TITLE:

D-GALACTOSAMINE/LIPOPOLYSACCHARIDE-INDUCED HEPATOTOXICITY DOWNREGULATES SIRTUIN 1 IN RAT LIVER: ROLE OF SIRTUIN 1 MODULATION IN HEPATOPROTECTION

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SHORT TITLE:

D-GALACTOSAMINE/LIPOPOLYSACCHARIDE-INDUCED HEPATOTOXICITY DOWNREGULATES SIRTUIN 1 IN RAT LIVER

SUMMARY

D-Galactosamine/Lipopolysaccharide (D-GalN/LPS) is a well known model of hepatotoxicity that closely resembles acute liver failure (ALF) seen clinically. The role of sirtuin 1 in this model has not yet been documented. However, there have been a number studies about the cytoprotective effects of resveratrol, a SIRT1 activator, in the liver. This study was aimed at elucidating the roles of SIRT1 protein expression or catalytic activity in D-GalN/LPS model of hepatotoxicity. ALF was induced in male Wistar rats by intraperitoneal injection of D-GalN and LPS. Some groups of animals were pretreated with resveratrol and/or EX-527 (SIRT1 inhibitor). The effects of these treatments were evaluated by biochemical and Western blot studies. D-GalN/LPS treatment was able to induce hepatotoxicity and significantly increase all markers of liver damage and lipid peroxidation. A dramatic decrease of SIRT1 levels in response to D-GalN/LPS treatment was also documented. Resveratrol pretreatment attenuated D-GalN/LPS-induced hepatotoxicity. EX-527 blocked the cytoprotective effects of resveratrol. However, both resveratrol and EX-527 pretreatments did not exhibit any significant effect on SIRT1 protein expression. Collectively, these results suggest that downregulation of SIRT1 expression is involved in the cytotoxic effects of D-GalN/LPS model and SIRT1 activity contributes to the cytoprotective effects of resveratrol in the liver.

KEYWORDS

- SIRT1
- Resveratrol
- EX-527
- D-galactosamine/Lipopolysaccharide
- Hepatotoxicity

INTRODUCTION

Liver is a metabolically active organ responsible for biotransformation and clearance of xenobiotics from the body. It is an important target of drugs and pathogens that may initiate liver cell damage and compromise its overall

function (Hong *et al*. 2009). Currently, there is no way to compensate for the absence of liver function in the long term and massive hepatic destruction often necessitates the need for liver transplantation (Chan *et al*. 2009). There is therefore an intensive search of safe, affordable and readily available agents that can protect the liver from fulminant damage (Cengiz *et al*. 2013).

The general strategy for prevention of liver damage includes reduction of reactive metabolites by using antioxidants (Bansal *et al*. 2005). Natural polyphenolic compounds such as resveratrol, quercetin, curcumin and silymarin possess antioxidant properties and anti-inflammatory effects and have been the subject of considerable research as liver protectants (Rivera *et al*. 2008, Haddad *et al*. 2011, Cerny *et al*. 2011, Lekic *et al*. 2013). Interest in resveratrol has skyrocketed over recent years due to its cytoprotective effects in many organs. For instance, it has been proven to be effective in attenuating vascular endothelial inflammation (Chen *et al*. 2013), diabetic nephropathy (Wen *et al*. 2013) and cholestatic liver injury (Ara *et al*. 2005). Moreover, our experimental studies, both in-vivo and in-vitro, demonstrated that resveratrol is effective in protecting hepatocytes against D-GalN/LPS-induced hepatotoxicity (Farghali *et al*. 2009). However, the exact mechanism by which resveratrol exerts its cytoprotective effects is still elusive.

One of the hypotheses is that resveratrol allosterically activates an NAD^+-dependent histone deacetylase SIRT1 which has multifaceted functions and plays a critical role in cellular stress responses (Howitz *et al*. 2003). On activation, SIRT1 can deacetylate and turn on anti-inflammatory and antioxidant factors such as FOXO (Brunet *et al*. 2004, Hasegawa *et al*. 2008, Tanno *et al*. 2010). The many positive health benefits of SIRT1 can also be explained in part by inhibition of pro-inflammatory factors such as NF- κB (Yeung *et al*. 2004, Farghali *et al*. 2013). This notion is also supported by the finding that SIRT1 deficiency in experimental animals exacerbates conditions such as nephrosclerosis and hyperglycemia which are normally ameliorated by resveratrol treatment (Wang *et al*. 2011, Vasko *et al*. 2014). Nonetheless, the validity of direct SIRT1 activation by resveratrol has been challenged by many researchers. Some studies suggest that activation of SIRT1 by resveratrol is an experimental artifact and resveratrol's health benefits and sirtuins are not related (Beher *et al*. 2009). Besides SIRT1, there are other potential

target molecules such as AMPK that may be involved in the aforementioned cytoprotective effects of resveratrol (Biasutto *et al*. 2012).

This ambiguity prevents development of more potent resveratrol-like compounds which are promising liver protectants. The goal of the present study was to elucidate the roles of SIRT1 protein expression and catalytic activity in D-GalN/LPS model of hepatotoxicity

MATERIALS AND METHODS

CHEMICALS

Lipopolysaccharide from Escherichia coli K-235 (LPS), D-galactosamine hydrochloride (D-GalN), Resveratrol (3,4′,5-Trihydroxy-trans-stilbene, 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol, ≥99% GC), EX-527(6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide, ≥98% HPLC), Tris–HCl, Nonidet P40 Substitute, dimethyl sulfoxide (DMSO), isopropyl alcohol, Tween 20, 2-thiobarbituric acid, tetraethoxypropane, trichloroacetic acid (TCA), sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N′,N″-tetramethylethylenediamine, 2-mercaptoethanol, bromophenol blue, glycerol, N,N′-methylenebis (acrylamide), NaCl, KCl, Na_2HPO_4, KH_2PO_4, ammonium molybdate tetrahydrate, hydrogen peroxide, filter paper, nitrocellulose membrane, anti-mouse IgG (whole molecule)-Peroxidase antibody and mouse monoclonal anti-B-Actin antibody were purchased from Sigma-Aldrich (Prague, Czech Republic). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology through Biotech A.S. (Prague, Czech Republic). Non-fat dry milk was from Biotech A.S. (Prague, Czech Republic). Water for injection 100% w/v was from Baxter (Czech Republic, Prague). Bio-Rad protein assay dye reagent was from Bio-Rad (Prague, Czech Republic).

ANIMALS

Male Wistar rats, 250-400g body weight, were purchased from Velaz-Lysolaje, Czech Republic. They were given water and a standard granulated diet ad libitum. They were maintained under standard conditions (12-hour light-dark

cycle, 22±2°C temperature and 50 ± 10% relative humidity). The animals received humane care in accordance with the ethical guidelines of the First Faculty of Medicine, Charles University in Prague.

EXPERIMENTAL DESIGN

The animals were allowed to acclimatize to the vivarium for seven days before being used in the experiments. Then they were randomly divided into five groups of six animals each and treated as follows:

- Group 1 –Control: DMSO (500µl/kg) + Physiologic solution (1000µl/kg)
- Group 2 –Resveratrol (2.3mg/kg)
- Group 3 –D-GalN (400mg/kg) + LPS (10µg/kg)
- Group 4 –Resveratrol (2.3mg/kg) + D-GalN (400mg/kg) + LPS (10µg/kg)
- Group 5 – EX-527 (1mg/kg) + Resveratrol (2.3mg/kg) + D-GalN (400mg/kg) + LPS (10µg/kg)

The above doses were selected based on our previous experimental studies (Farghali *et al*. 2009, Cerny *et al*. 2011, Lekic *et al*. 2013). All treatments were administered intraperitoneally. Group 1 received only DMSO and physiologic solution. Group 2 was given resveratrol dissolved in DMSO. Group 3 got D-GalN and LPS dissolved in physiologic solution. Group 4 was pretreated with resveratrol 60 minutes before induction of hepatic failure. Group 5 was pretreated with EX-527 30 minutes before resveratrol treatment that was followed 60 minutes later by D-GalN/LPS treatment. At the end of treatment period (6 hours), the animals were anesthetized with diethylether and then euthanized by exsanguination.Their blood samples were immediately collected into heparinized tubes for biochemical investigations.Their liver samples were excised and either homogenized for further biochemical analysis or snap-frozen in liquid nitrogen for Western blot studies.

BIOCHEMICAL INVESTIGATIONS

The extent of liver damage was assessed by detecting the levels of transaminases (ALT, AST) and bilirubin in plasma using commercially available diagnostic kits from Synlab (Prague, Czech Republic). Conjugated Dienes (CD) and Thiobarbituric acid reacting substances (TBARS) were measured in liver homogenate as previously described by Farghali *et al*. 2009.

IMMUNOBLOTTING

Liver samples were homogenized and lysed in NP40 lysis buffer supplemented with protease and phosphatase inhibitors. Equivalent amounts of lysate protein, 20µg of protein measured by the Bradford method, were then subjected to 10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. After blocking the nitrocellulose membranes by incubation with Tris-buffered saline containing 5% non-fat milk (for 1 hour at room temperature), the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used were SIRT1 (1:1000 dilution, Cell Signaling Technology) and Beta actin (1:5000, Sigma Aldrich). The following day, the membranes were washed in TBST and incubated with anti-mouse IgG (whole molecule)-Peroxidase antibody (1:80000, Sigma Aldrich) at room temperature for 1 hour. Proteins were visualized by enhanced chemiluminescence (GeneTiCAs.r.o. Prague, Czech Republic). Densitometric analysis was performed using the Quantity One software (Bio-Rad, Prague, Czech Republic).

STATISTICAL ANALYSES

All data are expressed as mean ±SEM of six animals used in each group. Statistical evaluation of the data was performed using one way ANOVA followed by Tukey-Kramer comparison test. $P < 0.05$ was considered to have statistical significance.

RESULTS

Effect of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury

We first sought to define the role of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury. For this, we measured the levels of ALT, AST and bilirubin in plasma (Figure 1). Treatment of animals with D-GalN/LPS was able to induce hepatotoxicity as evidenced by a significant increase in transaminases and bilirubin levels relative to the negative control groups (CO and RES). There was over 20-fold increase in ALT levels and slightly less with AST and bilirubin. Resveratrol alone had no significant effects on these markers ($^{a}P<0.05$). However, resveratrol pretreatment in D-GalN/LPS rats significantly lowered the ALT and bilirubin levels ($^{c}P<0.05$).

There was also the same trend with AST, despite the statistical non-significance ($^{c}P>0.05$). These findings demonstrate that resveratrol was effective in attenuating D-GalN/LPS induced hepatotoxicity. EX-527, on the other hand, blocked the effects of resveratrol and significantly increased the ALT and bilirubin levels ($^{d}P<0.05$). EX-527 is one of the few available SIRT1 inhibitors which combine high potency with specificity.Hence this finding provides a clear indication that the catalytic activity of SIRT1 is required for the cytoprotective effects of resveratrol.

Effect of resveratrol and EX-527 pretreatment on lipid peroxidation in D-GalN/LPS treated rats

To firmly establish the role of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury, we measured the levels of lipid peroxidation using TBARS and CD in homogenate (Figure 2). Both CD and TBARS were significantly enhanced after D-GalN/LPS treatment reflecting increased peroxidation ($^{a}P<0.05$, $^{b}P<0.05$). Resveratrol pretreatment reduced the levels of both markers by more than a fold ($^{c}P<0.05$). The anti-peroxidative effects of resveratrol were blocked by EX-527 as evidenced by a significant increase in both the TBARS and CD levels ($^{d}P<0.05$). The extent of lipid peroxidation corresponds to the liver function tests above (Figure 1) because lipid peroxidation is an index of oxidative stress (Niki *et al*. 2008).

Effect of resveratrol and EX-527 pretreatment on SIRT1 expression levels in D-GalN/LPS treated rats

A Western blot analysis was performed to confirm if SIRT1 is detected in the liver and how its expression is affected by resveratrol or EX-527 pretreatment. As shown in figure 3, we found that SIRT1 was ubiquitously expressed in liver samples from all the animal groups. Resveratrol alone, did not have any statistically significant effect on the total endogenous amount of SIRT1 ($^{a}P<0.05$). However, treatment with D-GalN/LPS dramatically decreased SIRT1 expression levels. In spite of an increasing trend on the blot, resveratrol pretreatment of D-GalN/LPS rats did not have any statistical significance on SIRT1 expression ($^{c}P>0.05$). Likewise, there was no significant change in SIRT1 expression levels in response to EX-527 pretreatment. This suggests that there may be other ways of modulating the aforementioned biochemical effects of resveratrol (Figure 1 and 2) in the liver rather than alterations in SIRT1 expression.

DISCUSSION

Acute liver failure is one of the most challenging conditions in internal medicine. It occurs when the previously healthy liver cells are seriously injured and die giving rise to complications such as jaundice, coagulopathy and encephalopathy within few days (McDowell *et al*. 2010). Most common causes of ALF are viral hepatitis and drug toxins (Gotthardt *et al*. 2007). Its prognosis is dismissal and in most cases orthotopic liver transplantation is the only definitive curative treatment (Russo *et al*. 2011). However, the scarcity of donors often precludes transplantation (Smith *et al*. 2008). There is therefore an intensive search of therapeutic strategies to prevent the onset of ALF by preventing apoptotic cell death of hepatocytes in experimental models (Hirono *et al*. 2001).

D-GalN/LPS-induced acute liver injury in experimental animals is a well-known in vivo model that closely resemble ALF seen clinically (Kosai *et al*. 1999). In this model, LPS, an endotoxin, activates macrophages and Kupffer cells to produce TNF-α. Through complex signaling cascades, TNF-α activates caspases and transcription factors such as NF-κB leading to cell demise (Silverstain *et al*. 2004, Bradham *et al*. 2008). D-GalN on the other hand selectively depletes uridine nucleotides in the liver, inhibits RNA synthesis in hepatocytes and potentiates the acute toxicity of LPS (Alcorn *et al*. 1992, Lekic *et al*. 2011). The combined effects of these two agents produce a more severe form of liver injury consistent with ALF (Leist *et al*. 1995). In this study, 10µg/kg of LPS and 400mg/kg of D-GalN markedly increased the plasma levels of transaminases confirming that fatal liver injury occurred within six hours of treatment. ALT is the most reliable, sensitive and specific marker of liver injury (Dufour *et al*. 2000). It is abundant in hepatocytes and is released into serum as a result of hepatocellular damage, so its level in plasma approximates the extent of liver damage (Chang-Jung *et al*. 2011). Likewise, D-GalN/LPS treatment augmented lipid peroxidation as shown by increase in the TBARS and conjugated dienes. Lipid peroxidation alters the physical and chemical properties of cell membranes and their fluidity resulting in cytolysis and cell death (Pradeep *et al*. 2009). The levels of bilirubin were also increased in response to D-GalN/LPS treatment. Bilirubin plays an important role as an antioxidant by scavenging peroxyl radicals and preventing oxidation of fatty acids and proteins (Mayer *et al*. 2000). Its activity is augmented in oxidative stress as an adaptive mechanism. Of interest, our Western blots revealed a significant and dramatic decrease in SIRT1 expression levels after D-GalN/LPS treatment (Figure 3). The precise

mechanism by which D-GalN/LPS treatment represses SIRT1 expression was not investigated in this study. However, several studies suggest that generation of ROS plays a key role in the cytotoxic effects of this model (Uchikura *et al*. 2003). For instance, LPS may execute induction of iNOS and subsequent peroxynitrite anion which can oxidize a wide array of molecules within cells including lipids and DNA (Szabó *et al*. 1997, Morikawa *et al*. 2004, Pacher *et al*. 2007, Lekic *et al*. 2013). Moreover, some recent studies have shown that microRNAs such as miR-34a can downregulate SIRT1 expression in response to oxidative stress and therefore augment liver damage (Yamakuchi *et al*. 2012, Choi *et al*. 2013). In brief, our studies add to the mounting evidence that SIRT1 expression is decreased to some extent by the degree of oxidative stress.

Pretreatment with resveratrol ameliorated D-GalN/LPS-induced liver damage as evidenced by a decrease in transaminases and other markers of oxidative stress. Interestingly, resveratrol pretreatment did not have any significant effect on SIRT1 expression level when compared to D-GalN/LPS treatment. This demonstrates that there are other ways in which resveratrol exerts its cytoprotective effects in the liver, beside upregulation of SIRT1 expression reported in some studies (Wang *et al*. 2013). SIRT1 expression and activity can be modulated at different levels. One school of thought is that resveratrol allosterically activates SIRT1. It binds to the non-catalytic N-terminus of SIRT1 to cause a conformational change that lowers its Michaelis constant (Howitz *et al*. 2003). SIRT1 in turn deacetylates and suppresses transcription factors such as NF-κB responsible for induction of pro-inflammatory cytokines and pro-apoptotic factors (Yeung *et al*. 2004). SIRT1 also upregulates FOXO-dependent antioxidants such as catalase and MnSOD which protect against oxidative stress-induced cellular apoptosis (Tanno *et al*. 2010, Hasegawa *et al*. 2008). However, the hypothesis that resveratrol is a bona fide SIRT1 agonist has been challenged by many authors (Beher *et al*. 2009, Baur *et al*. 2012). SIRT1 is not the only resveratrol-sensitive molecule that may have protective downstream effects. Another potential resveratrol target is the main metabolic regulator, AMPK (Centeno-Baez *et al*. 2011). SIRT1 and AMPK mutually coexist, share many common targets and have many overlapping cytoprotective effects (Ruderman *et al*. 2010, Farghali *et al*. 2013). It is also possible that SIRT1 and AMPK are interdependent and resveratrol activates SIRT1 through AMPK (Park *et al*. 2012). While the exact mechanism of resveratrol is yet unknown, within the experimental conditions of the present study, it seems that SIRT1 expression does not contribute to the cytoprotective effects of resveratrol in the liver.

To further demonstrate the role of SIRT1 catalysis in the cytoprotective effects of resveratrol, we pretreated another group of animals with a SIRT1 inhibitor, EX-527. EX-527 was chosen because it is more potent than other available SIRT1 inhibitors such as nicotinamide, splitomicin and sirtinol (Solomon *et al*. 2006). Furthermore, EX-527 is more selective for SIRT1 than other closely related histone deacetylases (Napper *et al*. 2005). However, its inhibition mechanisms are not fully understood. SIRT1 couples lysine deacetylation to NAD hydrolysis to yield nicotinamide and O-acetyl-ADP-ribose (Jackson *et al*. 2002, Blander *et al*. 2004). Kinetic analyses suggest that EX-527 binds to the SIRT1 C-pocket after release of nicotinamide and prevent the release of O-acetyl-ADP-ribose (Napper *et al*. 2005, Gertz *et al*. 2013). Despite non-significant/ negligible effects on SIRT1 expression levels, EX-527 significantly blocked the protective effects of resveratrol and augmented liver damage (Figure 1 and 2). Taken together, these findings confirm that the catalytic activity of SIRT1 plays a key role in the cytoprotective effects of resveratrol in the liver. If the enzymatic activity of SIRT1 is inhibited, then the protective effects of resveratrol are also concomitantly blocked.

In conclusion, we affirm our previous findings that resveratrol is protective against D-GalN/LPS induced hepatotoxicity in rodents. Resveratrol has antioxidant properties and protects cells against lipid peroxidation. Inhibition of SIRT1 by EX-527 renders resveratrol ineffective and exacerbates D-GalN/LPS-induced liver injury. According to our study, SIRT1 downregulation is an involved step in the hepatotoxic effects of D-GalN/LPS treatment but the roles of resveratrol and EX-527 on SIRT1 expression were not documented in this study.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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ABBREVIATIONS

AMPK, Adenosine monophosphate-activated protein kinase

D-GalN, D-Galactosamine

FLF, Fulminant liver failure

FOXO, Forkhead box-O

LPS, Lipopolysaccharide

MnSOD, Manganese Superoxide Dismutase

NAD, Nicotinamide adenine dinucleotide

NF-kB, Nuclear Factor-kappaB

ROS, Reactive oxygen species

SIRT1, Sirtuin 1, Silent information regulator T1

TNF-α, Tumor necrosis factor alpha

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LEGEND TO FIGURES

Figure 1: Effects of resveratrol and EX-527 pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma levels of alanine aminotransferase ALT (a), aspartate aminotransferase AST (b) and bilirubin (c). **CO**, control group; **RES**, 2.3 mg/kg Resveratrol; **D-GalN + LPS**, 400mg/kg D-galactosamine with 10 µg/kg Lipopolysaccharide; **RES + D-GalN + LPS** , 2.3 mg/kg Resveratrol + D-GalN + LPS; **EX-527 + RES + D-GalN + LPS** , 1mg/kg EX-527 plus combination of previous substances. Data are expressed as means ± SEM (n=6). [a]P<0.05 versus CO. [b]P<0.05 versus the RES. [c]P<0.05 versus D-GalN + LPS. [d]P<0.05 versus RES + D-GalN + LPS.

Figure 2: Effects of resveratrol pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (LPS/D-GalN) on the formation of a) Thiobarbituric acid reactive substances (TBARS) and b) Conjugated dienes (CD) in liver homogenate. **CO**, control group; **RES**, 2.3 mg/kg Resveratrol; **D-GalN + LPS**, 400mg/kg D-galactosamine with 10 µg/kg Lipopolysaccharide; **RES + D-GalN + LPS** , 2.3 mg/kg Resveratrol + D-GalN + LPS; **EX-527 + RES + D-GalN + LPS** , 1mg/kg EX-527 plus combination of previous substances. Data are expressed as mean ± SEM (n=6). [a]P<0.05 versus CO. [b]P<0.05 versus the RES. [c]P<0.05 versus D-GalN + LPS. [d]P<0.05 versus RES + D-GalN + LPS.

Figure 3: Effects of resveratrol and EX-527 pretreatment on SIRT1 expression. a) Quantification of SIRT1 expression levels by densitometry. Band intensity measurements were done using Bradford software. In each panel, the intensity of a given band was normalized to the intensity of the corresponding β-actin band. **CO**, control group; **RES**, 2.3 mg/kg Resveratrol; **D-GalN + LPS**, 400mg/kg D-galactosamine with 10 µg/kg Lipopolysaccharide; **RES + D-GalN + LPS** , 2.3 mg/kg Resveratrol + D-GalN + LPS; **EX-527 + RES + D-GalN + LPS** , 1mg/kg EX-527 plus combination of previous substances. Data are expressed as mean ± SEM (n=6). [a]P<0.05 versus CO. [b]P<0.05 versus the RES. [c]P<0.05 versus D-GalN + LPS. [d]P<0.05 versus RES + D-GalN + LPS. b) Representative Western blot images are shown below. **1**) CO; **2**) RES; **3**) D-GalN + LPS; **4**) RES + D-GalN + LPS; **5**) EX-527 + RES + D-GalN + LPS.

FIGURE 1:

Figure 1.a) ALT

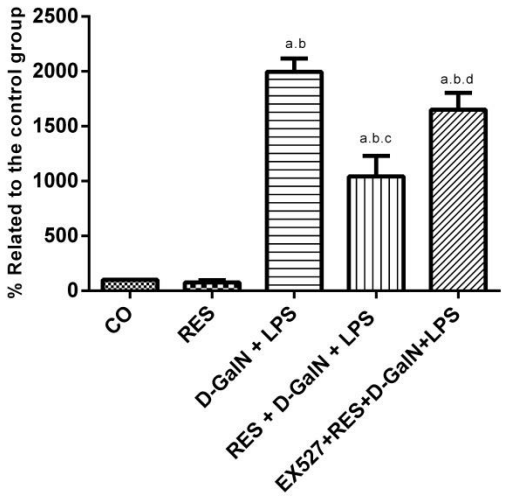

Figure 1.b) AST

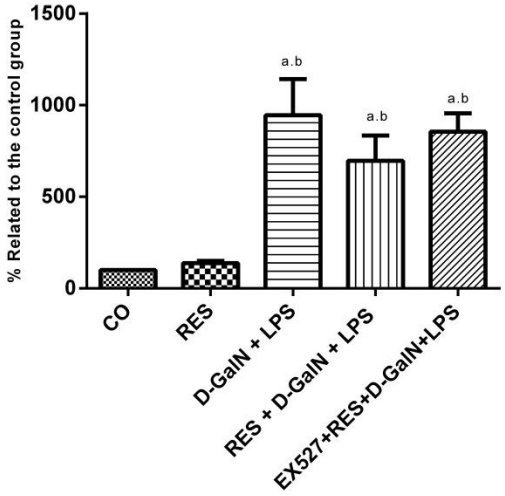

Figure 1.c) Bilirubin

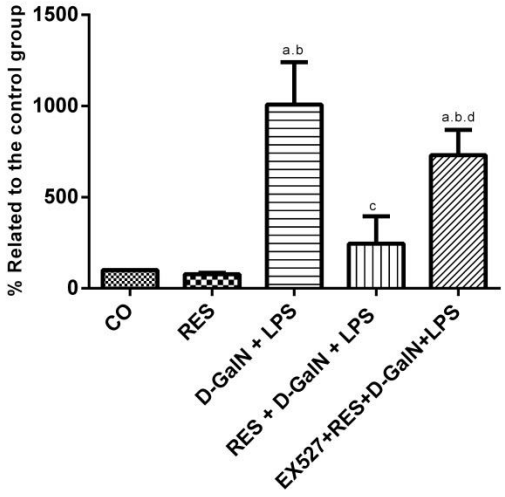

FIGURE 2:

Figure 2.a) Conjugated dienes

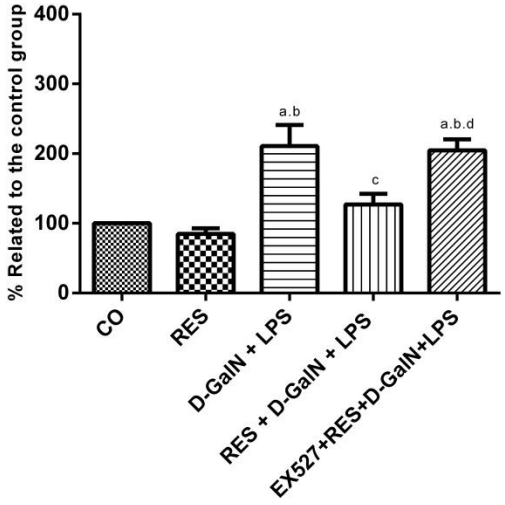

Figure 2.b) TBARS

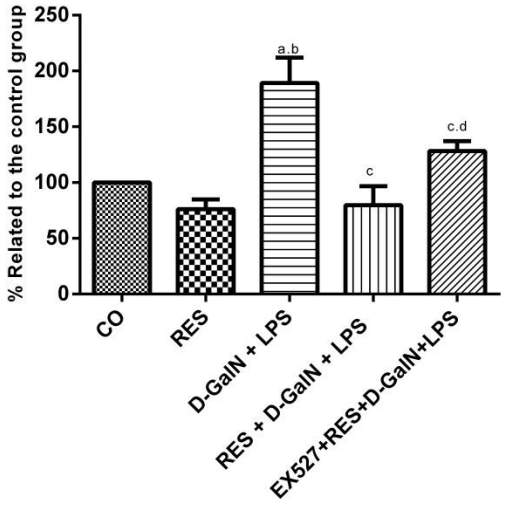

Figure 3:

Figure 3.a) Relative percentage of SIRT1 expression

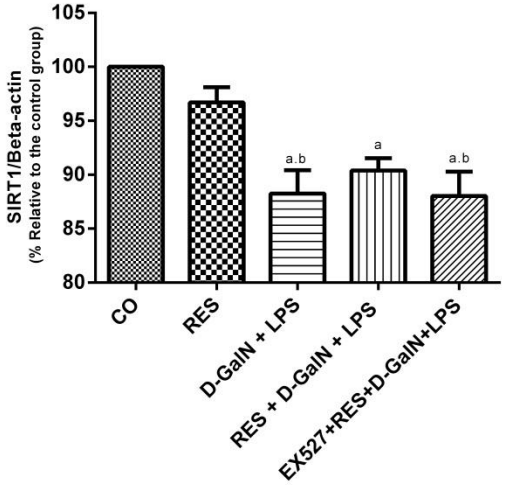

Figure 3.b) Representative Western blot image