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Differential Oxidative Stress Responses to D-Galactosamine-Lipopolysaccharide Hepatotoxicity Based on Real Time PCR Analysis of Selected Oxidant/Antioxidant and Apoptotic Gene Expressions in Rat

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Short title- RT-PCR analysis of D-GalN/LPS hepatotoxicity

Summary

Oxidative stress and apoptosis are proposed mechanisms of cellular injury in studies of xenobiotic hepatotoxicity. This study is focused on addressing the mutual relationship and early signals of these mechanisms in the D-Galactosamine and lipopolysaccharide (D-GalN/LPS) hepatotoxicity model, with the help of standard liver function and biochemistry tests, histology, and measurement of gene expression by RT-PCR. Intraperitoneal injection of 400 mg/kg D-GalN and 50 µg/kg LPS was able to induce hepatotoxicity in rats, as evidenced by significant increases in liver enzymes (ALT, AST) and raised bilirubin levels in plasma. Heme oxygenase-1 and nitric oxide synthase-2 gene expressions were significantly increased, along with levels of their products, bilirubin and nitrite. The gene expression of glutathione peroxidase 1 remained unchanged, whereas a decrease in superoxide dismutase 1 gene expression was noted. Furthermore, the significant increase in the gene expression of apoptotic genes Bid, Bax and caspase-3 indicate early activation of apoptotic pathways, which was confirmed by histological evaluation. In contrast, the measured caspase-3 activity remained unchanged. Overall, the results have revealed differential oxidative stress and apoptotic responses, which deserves further investigations in this hepatotoxicity model.

Key words: Hepatotoxicity, D-Galactosamine/Lipopolysaccharide, Apoptosis, Oxidative stress, RT-PCR

Introduction

Liver is vulnerable to cellular damage, due to its extensive exposure to high concentrations of xenobiotics. Fulminant hepatic failure (FHF) can be induced by viral infection or xenobiotic injury and its incidence in population is low: however, unless a liver transplantation is carried out the rates of mortality are high (Chan *et al.* 2009). Combination of D-Galactosamine and lipopolysaccharide (D-GalN/LPS) is a well established experimental model for studies of FHF (Feng *et al.* 2007, Silverstein 2004). Administration of D-GalN/LPS causes cytokine release that contributes to increased oxidative stress and formation of reactive oxygen species, which are fatal to the cell and result in hepatocyte death (Liu *et al.* 2008, Oberholzer *et al.* 2001). In addition, D-GalN inhibits mRNA and protein synthesis as it depletes the uridine triphosphate pool (Stachlewitz *et al.* 1999). The exact mechanism of cellular damage in FHF remains unclear. Identifying novel and sensitive early markers in this model of hepatotoxicity that can be used to complement conventional liver function tests is still needed.

Furthermore, the oxidative stress causes a misbalance in pro-oxidant/antioxidant steady state due to generation of increased amount of oxidants resulting in cellular damage as manifested by apoptosis and/or necrosis (Hong *et al.* 2009). Oxidative stress can be induced by toxins and it causes accumulation of reactive oxygen/nitrogen species, by activation of inducible nitric oxide synthase (NOS-2) (Diesen and Kuo 2010). Heme oxygenase-1 (HO-1), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (Gpx1) and catalase are major antioxidant enzymes, which along with the reactions that they catalyze, play important roles in defense against oxidative stress induced by toxins (Farombi and Surh 2006, Mari *et al.* 2009, Valdivia *et al.* 2009). Oxidative stress can induce a TNF-α mediated apoptosis that involves the activation of executive caspases and the members of Bcl-2 family proteins BH3 interacting domain death agonist (Bid) and Bcl-2 -associated X protein (Bax) (Morgan *et al.*

2010, Van Herreweghe *et al.* 2010). Clarifying the steps involved in the complex interaction between the oxidative stress and apoptotic mechanisms is of great value in identifying early markers of cell injury.

One of the approaches to methods in toxicity research that has gained popularity in recent decades is study of toxicogenomics, which focuses on gene and protein activity responses to toxic substances (Gatzidou *et al.* 2007). Real time PCR analysis is one of the methods that has been proven reliable in verification of gene expressions in this field. As well, this method in combination with histopathology and biochemistry provides a further mechanistic approach to research in toxicology (Harril and Rusyn 2008). Our previous research work addressed the mutual cross talk of CO/HO-1 and NO/NOS-2 systems in the D-Galactosamine (D-GalN)/Lipopolysaccharide (LPS) hepatotoxicity (Farghali *et al.* 2009) with the use of these three before mentioned methods. The aim of this study is to provide further insight into the mechanisms of cellular injury in this model, by focusing on involvement of several other major antioxidant enzymes and apoptotic mediators. By analysis of their gene expressions we will attempt to address potential early signals of cell injury and existence of a relationship between conventional liver dysfunction markers and the select gene expression responses.

Materials and Methods

Animals and experimental design

This study was performed on male Wistar rats of 200–300 g body weight obtained from Velaz-Lysolaje, Czech Republic. They were given water and standard granulated diet ad libitum and were maintained under standard conditions; light (i.e. 12 h light and 12 h dark); temperature (22 ± 2ºC); relative humidity (50 ± 10%). All rats received humane care

according to the general guidelines and approval of the Ethical Committee of the First Faculty of Medicine, Charles University in Prague. Rats in the D-GalN/LPS group were injected intraperitoneally with a dose of 400 mg/kg D-GalN (D-Galactosamine hydrochloride) and 50 μg/kg LPS (Lipopolysaccharide from Escherichia coli K-235) dissolved in dimethyl sulfoxide (DMSO), the control group received the equal volume of vehicle only. Eight animals of each group were killed at twenty four hours after injection under light ether anasthesia, after which the blood samples were collected. Following this, livers were excised quickly and perfused for morphological evaluation, preserved in liquid nitrogen for RT-PCR studies, and homogenized for biochemical study.

Measurements of liver enzymes and bilirubin

Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit and/or BioLATest® ALT UV Liquid 500 tests. Fluitest® GOT AST kit by Analyticon and/or BioLATest® AST UV Liquid 500 tests were used in determination of aspartate aminotransferase (AST) plasma levels. Total bilirubin in plasma was measured using Fluitest® BIL-Total kit.

Determination of NO_2^-/NO_3^- , reduced glutathione (GSH) and catalase levels

Assessment of plasma NO_2^-/NO_3^- was carrried out using a nitrate/nitrite colourimetric assay kit of Cayman Chemical Company (An Arbor, MI) and a microplate reader according to manufacturer's instructions. In short, this method is based on a colourimetric conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. The addition of the Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H3PO4) converts nitrite into a coloured azo compound. Spectrophotometrical measurement of absorbance at 540 nm determines the nitrite concentration, using the appropriate standard curve. Assessment

of reduced gluathione in homogenate was based on the method that depends on a reaction between thiol group with 5,5-dithio-2-nitrobenzoic acid (DTNB) which can be measured spectrophotometrically (Sedlak and Lindsay 1968). The measurement of catalase in plasma was performed according to the reaction between H_2O_2 and molybdenium ammonium as previously reported (Aebi 1984).

Lipid peroxidation: The thiobarbituric acid reacting substances (TBARS) and conjugated dienes (CD) measurement

D-GalN/LPS lipid peroxidation of the rat liver was assayed by the thiobarbituric acid (TBARS) method, and the spectrofluorometric assay for conjugated dienes (CD) was carried out as described earlier (Yokode *et al*. 1988). The results were expressed in nmol/mg of total protein.

Select gene expression measurements with the use of real-time PCR method

Twenty-four hours following drug administration, the liver samples were obtained to be used for total RNA isolation according to the manufacturer's instructions of the Qiagen® RNeasy plus kit (Bio-Consult Laboratories). Following total RNA isolation, the reverse transcription from total RNA to cDNA was processed by universal kit GeneAmp® RNA PCR using a murine leukemia virus (MuLv) reverse trancriptase (RT). Reverse transcription included the following three phases: 10 min at 25ºC for RT enzyme activation, 30 min at 48ºC for PCR amplification and 5 min at 95ºC for denaturation.

Expressions of select genes were evaluated using real-time polymerase chain reaction (RT-PCR) of cDNA originating from total RNA, with the help of ABI PRISM 7900, and TagMan ® Gene Expression master mix (Applied Biosystems). Total of eight genes were evaluated using the TagMan ® Gene Expression Assays Kit - nitric oxide synthase-2 (NOS-

2), heme oxygenase-1 (HO-1), glutathione peroxidase 1 (Gpx1), superoxide dismutase 1 (SOD1), BH3 interacting domain death agonist (Bid), Bcl-2 -associated X protein (Bax), caspase 3 (Casp 3)- as genes of interest (target genes) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) gene as a control (endogenous or housekeeping) gene, using the FAM coloured primes and probes. Housekeeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. Thermal cycling conditions for primer and probes optimization were 10 min at 90–95 ºC for Taq polymerase activation, followed by 15 s at 95–99 ºC for DNA denaturation and 1 min at 60 ºC for annealing. The obtained Ct values were used in relative quantification of gene expression measurements relative to the endogenous gene control Ct measurements, and the relative gene expression was calculated using the ΔΔCt method. (Arocho *et al.* 2006).

Measurement of caspase 3 activity and morphological evaluation

Cell lysates were prepared according to the instructions of Sigma-Aldrich (Prague, Czech Republic) fluorometric caspase 3 assay kit. The results were expressed as percentage of caspase 3 activity in the treated group relative to the control. The protein concentration in the supernatant was determined using an Bio-Rad protein assay kit according to the manufacturer’s instructions.

Morphological evaluation of hepatocytes at the light microscopical level was done on semithin epon sections (1 – 2 µm thick) stained by toluidine blue using Leica IM 500 program for digital recording and measurements.

Statistical examinations

All experiments were performed in two groups of eight rats with the reported results stated as ± standard error of mean. The statistical analysis was performed using unpaired T-test with Welch correction. The p-values less than 0.05 were considered significant.

Results

Effects of D-GalN/LPS treatment on liver function, lipid peroxidation and oxidative stress parameters

The combination D-GalN/LPS treatment in rats has produced hepatic failure, which can be seen by highly significant ($p < 0.001$) increases in levels of aminotransferases in plasma. A two hundred fold increase in AST level and one hundred fold increase in ALT level compared to those of the control group was observed (Table 1). The extent of lipid peroxidation as measured by formation of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) did not show any statistically significant differences between the two groups ($p > 0.05$). Furthermore, Table 1 shows highly significant ($p < 0.001$) increase in antioxidant enzyme catalase (CAT) in plasma of D-GalN/LPS treated rats compared to control: however, there was no measurable change in its level in homogenate (data not shown). There was no significant ($p > 0.05$) difference between the two groups in the measurement of reduced glutathione (GSH) level in homogenate.

Fig. 1 further demonstrates changes in the gene expression of selected antioxidant enzymes as measured by the RT-PCR method. Glutathione peroxidase 1 (Gpx1) and superoxide dismutase 1 (SOD1) gene expressions were related to Gadph as the endogenous control, and measured in both D-GalN/LPS and the control groups. The increase of Gpx1 gene expression in the treated group is non significant as $p > 0.05$: however, D-GalN/LPS

treatment has caused a highly significant decrease of SOD1 gene expression in comparison to the untreated control group.

The extent of heme catabolism as shown in the Fig. 2a, illustrates significantly higher levels of bilirubin in plasma of D-GalN/LPS treated rats compared to that of the control group. The same trend is observed in the inducible HO-1 gene expression (Fig. 2b) relative to the Gapdh, where the seven fold increase in the D-GalN/LPS treated group is highly significant. In comparison to the untreated control animals, D-GalN/LPS treatment induced simultaneous statistically significant increase in both plasma NO_2^- levels (Fig. 3a) and NOS-2 expression relative to Gapdh as endogeneous control (Fig. 3b).

Effects of D-GalN/LPS treatment on apoptotic markers and morphological findings

Measurements of selected apoptotic parameters are illustrated in the Fig. 4. Caspase 3 activity, although slightly increased in the D-GalN/LPS group, is not significantly different from the control group (Fig. 4a): however, the expression of Casp 3 gene did show a significant increase. The same trend is also observed in the expressions of Bid and Bax genes, where the increase in the D-GalN/LPS treated group was statistically significant. Bax gene expression was more than two-fold and thus the highest of the three apoptotic genes that were measured.

The morphological evaluation has shown the well preserved cytological features of the control liver tissues. Specifically, the periphreal region of the central vein lobules consists of radially arranged cords of hepatocytes with more or less comparable cytological features, such as stainability of the cytoplasm and distribution of cell organelles (Fig. 5a). The administration of D-GalN/LPS has significantly affected morphological parameters of the rat livers. Even at the lower magnification (Fig. 5b) striking necrotic lesions can be observed in the peripheral and intermediate regions of the central vein lobules. Typical changes and

aggregation of heterochromatin near the nuclear envelope confirm the occurence of apoptosis in some hepatocytes (Fig. 5c). At the peripheral region of some injured lobules transitional change of aponecrosis can be detected and the presence of pycnotic nuclei is clearly visible (Fig. 5d).

Discussion

Understanding the exact mechanism of xenobiotic hepatotoxicity is one of the major challenges hepatologists are faced with today. Recent advances in the studies of toxicogenomics have been useful in elucidating several different pathways of hepatotoxicity. Further research is needed to confirm these results as well to gain a mechanistic understanding of toxic changes that occur in the liver. As before mentioned, combination of D-GalN/LPS is a useful model for hepatotoxicity research that resembles fulminant hepatic failure. In this study, the administration of D-GalN/LPS significantly increased the levels of ALT and AST, which are indicative of failing liver function and are a cardinal feature in the FHF. The impairment of biliary function has been seen by the raised levels of bilirubin in the D-GalN/LPS treated animals. The present study revealed that the extent of lipid peroxidation in this model seems to be non significant, since the levels of conjugated dienes and the measured TBARS in plasma of D-GalN/LPS treated animals were not different from those of the control group.

Heme oxygenase -1 is the inducible isoform that is activated in response to cellular stress, playing a main role in degradation of heme into carbon monoxide, free iron and biliverdin. In turn, the enzyme biliverdin reductase converts biliverdin into bilirubin, a powerful antioxidant with cytoprotective capabilities that has been linked to increased heme oxygenase activity (Baranano *et al.* 2002, Clark *et al.* 2000). As well, the other heme

degradation pathway products, biliverdin and carbon monoxide, play a protective role against oxidative stress which may explain the observed increase in HO-1 expression (Lehmanne *et al*. 2010, Zhu *et al.* 2008). One of the ways lipopolysaccharide exerts its inflammatory action is by stimulation of production of pro-inflammatory cytokine TNF-α by the Kupffer cells. (Lichtman *et al.* 1994). The cooperative action of biliverdin/bilirubin and CO was reported to be responsible for the prolonged survival of mice in the D-GalN/LPS model of hepatotoxicity due to cytokine reduction, specifically TNF-α (Sass *et al*. 2004). This is relevant to the observed parallel increase in both HO-1 expression and bilirubin levels in this experiment.

Oxidative stress causes an increase in production of nitric oxide, a molecule which plays a complex role in both oxidative stress and cell death responses. The activity of NO in this study was reflected in the measurement of its oxidation end product nitrite in plasma, which has been significantly increased in parallel with the gene expression of NOS-2 enzyme in the D-GalN/LPS group. One of the important influences of NOS-2 enzyme is that once it is induced by increased levels of TNF- α, it produces nitric oxide that in turn stimulates additional production of TNF- α resulting in inflammatory injury (Sass *et al.* 2001). Furthermore, nitric oxide is thought to play a dual role in apoptosis acting as both pro-apoptotic and anti-apoptotic mediator depending on various cellular conditions and cell types (Chung *et al.* 2001, Brune 2005). Some studies have shown that this inducible isoenzyme in certain cell types contributes to cell death by increasing caspase 3 activity due to increased cytokine levels such as TNF- α (Obara *et al.* 2010). Earlier studies on hepatocytes however have shown that nitric oxide exerts anti-apoptotic action through direct inhibition of caspase activity by S-nitrosylation, resulting in prevention of Bcl-2 cleaveage and cytochrome C release (Li *et al*. 1997, Kim *et al*. 1998). Although we have not been able to observe any significant change in Caspase 3 activity in D-GalN/LPS treated animals, there was a significant increase in Casp 3 and NOS-2 gene expressions. Furthermore, some studies have

shown that the induced HO-1 enzyme exerts its cytoprotective action through inhibition of inflammatory NOS-2 induction, decrease in levels of cytokines and decreased Caspase 3 activity (Sass *et al.,* 2003, Wen *et al.* 2003). Therefore, the last reports support our present findings in so far as the relationship between HO-1 and billirubin from one hand and NOS-2 and Casp 3 gene expresion on the other hand.

It is well established that interdependence of members of Bcl-2 pro-apoptotic and antiapoptotic proteins plays a major role in apoptotic cell death, through their action on mitochondrial permeability pores, cytochrome C release and activation of caspases (Garcia-Saez *et al.* 2010, Chipuk and Green 2008) . The increases in measured gene expressions of Bcl-2 pro-apoptotic members, Bid and Bax, as well as that of Casp 3 gene expression signify an early initiation of the apoptotic pathways. Furthermore, the morphological evaluation of D-GalN/LPS treatead rats has shown the presence of pycnotic nuclei, which in those cells support a classification of running apoptotic process. In addition, the simultaneously marked disintegration of their cytoplasm shows a necrotic continuation, apparently, following process of apoptotic cell death. Typical changes and aggregation of heterochromatin near the nuclear envelope testify an occurence of apoptosis in some hepatocytes. These findings support the concept of the presence of apoptosis which was followed by necrotic changes, in other words aponecrotic cell death.

Reduced glutathione (GSH) is a powerful antioxidant that protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species and a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins (Ballatori *et al.* 2009). In this study, the GSH levels were similar between the control and D-GalN/LPS group, indicating that the GSH pool has remained intact. In addition to antioxidant action of GSH, the antioxidant enzymes SOD1, Gpx1 and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing ROS from the cell (Yuan and Kaplowitz 2008).

Specifically, SOD reduces superoxide into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase (Valdivia *et al.* 2009). It is noteworthy that within the present experimental conditions gene expression of SOD1 decreased significantly, while that of the Gpx1 remained unchanged. Catalase was significantly induced by D-GalN/LPS as was seen by significantly increased levels in plasma. It might be expected that under the present experimental conditions, the responses of these three parameters would be increased in parallel, however, under D-GalN/LPS toxicity the expected mutual relationship of these antioxidant enzymes was not seen. Differential response of these enzymes may be dependent on the dictating cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states (Djordjevic *et al*. 2010).

In summary, D-GalN/LPS induced hepatotoxicity has resulted in a differential oxidative stress response as reflected by the alterations in expressions of certain oxidant/antioxidant genes, while the expression of others remained unchanged. Even though our findings were not able to confirm a direct relationship between the oxidative and apoptotic parameters that were tested, a parallel relationship between selected enzymes' gene expressions and their respective biochemical markers was seen. Thus, the real time PCR analysis of certain genes, which according to the present conditions is extremely sensitive, combined with conventional biochemical markers and morphology is potentially a very useful tool in understanding various steps involved in D-GalN/LPS induced fulminant hepatic injury.

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Legend to Figures

Fig. 1. Effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on Gpx1 and SOD1 gene expressions relative to Gapdh as the endogenous control 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n = 8; *ns* non-significant value compared to the negative control group (Control) $p > 0.05$; ** value significant compared to Control $p \leq 0.01$**.

Fig. 2. Effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on plasma bilirubin (a) and on HO-1 gene expression relative to Gapdh as the endogenous control (b) 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n = 8; *,** value significant compared to Control $p \leq 0.05$*, $p \leq 0.01$**.

Fig. 3. Effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on plasma NO_2^- (a) and on NOS-2 gene expression relative to Gapdh as the endogenous control (b) 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n = 8; * value significant compared to Control $p \leq 0.05$*.

Fig. 4. Effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on Caspase 3 activity (a) and on Bid, Bax and Casp 3 gene expressions relative to Gapdh as the endogenous control (b) 24h after injection. Control: saline injection only; D-

GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n = 8; *ns* non-significant value compared to the negative control group (Control) $p > 0.05$; *,** value significant compared to Control $p \leq 0.05$*, $p \leq 0.01$**.

Fig. 5. Light microscopy morphological findings of rat liver of control and D-GalN/LPS treated samples: a) control hepatocyte liver- peripheral region of the central vein lobule; trabecular arrangement of polyhedral hepatocytes demonstrates well preserved cytological features; bar 50 µm. b) the effect of D-GalN/LPS treatment (low magnification)- striking necrotic lesions can be seen in the peripheral and intermediate (arrows) regions of a central vein lobule of the liver; many transparent pseudovacuoles are visible; bar 100 µm; c) the effect of D-GalN/LPS treatment (higher magnification)- increased number of neutral lipid droplets, increased distribution of dark granular accumulations in the cytoplasm and activated lysosomal apparatus of injured hepatocytes are seen; typical changes of demilunar apoptotic heterochromatin arrangement in the nucleus are indicated by an arrow; bar 50 µm; d) peripheral region of injured lobule- some transitional stages of aponecrosis can be detected; presence of pycnotic nuclei (arrows); marked disintegration of the cell cytoplasm; bar 50 µm; All samples prepared by semithin epon section, toluidine blue.

Table 1 Effects of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), catalase (CAT), conjugated dienes (CD), reduced glutathione (GSH) and formation of thiobarbituric acid reactive substances (TBARS) 24h after injection.

	CONTROL	D-GalN/LPS
ALT		
Plasma	0.8225 ± 0.05	166.948 ± 12.42***
AST		
Plasma	2.016 ± 0.09	254.802 ± 4.85***
CAT		
Plasma	51.24 ± 6.55	156.00 ± 1.88 ***
CD		
Homogenate	2.45 ± 0.57	2.94 ± 0.54 *ns*
GSH		
Homogenate	458.36 ± 19.35	513.06 ± 49.89 *ns*
TBARS		
Homogenate	197.84 ± 22.24	261.12 ± 6.10 *ns*

CONTROL: negative control group receiving vehicle only. D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Units: ALT and AST µcat/l; CAT- µmol/ml; CD and TBARS- nmol/mg protein; GSH- µmol/mg protein; Values are mean ± S.E.M., n = 8; *ns* non-significant value compared to the negative control group (CONTROL) $p > 0.05$; *, **, *** value significant compared to CONTROL $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***.

Fig. 1.

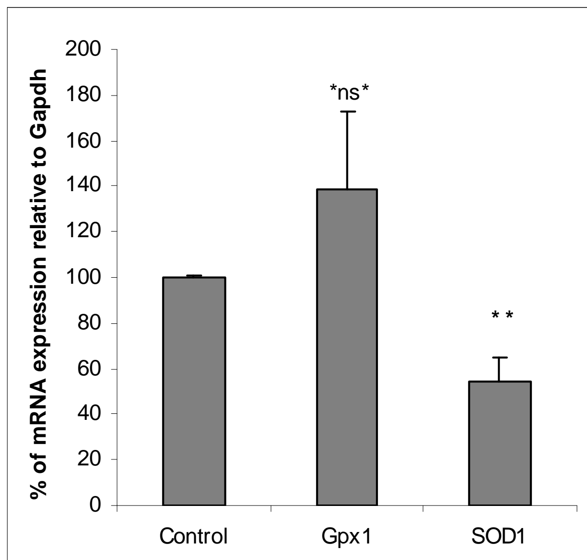

Fig. 2.

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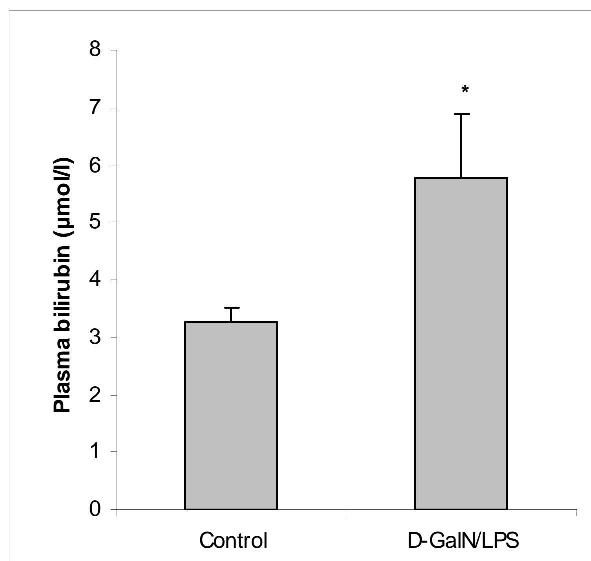

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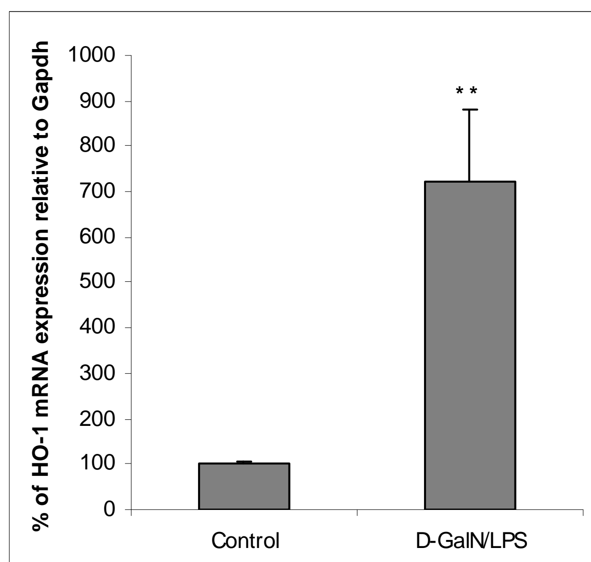
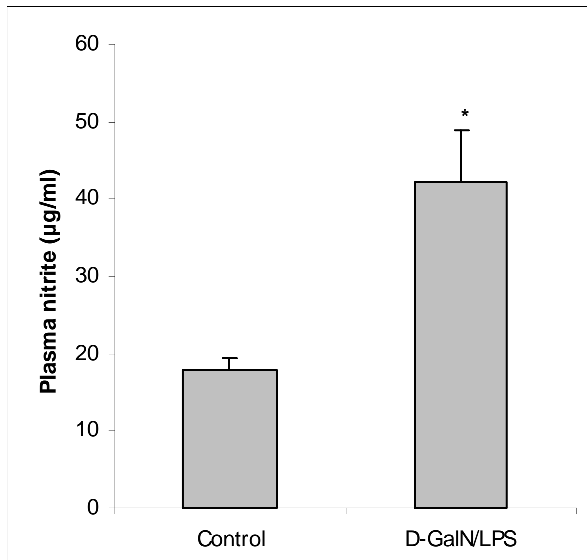


Fig. 3.

a



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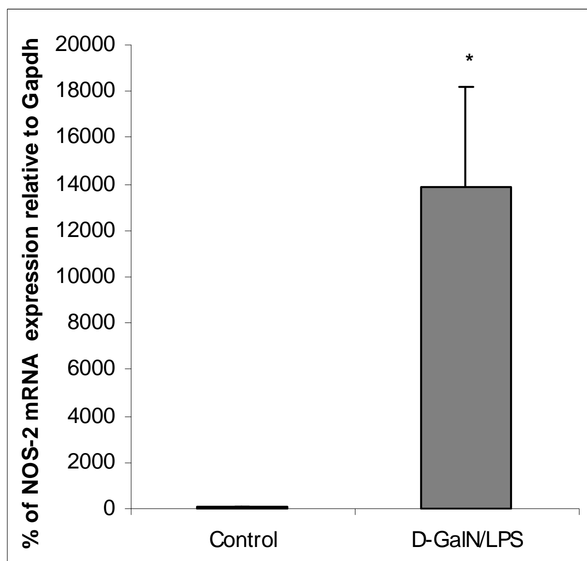
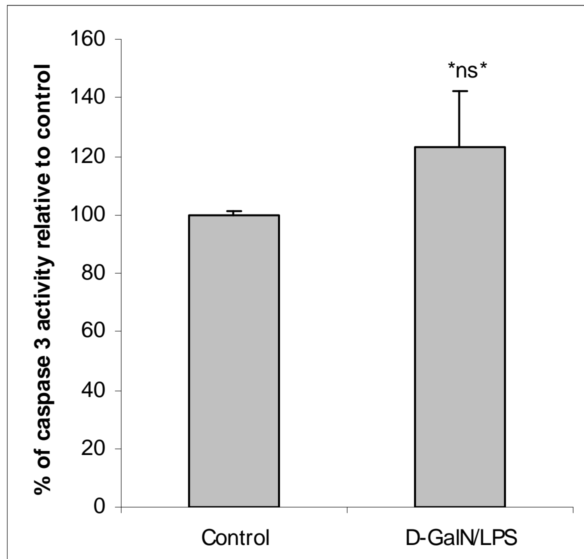


Fig. 4.

a



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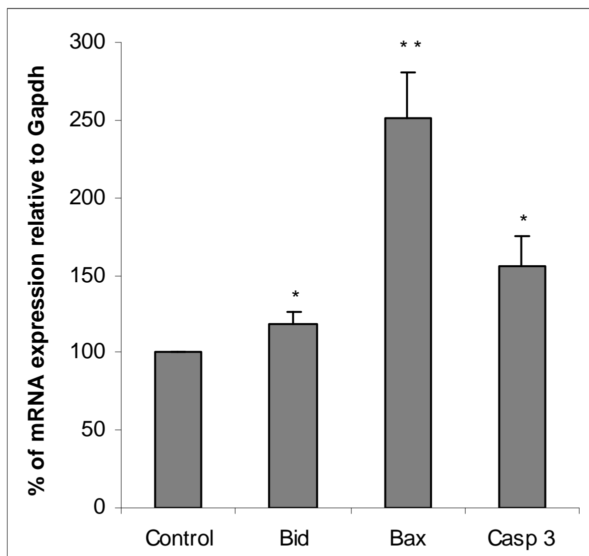

Fig. 5.