50 µg) were separated by 10% (if not otherwise stated) SDS-PAGE and
then transferred to nitrocellulose membranes (Ju et al. 2014; Rosner and Hengstschlager 2008). Immunoblotting was conducted with primary antibodies against iNOS (1:1000; Chemicon International, USA); 3-nitrotyrosine (1:4000; Millipore, USA); full length poly(ADP-ribose)polymerase 1 (116 kDa) including its large (89 kDa) fragment (1:1000; Cell Signaling Technology, USA); caspase-3 active subunit p17 (1:200; Santa Cruz Biotechnology, USA); myeloperoxidase (1:500; Abcam, USA); NF-κB (1:1000; Cell Signaling Technology, USA); Ser311 phosphorylated NF-κB p65 (1:500; Santa Cruz Biotechnology, USA); inhibitory κB-α (1:1000; Santa Cruz Biotechnology, USA); VDR (1:200; Santa Cruz Biotechnology, USA); β-actin (1:50000; Sigma, USA) and lamin B1 (1:1000; Sigma, USA). Primary-antibody-bound membranes were incubated with peroxidase-conjugated secondary antibodies: anti-mouse IgG (Fab Specific)-Peroxidase (Sigma, USA) or anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc., USA). Thereafter the membranes were developed with chemiluminescent agents: p-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany). Tissue levels of proteins mentioned above were normalized to β-actin or lamin B1. The immunoreactive bands were quantified with Gel-Pro Analyzer 32.

**Quantitative RT-PCR**

The liver tissue with weight 0.5 g was collected and total RNA was extracted by Trizol RNA Prep kit (NEOGENE, Ukraine). Single standard cDNA templates subsequently were synthesized using Maxima H Minus First Strand cDNA
Synthesis Kit (Thermo Fisher Scientific Inc., USA). Real-time polymerase chain reactions were undertaken for the VDR and NF-κB p65 with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. Primer sequences for the VDR, NF-κB and GAPDH were designed by Primer BLAST program as follows: VDR – forward 5´-TCATCCCTACTGTGTCCCGT-3´; reverse 5´-TGAGTGCTCCTTGGTTCGTG-3´; product length – 161 bp; NF-κB p65 – forward 5´-GTACTTGCCAGACACAGACGA-3´; reverse 5´-CTCGGGAAGGCACAGCAATA-3´; product length – 149 bp; GAPD – forward 5´-TGAACGGGAAGCTCACTGG-3´; reverse 5´-TCCACCACCCTGTGCTGTA-3´; product length – 307 bp. Genes were amplified for 60 cycles using Maxima SYBER Green/ROX qPCR Master Mix (2X), (Thermo Fisher Scientific Inc., USA). Data were calculated using ΔΔCt method.

**Serum 25OHD₃ measurement**

The content of 25-hydroxyvitamin D₃ (25OHD₃) in serum was assayed using a commercial ELISA kit (The IDS 25-Hydroxy Vitamin D EIA, USA) as described by the manufacturer.

**Statistics**

All data are expressed as mean ± SEM and statistical analyses were carried out using the Student’s t test. Differences were considered to be statistically significant when p<0.05.
Results

Prednisolone-induced liver injury and protective effect of vitamin D$_3$ treatment

Histopathological observations revealed disordered hepatic lobules, swelling cells and vacuoles in the liver specimens from rats administered with prednisolone (Fig. 1a). After treatment with vitamin D$_3$, the most of these abnormal changes disappeared, however hepatocytes still had some signs of hydropic dystrophy, indicating that the normal cell structure was not fully restored. Consistent with hepatic damage, prednisolone caused a significant increase in serum ALT and AST levels that were reversed by vitamin D$_3$ treatment (Table 1).

In addition, propidium iodide staining and flow cytometry showed an increase in the number of dead (mostly necrotic) cells among isolated hepatocytes to 12.8% compared with 7.5% in the control (Fig. 1b). Besides necrotic cells, PI-positive hepatocytes may include cells at late stage of apoptosis; therefore we concomitantly studied whether prednisolone induces apoptosis in the liver. As the sequential activation of caspases plays a key role in the execution phase of cell apoptosis, the levels of caspase-3 active subunit (p17) and cleaved PARP-1 were examined by western blot analysis. The level of cleaved caspase-3 was shown to be more elevated (1.34 fold) in liver tissue of prednisolone-administered rats than was seen in the control animals (Fig. 1c). Although there were no significant changes of the full-length PARP-1 protein content in liver tissue, the level of the 89 kDa cleaved fragment of PARP-1 was appreciably higher (1.64 fold) in the GC-administered animals (Fig. 1d). These findings suggest that chronic prednisolone
administration activates caspase-3-mediated apoptotic death pathway in the liver that presumably occurs in parallel with necrotic cell death. When compared with the prednisolone-administered group, vitamin D$_3$ supplementation led to a substantial decrease in the number of PI-positive cells. Vitamin D$_3$ treatment also lowered the expression of pro-apoptotic proteins by 1.60 and 1.21 fold respectively as compared with the effect of the glucocorticoid hormone.

**Vitamin D$_3$ reverses NO overproduction and nitrotyrosine accumulation in liver associated with prednisolone administration**

To evaluate the possible involvement of nitric oxide and nitrogen metabolites in the mechanism of GC-induced damage to liver tissue we examined the levels of NO formation and reactive nitrogen species (RNS)-mediated posttranslational modification of proteins. The quantities of nitric oxide were measured using a NO-specific fluorescence indicator, DAF-2DA. It was found that chronic prednisolone administration stimulated NO production in isolated hepatocytes by 1.88 fold compared with the control animals (Fig. 2). Vitamin D$_3$ treatment reduced the level of NO formation in hepatocytes compared with the prednisolone-administered group.

Cytotoxic and mutagenic effects of ROS/RNS are mediated by free radical oxidation of physiologically important macromolecules (Reiniers et al. 2014). Nitric oxide can interact with superoxide anion radicals to form unstable and highly reactive compound – peroxynitrite (ONOO$^-$). Consistent with the ability of NO to covalently modify protein tyrosine residues synergistically with reactive
oxygen species, the content of protein 3-nitrotyrosine is considered to be a specific marker of peroxynitrite activity and nitrosative stress (Yang et al. 2013). Fig. 3 depicts the immunoblotograms of nitrated proteins and their relative content in liver tissue of the GC-administered rats with or without vitamin D₃ treatment. It was found that prednisolone increases 1.55 fold the level of nitrated proteins as compared with the control. Vitamin D₃ administration normalized the content of nitrated proteins almost to the level of the control animals.

**Myeloperoxidase is not involved in prednisolone-induced nitrative modifications of hepatic proteins**

Since new evidence is provided for proteins as a susceptible target for nitration by myeloperoxidase (MPO)/nitrite/H₂O₂ system (Lu et al. 2014), we also examined the protein level of this enzyme in liver tissue. It was observed 1.25-fold lowering of hepatic MPO expression after prednisolone administration that apparently exclude MPO involvement as a likely alternative mechanism for steroid-induced nitrative modification of proteins in the liver (Fig. 4). Vitamin D₃ treatment slightly increased MPO protein level as compared with the effect of prednisolone.

**Modulation of hepatic NF-κB/iNOS and protective effect of vitamin D₃ in prednisolone-administered rats**

As inducible nitric oxide synthase is normally detectable in the three main types of liver cells (hepatocytes, Kupffer and stellate cells) (Muriel 2000), it was
anticipated that the increase in NO production may result from an accompanying iNOS upregulation in hepatic tissue. The effect of prednisolone on iNOS at translational level is shown in Fig. 5a. Not surprisingly, the expression of iNOS protein was increased by 1.35 fold in rat livers after GC administration. After vitamin D$_3$ treatment a slight decrease in the protein level of iNOS was observed as compared with prednisolone administration.

Transcription factor NF-κB is essential for the control of a large variety of cellular functions including inflammatory response, apoptosis and cell survival pathways (Luedde and Schwabe 2011). In different tissues, the most common NF-κB consists of a large subunit RelA (p65), which forms either a homodimer or a heterodimer with the structurally related p50 protein. As NF-κB activation is one of the likely events to mediate iNOS expression (Ma et al. 2008), the levels of total and phosphorylated NF-κB p65 were assayed in liver tissue by different methods. Fig. 5b shows that prednisolone administration results in a 2.90 fold increase in NF-κB p65 mRNA level compared with that of the control group, while vitamin D$_3$ treatment significantly attenuates the effect of steroid hormone on the transcription of hepatic NF-κB p65 gene. The pattern of NF-κB p65 protein expression differs from that of its mRNA expression. When compared with the control rats, none of the animals displayed remarkable changes in hepatic NF-κB p65 protein levels after prednisolone administration (Fig. 5c). Vitamin D$_3$ exerted lowering effect (by 1.34 fold) on this parameter as compared with GC action.

Consistent with a critical role of phosphorylation in the transcriptional activation of NF-κB downstream of various stimuli, western blot analysis showed
that hepatic protein level of Ser311 phosphorylated NF-κB p65 in prednisolone-administered group was 2.10 fold elevated in total liver lysates as compared with the control animals (Fig. 6a). Recently, atypical protein kinase (PKC) ξ has been proposed as the kinase phosphorylating Ser311 that requires prior release of p65/p50 from inhibitory κB-α (IκB-α) complex (Moscat and Díaz-Meco 2011). In keeping with this observation, prednisolone reduced the level of IκB-α protein by 1.22 fold, suggesting increased proteolytic degradation of IκB-α (Fig. 6c). The lack of IκB-α may contribute to the activation and nuclear translocation of NF-κB that binds to the promoter site of specific genes and induces their transcription. Furthermore, we also found that prednisolone increased protein level of nuclear NF-κB p65 phosphorylated at Ser311 (Fig. 6b) probably due to enhanced NF-κB p65 translocation from the cytoplasm to the nucleus and its subsequent activation. Vitamin D₃ treatment partially suppressed (1.35 fold) GC-evoked translocation to the nucleus and phosphorylation of NF-κB p65 at Ser311 with simultaneous increase in IκB-α level to a value 1.50 fold higher than in the normal rats. These data clearly demonstrate the inhibitory effect of vitamin D₃ on prednisolone-induced NF-κB activation and iNOS expression.

**Prednisolone-evoked hepatotoxicity is accompanied by vitamin D₃ deficiency, decline in VDR expression and is reversed by vitamin D₃ treatment**

The alterations associated with the long-term administration of prednisolone were accompanied by vitamin D₃ deficiency in rats. This is evidenced by 3.0 fold reduction in the concentration of vitamin D₃ prohormone – 25-hydroxyvitamin D₃.
(25OHD$_3$), in blood serum of glucocorticoid-treated animals compared with the controls (Fig. 7a). The content of 25OHD$_3$ in circulating blood is known to be a reliable marker of vitamin D$_3$ bioavailability (Romagnoli et al. 2014). Vitamin D$_3$ treatment completely restored the 25OHD$_3$ content to the control values compared with the effect of prednisolone.

To test whether vitamin D$_3$ deficiency correlates with the impairment of VDR signalling pathway in the mechanism of prednisolone-induced hepatic damage, we finally examined VDR expression at transcriptional and translational levels. Chronic prednisolone administration was associated with a significant decrease in both VDR mRNA (about 10 fold) and protein expression (by 1.20 fold) in liver tissue compared with the control animals (Fig. 7b and 7c). On the background of 25OHD$_3$ restoration in blood serum, it was revealed partial normalization of hepatic VDR mRNA and protein levels in the animals treated with vitamin D$_3$ compared with prednisolone action.

**Discussion**

Hepatic damage has been recognized as one of the major complications of prolonged glucocorticoid therapy; however, the underlying mechanisms remain largely unknown. In this study, elevated amounts of NO, a free-radical molecule produced by NOS, 3-nitrotyrosine accumulations in liver tissue and increased incidence of cellular death have been identified in the experimental model of GC-related hepatotoxicity. Generally, NO becomes dangerous when coexistence with augmented superoxide formation causes the synthesis of peroxynitrite, which is a
potent oxidant and nitrating agent (Pisoschi and Pop 2015). Prednisolone-induced alterations of oxidative metabolism in the liver and excessive formation of ROS that leads to oxidative stress have been recently reported in our group (Shymanskyy et al. 2014). Although the events that promote GC-induced production of hepatocellular oxidants are poorly understood, impaired activity of mitochondrial respiratory chain may play a crucial role as a source of superoxide leading to increased peroxynitrite levels and tyrosine nitration (Tang et al. 2013). GCs are also referred to as strong inducers of cytochrome P\textsubscript{450} isoforms, which can be an additional source of free radicals (Matsunaga et al. 2012).

It is likely that multiple mechanisms contribute to prednisolone-induced liver injury, but overproduction of superoxide and other oxidants in hepatocytes seems to play a role as the critical initiating step that leads to the development of NO-mediated prednisolone hepatotoxicity. A key reason for increased NO production in primary hepatocytes is that ROS can regulate redox-sensitive transcription factors, including NF-κB, which activation promotes iNOS expression (Das et al. 2012). In the present study, a probable reduction of sequestered NF-κB in the cytoplasm after IκB-α degradation has been ascertained as one of the mechanisms of hepatic NF-κB activation after chronic prednisolone administration. Additional evidence for the induction of NF-κB pathway is that Ser 311 phosphorylation of the large NF-κB subunit (p65) and its nuclear localization were substantially increased. Importantly, phosphorylation of NF-κB subunits containing transactivation domains strongly regulates transcription in a gene-specific manner (Christian et al. 2016). A number of research efforts indicate that Ser311
phosphorylation of NF-κB p65 subunit mediated by PKCζ significantly contributes to controlling transcriptional responses (Moscat and Diaz-Meco 2011). Our findings have confirmed that prednisolone-evoked NF-κB activation was accompanied by the elevation of iNOS protein expression in liver tissue and NO synthesis in primary hepatocytes. Yuan et al. (2000) have also reported the induction of hepatic iNOS in dexamethasone-treated mice. Involvement of other cell types in GC-induced hepatic activation of NF-κB/iNOS/NO pathway appears to be less likely. In particular, Ikeda et al. (1996) have shown that GCs significantly inhibited iNOS expression and NO formation in Kupffer cells, suggesting that iNOS/NO pathway cannot be related to activation of phagocytic macrophages in the liver. Moreover, decreased level of hepatic myeloperoxidase, a marker of neutrophil/macrophage recruitment and activation, is additional evidence obtained in our study that excess amounts of NO are not produced by inflammatory cells of the prednisolone-administered animals. Generally, these findings are in accordance with the anti-inflammatory effects of GC therapy.

NO overproduction, through upregulation of NF-κB/iNOS, has implication for cellular dysfunction and apoptosis in a variety of cell types, including hepatocytes (Weidinger 2015). In parallel with markedly enhanced oxidative/nitrosative stress and hepatocellular necrosis, prednisolone-administered rats also displayed apoptosis assessed by the increase in caspase-3 p17 expression and subsequent cleavage of PARP-1 in the liver. Caspase-3 appears to be among central executioners for mitochondria-dependent death pathway (Fulda 2015). Its involvement indicates severe GC-induced disturbances of mitochondrial function.
Given that the tumor necrosis factor α (TNF-α) is able to activate NF-κB by PKCζ-mediated phosphorylation of serine 311 (Kai et al. 2009), we may also suggest the involvement of this pro-inflammatory cytokine in the mechanism of prednisolone-induced NF-κB/iNOS response and liver damage. TNF-α is one of the major cytokines, which can either promote NF-κB activation and cell survival and/or initiate the caspases-activated death pathway (Van Herreweghe et al. 2010). Nevertheless, this assumption raises the question concerning the type of cells that could be responsible for increased expression of TNF-α. While contribution of immune cells is very elusive due to anti-inflammatory properties of GCs, it has been previously found that hepatocytes can also produce and release a number of inflammatory cytokines, including TNF-α (Kostecká et al. 2012). Furthermore, elevation of TNF-α in the liver of GC-treated rats has been recently detected by Aboelwafa and Yousef (2015). Consistent with the notion that stimulation of iNOS expression is dependent on oxidant-sensitive NF-κB activation, we may propose the following events that ultimately lead to cell death associated with prednisolone action. ROS formation can be the primary cause of GC-induced iNOS/NO responses, most likely protective, to hepatocyte damage. Increased ROS/RNS production leads to initial cell death and those ROS/RNS together with inflammatory cytokines from destroyed cells may induce secondary hepatocellular damage through the activation of NF-κB/iNOS/NO pathway.

In the present investigation we further confirmed the importance of sufficient vitamin D₃ availability in maintaining proper function of hepatic cells in normal and diseased conditions. It has been demonstrated that prednisolone-induced
oxidative/nitrosative stress and liver injury may be related to established inadequate circulating level of 25OHD₃, because prohormone repletion after vitamin D₃ treatment resulted in partial or complete normalization of the most detrimental alterations associated with GC hepatotoxicity. Interestingly, vitamin D₃ deficiency was shown to be correlated with the elevation of 3-nitrotyrosine that was attributable to extensive nitrosative stress in different tissues (Codoñer-Franch et al. 2012; Keeney et al. 2013). One of the explanations for the 25OHD₃ deficiency elicited by prednisolone can be ascribed to diminishing of vitamin D₃ 25-hydroxylation in hepatocytes (Khomenko 2013). It has been also established that GCs can increase VDR-mediated transcription of the Cyp24A1 and 1,25-dihydroxyvitamin D₃ 24-hydroxylase activity. This enzyme participates in the degradation of calcitriol and limits its hormonal function (Hidalgo et al. 2010). mRNA and protein levels of VDR were currently shown to be significantly reduced in the liver tissue of prednisolone-administered rats, possibly indicating decreased cell sensitivity to vitamin D₃. Moreover, ROS and RNS can modulate effects of vitamin D₃ by inhibiting association of VDR with DNA (Kroncke 2002).

More recently, we have clearly detected antioxidant effects of vitamin D₃ in liver tissue of GC-administered rats (Shymanskyy et al. 2014). The potent antioxidant activity of vitamin D₃ had also been reported in other publications (Luong and Nguyen 2013). The suppressive VDR-mediated effect of cholecalciferol on NF-κB activation and iNOS induction revealed in this study can be proposed as a key mechanism to explain the efficacy of vitamin D₃ in the prevention of oxidative/nitrosative stress and cell death. Positive effect of
cholecalciferol can be achieved by the action of its hormonally active metabolite – 1,25(OH)\(_2\)D\(_3\). The VDR binds 1,25(OH)\(_2\)D\(_3\) with the high affinity and elicits its action to regulate gene expression in target cells by binding to vitamin D-responsive elements (De Mattia et al. 2013). As the result, vitamin D\(_3\) can regulate the rate of ROS/RNS formation in liver cells and their survival via VDR-mediated inhibition of NF-κB transcriptional activation that leads to the changes in the expression of various pro/antioxidant and pro/anti-inflammatory genes, including \(iNOS\) (Hamden et al. 2009; Li et al. 2013). We can assume that vitamin D\(_3\) efficacy to counter the negative consequences of GC action in the liver is probably related to the ability of cholecalciferol to compete with prednisolone, structurally similar steroid, for binding to the consensus sequence in the promoters of target genes. Notably, vitamin D\(_3\) has been reported to reduce NF-κB nuclear translocation by upregulating \(IκB-α\) levels via increasing mRNA stability and decreasing \(IκB-α\) phosphorylation (Cohen-Lahav et al. 2006). Our results also show that downregulation of NF-κB/\(iNOS/NO\) pathway in liver tissue of the animals treated with vitamin D\(_3\) may be associated with increased inhibitory control of \(IκB-α\).

In conclusion, the relevance of our study is to provide further knowledge about the mechanisms that account for the GC-induced liver complications. They are associated with the activation of hepatic NF-κB/\(iNOS/NO\) pathway and vitamin D\(_3\) deficiency. Augmentation of cholecalciferol metabolism was shown to be an effective therapeutic tool for weakening hepatotoxic effects of GCs.
Acknowledgements

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References


Figure captions

**Fig. 1.** Evaluation of pathological changes in liver tissue: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D₃ administration. Histological analysis was performed in liver sections (5 µm) stained with hematoxylin and eosin. Typical images were chosen from each experimental group (magnification 100 x) (a). Primary hepatocytes were stained with propidium iodide and percentage of PI-positive cells was analyzed by flow cytometry. Values represent percentages from at least 10000 counted cells (b). Expression of hepatic proteins involved in cell apoptosis was assessed by western blot analyses. Representative blots (upper panel) show immunoreactive bands for caspase-3 active subunit (p17) (c) and PARP-1 (d) from total liver lysates and nuclear extracts respectively. 15% SDS-PAGE was used for cleaved caspase-3 detection, while 10% gel was used for PARP-1. Representative histograms (lower panel) show the band densities with relative β-actin or lamin B1. All data are shown as mean ± SEM and representative of three independent experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

**Fig. 2.** Nitric oxide generation in primary hepatocytes. Representative histograms of DAF fluorescence (count – the number of events; FL1 LOG – fluorescence intensity) (a); quantification of DAF oxidation documented by flow cytometry analysis: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D₃ administration (a.u. – arbitrary units) (b). All data are shown as mean ± SEM and representative of three independent experiments done in triplicate; *
p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

**Fig. 3.** Effects of prednisolone and vitamin D\textsubscript{3} administration on the levels of nitrated proteins in liver tissue: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D\textsubscript{3} administration. Proteins from total liver lysates of rat livers were separated by SDS-PAGE and western blot analysis was performed using the antibodies against 3-nitrotyrosine. Representative immunoblot (a) and quantification of three experiments (b) are shown. The mean protein expression level was quantified as the sum of the densities of immunoreactive bands in each track corresponding to proteins with molecular masses from 50 to 150 kDa. Protein levels were normalized to β-actin. All data are shown as mean ± SEM of three independent experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

**Fig. 4.** Western blot analysis of myeloperoxidase expression in liver tissue: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D\textsubscript{3} administration. Representative immunoblot (a) and quantification of three experiments (b) are shown. Protein levels were normalized to β-actin. All data are shown as mean ± SEM of three independent experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

**Fig. 5.** Hepatic expressions of iNOS and NF-κB. Protein levels of iNOS (a) and p65 subunit of NF-κB (c) were determined by western blot analysis in total liver lysates obtained from three animal groups: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D\textsubscript{3} administration. Representative immunoblots are shown next to the bar charts and quantified using β-actin as a
loading control. Quantitative RT-PCR was used to determine NF-κB p65 mRNA expression (b) in rat livers of three animal groups mentioned above. Levels of mRNA were normalized to GAPDH expression. All data are shown as mean ± SEM of three independent experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

Fig. 6. Levels of IκB-α and phosphorylated NF-κB p65 (Ser311) in liver tissue. Proteins from (a) total liver lysates and (b) nuclear fractions of 1 – control, 2 – prednisolone-administered, 3 – prednisolone- and vitamin D₃-administered rats were subjected to western blot analysis with antibodies against phosphorylated NF-κB p65 (Ser311). The protein level of IκB-α (c) was analyzed in total liver extracts. Representative immunoblots are shown above the bar charts and quantified using β-actin and lamin B1 as a loading controls for total liver lysates and nuclear extracts respectively. All data are shown as mean ± SEM of three independent experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

Fig. 7. Vitamin D₃ bioavailability and VDR expression in liver tissue. 25OHD₃ concentration was measured by ELISA (a). VDR mRNA (b) and protein (c) levels were determined by quantitative RT-PCR and western blot respectively in rat livers of three animal groups: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D₃ administration. Representative immunoblot is shown above the bar charts. Protein levels were normalized to β-actin and mRNA level to GAPDH expression. All data are presented as mean ± SEM of three independent
experiments done in triplicate; * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.

**Table 1.** Effects of prednisolone and vitamin D₃ administration on serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78,45±6,80</td>
<td>17,71±1,42</td>
</tr>
<tr>
<td>Prednisolone administration</td>
<td>112,9±11,03*</td>
<td>29,28±1,96*</td>
</tr>
<tr>
<td>Prednisolone and vitamin D₃ administration</td>
<td>85,34±1,45#</td>
<td>20,05±1,12#</td>
</tr>
</tbody>
</table>

**Note:** results are shown as mean ± SEM (n=10 animals per group); * p<0.05 vs. control, # p<0.05 vs. prednisolone administration.
(a) [Graph with different fluorescence trace lines labeled as autofluorescence, 1, 2, and 3.]

(b) [Bar graph showing relative fluorescence (fold) for samples 1, 2, and 3. Sample 2 has a significant increase marked with an asterisk (*), and sample 3 has a significant decrease marked with a hash mark (#).]
Relative expression level of nitrated proteins (fold)

(a) Image of gel bands with molecular weight markers:
- 130 kDa
- 100 kDa
- 70 kDa
- 55 kDa
- β-actin (42 kDa)

(b) Bar chart showing relative expression levels:
- Sample 1: 1.0
- Sample 2: 1.5
- Sample 3: 1.0

Significance:
- * indicates a significant difference.
- # indicates another level of significance.
Relative expression level of MPO (fold)
Relative expression level of pNF-κB p65 (fold)

(a) Total pNF-κB, 65 kDa

(b) Relative expression level of nuclear pNF-κB p65 (fold)

(c) Relative expression level of IκB (fold)

- * indicates significant difference compared to control
- # indicates significant difference compared to treatment 1