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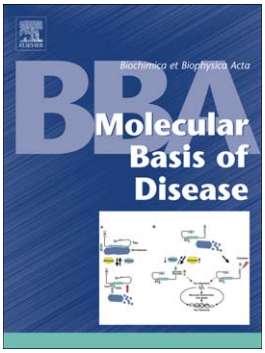

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MARINOBUFAGENIN IS AN UPSTREAM MODULATOR OF GADD45A STRESS SIGNALING IN PREECLAMPSIA

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ABSTRACT

Preeclampsia (PE) is a hypertensive disorder of pregnancy, in which marinobufagenin (MBG), a circulating cardiotonic steroid, is increased. The Gadd45a stress sensor protein is an upstream modulator of the pathophysiological changes observed in PE. However, the effects of MBG on Gadd45a stress signaling remain unknown. We examined the expression of Gadd45a, the sFlt-1 receptor, and p38, as well as caspase 3 and 8 activities in placental samples from four groups of rats. These were: normal pregnant (NP, $n = 8$); pregnant rats which received weekly injections of desoxycorticosterone acetate and 0.9% saline as their drinking water (PDS, $n = 9$); normal pregnant rats injected with MBG (NPM, n = 8); and PDS rats injected with resibufogenin (RBG), an *in vivo* antagonist of MBG, (PDSR, n = 8). Utilizing human cytotrophoblast (CTB) cells, we examined the effect of MBG on these stress signaling proteins *in vitro*. Placental Gadd45a expression, caspases 3 and 8 activities, sFlt-1 concentrations, and sFlt-1 receptor expression were significantly higher in PDS and NPM compared to NP and PDSR rats. Gadd45a protein was significantly upregulated in the CTB cells when MBG was present in concentrations ≥1 nM. Treatment with MBG (≥1nM) also significantly arrested cell cycle progression and activated the expression of the Gadd45a-mediated stress signaling proteins. Inhibition of Gadd45a through RNAi-mediation attenuated MBG-induced CTB cell stress signaling. In conclusion, MBG is involved in the alteration in Gadd45a stress-signaling both in vivo and in vitro and RBG prevents these changes when administered in vivo.

Key Words: Preeclampsia, MBG, RBG, Gadd45a, Apoptosis, Cellular regulation

1. Introduction

Marinobufagenin (MBG) is an endogenous mammalian cardiotonic bufadienolide [1] that inhibits the membrane-bound sodium pump Na^+/K^+-ATPase [2, 3] and has vasoconstrictive properties [4]. MBG secretion occurs in response to increased plasma volume, and circulating levels of MBG have been reported to be increased in patients with volume expansion-mediated hypertension and preeclampsia (PE) and in experimental animal models of PE [5–8]. PE is a syndrome which occurs in 3-10% of pregnancies and is a leading cause of maternal and fetal morbidity and mortality [9-11]. This syndrome is characterized by the *de novo* development of hypertension and proteinuria after 20 weeks of gestation [9, 12]. The precise etiology (etiologies) of this syndrome remain(s) unknown.

In a rat model of PE in which excessive volume expansion was induced [13], we have shown that the urinary excretion of MBG is elevated *prior* to the development of hypertension and proteinuria. These findings implicate MBG as an important factor in the pathogenesis of this animal model which evidences many of the characteristics of human PE [7]. We have also demonstrated that MBG inhibits first trimester cytotrophoblast (CTB) cell function [14-16]. Additionally, we have determined that MBG causes an increase in vascular permeability [17], leading to hemoconcentration, which occurs in human PE [18, 19]. Recently, we reported the discovery of an antagonist to MBG, resibufogenin (RBG), which has a similar chemical structure to MBG [20-22]. When injected into "preeclamptic" rats [20], RBG restored blood pressure to normotensive levels and corrected the proteinuria. [1]

[1] **Abbreviations:**
(PE) preeclampsia; (sFlt-1) sfms-like tyrosine kinase; (Gadd45) growth arrest and DNA damage-inducible family of genes; (MBG) marinobufagenin; (RBG) resibufogenin; (DOCA) desoxycorticosterone acetate; (PDS) pregnant + DOCA + saline; (NPM) normal pregnant rats injected with MBG; (PDSR) PDS rats injected with RBG; (IUGR) intrauterine growth restriction; (CTB) cytotrophoblast; (IL-6) interleukin-6; (MAPK) mitogen-activated protein kinase; (DMSO) dimethyl sulfoxide; (ELISA) enzyme linked immunosorbent assay; (EIA) enzyme immunometric assay.

When given early in pregnancy, RBG prevented the development of hypertension, proteinuria, and intrauterine growth restriction (IUGR) [22]. PE is associated with inflammatory changes in placental tissues [23] and accumulation of circulating inflammatory cytokines [24]. Placental stresses during pregnancy, notably hypoxia, ischemia and an increase in soluble fms-like tyrosine kinase-1(sFlt-1) are important in the etiopathogenesis of PE [25-28].

The growth arrest and DNA damage-inducible (Gadd45) family of genes are known to be associated with cell growth control, cell cycle regulation, apoptotic cell death and the cellular response to DNA damage [29-31]. Gadd45 is an important sensor of oxidative stress and hypoxia [32, 33]. Gadd45 proteins are signal transducers that convert environmental and physiological stresses into various cellular stress responses including inflammation, innate immunity and autoimmune diseases [34-36]. The Gaad45 family genes encode for small proteins (~18 kDa) that bind to and regulate the activity of several downstream stress response proteins that ultimately mediate activation of both p38 and Jnk stress response kinases [33, 37-40]. The Gadd45a stress sensor protein has been reported to be an upstream modulator of pathophysiological changes observed in human PE [41].

PE is a syndrome, not a single disease entity, which involves more than one pathogenetic mechanism [9, 10]. It has been postulated that a significant segment of the PE population is responding to the secretion and elaboration of an excessive amount of MBG [42]. To date, no study has focused on the role that MBG plays in the regulation of Gadd45a stress signaling in the pathogenesis of PE. In addition, the effects of RBG on Gadd45a have not previously been studied. The purpose of this study was to investigate the relationship/interactions between MBG and Gadd45a stress signaling in the development of PE in our rat model. We, therefore, examined the role of MBG in Gadd45a stress

signaling in this model of PE and determined whether RBG antagonized the actions of MBG on Gadd45a. In addition, we evaluated the effects of MBG on Gadd45a stress signaling in CTB cells. We also determined the effect of RNAi-mediated inhibition of Gadd45a expression on stress signaling in CTB cells.

2. Materials and Method:

2.1 Animals

The surgical procedures and experimental protocol were conducted at Texas A&M University Health Science Center/Scott and White Hospital after approval by the Institutional Animal Care and Use Committee. The facility is approved by the American Association for Accreditation of Laboratory Animal Care in accordance with National Institutes of Health guidelines.

2.2 Animal Preparation

Male (275-325g) and female (200-250g) Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). These animals were housed in the institutional animal facility and allowed free access to standard rat chow (Lab Diet 5001 Laboratory Rodent Diet) and tap water. They were maintained on a 12:12 hour dark /light cycle. The room temperature and humidity were maintained at 25° ± 2° C and 55% respectively. Four groups of animals were studied: normal pregnant (NP, $n = 8$); pregnant rats which received weekly injections of desoxycorticosterone acetate and whose drinking water was replaced with 0.9% saline (PDS, $n = 9$); normal pregnant rats injected with MBG (7.65 µg/kg/d, NPM, n = 8); and PDS rats injected daily with RBG (30 µg/kg/d, PDSR, n = 8) beginning once pregnancy had been established. Systolic blood pressure was measured by the tail-cuff method (IITC Inc., LifeScience Instruments, model 59). At 18-20 days of pregnancy, 24-h urine was collected in the absence of food. Each animal was housed separately in a metabolic cage. The animals were then sacrificed on days 18-20 and blood samples were taken. Pups were counted and examined.

2.3 Urine and Blood Analyses

The 24-hr protein excretion was measured using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method, Sigma). Creatinine was measured in the blood and urine on a Nova 16 Analyzer (Waltham, MA) and the creatinine clearance was calculated. Hematocrit was measured using a StatSpin MP Multipurpose Centrifuge (Norwood, MA).

2.4 Harvesting and Preparation of Placental Tissue Homogenates and Measurement of Placental Gadd45a Protein Expression

Placental tissue was dissected, weighed and homogenized in a cold buffer preparation (10 mM Tris-HCl pH = 7.5, 0.3 M sucrose, 10 µM apoptinin, 10 µM pepstatin, 10 µM leupeptin, 1mM PMSF). The tissue homogenates were centrifuged (10,000 X g for 60 min at 4°C) and the supernatant was collected and analyzed for protein estimation [43]. The Gadd45a protein was measured by gel electrophoresis of the placental homogenate followed by detection with immunoblotting (Western blotting) using anti-Gadd45a antibody as described previously [41].

2.5 Caspase 3 and 8 Activities in the Placental Tissue Homogenates

Caspase 3 and 8 activities were determined using commercially available assay kits (Calbiochem, La Jolla, CA) as described previously [17]. The tissue homogenates were treated with the substrate conjugate and incubated for 2 h at 37°C. The DEVD substrate (caspase 3 assay) and IETD substrate (caspase 8 assay) provided in the assay kits were labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin. The resulting fluorescence was measured in a fluorescent plate reader, which measured excitation at 400 nm and emission at 505 nm.

2.6 sFlt-1 Concentration in the Placental Tissue Homogenates

sFlt-1 was measured by commercially available kits (R & D Systems) according to the instructions provided by the manufacturer as described previously [25]. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF R1 (sFlt-1) was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF R1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF R1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. Color then developed in proportion to the amount of VEGF R1 bound in the initial step. Color development was then stopped and the intensity of the color was measured.

2.7 Cytotrophoblast Cell culture

The human extravillous CTB cell line SGHPL-4 utilized in these studies was derived from first trimester extravillous tissue [44], and was kindly provided by Dr. Guy Whitley (St. George's Hospital Medical School, London, UK). These cells are well characterized and share many characteristics with isolated primary cells, including the expression of cytokeratin-7, HLA class I antigen, HLA-G, BC-1, CD9, human chorionic gonadotropin and human placental lactogen [45-48].

SGHPL-4 cells were cultured in Ham's F10 Nutrient Mix supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 mg/ml) and 2 mM L-glutamine (Sigma). Cells were incubated at 37^0C, 5% CO_2, and 99% humidity (Fisher Scientific, Isotemp CO_2 incubator). CTB cells were seeded (7,000 cells/well) onto 6-well plates in complete medium and allowed to adhere overnight at 37°C. Cells were then serum-starved in medium containing 0.5% FBS for 24 h and washed twice with 1X phosphate buffered saline (PBS). Four replicates were subsequently treated with 10% FBS/Ham F12 containing DMSO (vehicle) or 0.1, 1, 10 or 100 nM MBG. Cells were

incubated for 24 h with the respective treatments and were then collected for the measurement of Gadd45a protein expression.

2.8 Gadd45a mRNA Knockdown

CTB cells were sub-cultured to 30-50% confluence 24 h before transfection. Either 60 pmol Gadd45a RNAi or RNAi negative control (Invitrogen) were added into the culture medium together with 10 µl Lipofectamine RNAiMAX (Invitrogen). CTB cells were cultured with the transfection reagents for 4-6 h. The transfection media was replaced with normal media for 48 h prior to the indicated treatments. The sense-strand sequence for two siRNAs is described (a complementary oligonucleotide was synthesized) as follows: GADD45a siRNAs 5'-AACGTCGACCCCGATAACGTG and 5'-AACATCCTGCGCGTCAGCAAC. Chemically synthesized RNA oligonucleotides were annealed, deprotected, and desalted as recommended by the manufacturer.

2.9 Effect of MBG on Cell Cycle Phase Determination Before and After Gadd45a mRNA Knockdown

Cell cycle progression was evaluated by Accuri's Cell Cycle Phase Determination Kit (Cayman Chemical Company, Ann Arbor, MI). CTB cells were treated with DMSO (vehicle), 0.1, 1, 10 or 100 nM MBG before and after Gadd45a mRNA knockdown. The cells were harvested by trypsinization, and fixed with 70% cold ethanol. Prior to flow cytometric analysis, the cells were washed and resuspended in PBS containing 20 µg/ml of RNase A and 50 µg/ml propidium iodide. Cell cycle analysis was performed on an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI), and cellular DNA content was analyzed with FCS Express software (De Novo Software, Los Angeles, CA). Experiments were repeated at least four times.

2.10 Effect of MBG on Apoptotic Signaling of CTB Cells Before and After Gadd45a mRNA Knockdown

Apoptosis was evaluated by Accuri's Apoptotic Blebs Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The kit employs a recombinant protein containing single-chain variable fragments fused to the protein A, domain B fraction as an antibody for apoptotic blebs. The protein A portion of this fusion protein is recognized and visualized by fluorescein-conjugated rabbit IgG. Propidium iodide is included in the kit for detection of dead cells. CTB cells were treated with DMSO (vehicle), 0.1, 1, 10 or 100 nM MBG before and after Gadd45a mRNA knockdown. The apoptotic blebs assay was performed on an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI), and staining intensity was analyzed with FCS Express software (De Novo Software, Los Angeles, CA). Experiments were repeated at least four times.

2.11 Effect of MBG on p38 Phosphorylation Before and After Gadd45a mRNA Knockdown

The effect of MBG on p38 phosphorylation was evaluated by immunoprecipitation and Western blot analysis. CTB cells were treated with DMSO (vehicle) or 0.1, 1, 10 or 100 nM MBG for 60 min. After each treatment, total cellular protein was obtained in lysis buffer containing 50 mM Tris at pH 7.4, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.3 mM Na-orthovanadate, 50 mM NaF, 1 mM DDT, 10 µg/ml leupeptin and 5 µg/ml aprotinin. Protein concentrations were determined by BCA reagent (Pierce). An equal amount of protein in each sample was separated by 12% SDS/PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 20 mM Tris (pH 7.6), 250 mM NaCl containing 5% BSA and probed with mouse anti-p38 polyclonal antibody (Santa Cruz). Following incubation with peroxidase- or alkaline phosphatase-conjugated donkey anti-mouse secondary antibody (Santa Cruz), proteins were visualized with a chemiluminence detection system (GE Healthcare). The intensity of the bands was determined by scanning video densitometry using a

phospho-imager (Storm 860) and ImageQuant TL software version 2003.2 (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.12 Effect of MBG on sFlt-1 Concentration Before and After Gadd45a mRNA Knockdown

The concentration of sFlt-1 in CTB cell culture medium was measured by commercially available kits (R & D Systems) according to the instructions provided by the manufacturer as described previously [25].

2.13 Effect of MBG on the sFlt-1 receptor (VEGFR-1) Before and After Gadd45a mRNA Knockdown

The effect of MBG on the sFlt-1 receptor was evaluated by immunoprecipitation and Western blot analysis. CTB cells were treated with DMSO (vehicle) or 0.1, 1, 10 or 100 nM MBG for 24 h. The sFlt-1 receptor was measured by gel electrophoresis of the CTB cell lysates followed by detection with immunoblotting (Western blotting) using anti- VEGFR-1 antibody as described previously [41].

2.14 Effect of MBG on 8-isoprostane (8IP) Concentration Before and After Gadd45a mRNA Knockdown

CTB cells were treated with DMSO (vehicle), 0.1, 1, 10 or 100 nM MBG before and after Gadd45a mRNA Knockdown for 24 h. The levels of 8IP in the culture media of MBG-treated CTB cells were measured by a commercially available ELISA kit (Cayman Chemical Company, Ann Arbor, MI).

2.15Effect of MBG on IL-6 Secretion Before and After Gadd45a mRNA Knockdown

IL-6 concentrations in the serum-free media of CTB cells after treatment with 0.1, 1.0, 10 or 100 nM MBG were determined by a human IL-6 TiterZyme Enzyme Immunometric Assay (EIA) kit (Assay Designs) [49, 50]. The kit employs a monoclonal antibody to human IL-6 immobilized on a microtiter plate to bind human IL-6 in standards or experimental samples. CTB cells were treated with 0.1, 1.0, 10 nM or 100 nM MBG in serum-free medium. After 24 h treatment, media were collected from the

flasks seeded with CTB cells. Cells treated with DMSO were used as controls. All of the samples were collected from flasks after treatment for determination of IL-6 concentration. After a short incubation, the excess sample or standard was washed out and an antibody to human IL-6 was added. Following a short incubation [14], the excess antibody was washed out and donkey anti-rabbit IgG conjugated to HRP was added. After a short incubation [14], the enzyme reaction was stopped and the color generated was read at 450 nm.

2.16 Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons for multiple determinations were performed using a one-way ANOVA analysis of variance with Tukey's *post hoc* test. A *p* value of < 0.05 was considered significant.

3. Results

3.1 Blood Pressure, Urine and Blood Analyses

Data for blood pressure (BP) measurements, the urinary excretion of protein, creatinine clearance, hematocrit levels and number of pups are presented in Table 1. BP rose in the PDS and NPM animals compared to NP and PDSR ($p<0.05$). BP in the NP animals fell over the course of gestation, as was also the case in the PDSR rats. The PDS and NPM animals showed a statistically significant ($p<0.05$) increase in protein excretion when compared with the NP and PDSR groups. The mean hematocrit values for the PDS and NPM groups were statistically significantly lower than those obtained in the NP and PDSR groups ($p <0.05$ in each case). The mean number of pups for the PDS and NPM groups was significantly lower than those obtained in the NP and PDSR groups ($p <0.05$ in each case). There were no statistically significant differences in creatinine clearance between the groups. These data reproduce the observations obtained in previous studies in this animal model of preeclampsia [7, 13]. Thus, the PDS and NPM animals became hypertensive and demonstrated an increase in protein

excretion which exceeded that seen in the other two groups of animals. As is the case in PE patients, the hematocrit values in our PDS and NPM rats were higher than in the NP group (Table 1). Likewise, the PDS animals and rats given MBG (NPM) demonstrated hemoconcentration [51]. The lower number of pups in the PDS and NPM groups compared to NP and PDSR rats reflect the fact that PDS ("preeclamptic") and NPM rats develop IUGR, while RBG prevents the development of IUGR [22]. There were no malformed pups in the NP and PDSR groups. The percentage of malformed pups for PDS and NPM rats were 18% and 6% respectively (Table 1). This finding indicates that RBG prevented the development of IUGR.

3.2 Gadd45a Protein Expression in the Placental Tissues

Western Blot analysis was performed to evaluate the expression of Gadd45a in the placental tissues from the four groups of rats. Gadd45a protein expression was significantly higher in the PDS and NPM groups compared to the NP and PDSR animals (Figure 1). The stress sensor protein Gadd45a was upregulated in the placental tissues of our rat model and in the normal pregnant rats injected with MBG. RBG prevented this alteration in the placental tissues of the PDSR animals.

3.3 Caspases 3 and 8 Activities in the Placental Tissues

Caspases are the molecular instigators of apoptosis. Caspase 3 and 8 activities were significantly higher in the PDS and NPM groups compared to the NP and PDSR animals (Figure 2). Apoptotic signaling was observed in the placental tissues of our rat model and in the NPM rats. This signaling was prevented in the placental tissues of RBG-injected PDS animals.

3.4 sFlt-1 Concentration in Placental Tissues

sFlt-1 was significantly higher in the PDS and NPM groups compared to the NP and PDSR animals (Figure 3). sFlt-1, a downstream stress response protein of Gadd45a was upregulated in our rat model and in the NPM rats. This alteration was attenuated in the placental tissues of the PDSR rats.

3.5 sFlt-1 (VEGFR-1) Receptor Expression in Placental Tissues

VEGFR-1 was significantly upregulated in our rat model of PE and normal pregnant rats injected with MBG. This alteration was attenuated in the placental tissues of the RBG-injected PDS rats (Figure 4).

3.6 Effect of MBG on Gadd45a Protein Expression in CTB cells

Gadd45a protein expression was significantly increased in 1, 10 and 100 nM MBG- treated CTB cells when compared to the DMSO (vehicle)-treated cells. The 0.1 nM MBG treatment had no effect (Figure 5A).

3.7 Gadd45a Protein Knockdown

The RNAi-mediated inhibition of Gadd45a expression completely knockdown the Gadd45a protein in CTB cells (Figure 5B).

3.8 Effect of MBG on Cell Cycle Progression

Gadd45a is an upstream activator of cell cycle arrest. MBG (1, 10 or 100 nM) significantly arrested progression of the cell cycle compared to DMSO (vehicle)-treated cells, whereas 0.1 nM MBG had no effect (Figure 6A). There was a significantly higher percentage (~80%) of cells in the G0/G1 phase in the MBG 1, 10 and 100 nM-treated cells compared to basal and to 0.1 nM treated cells (~ 55%) ($p< 0.05$). The effect of MBG on cell cycle progression in CTB cells was prevented by Gadd45a knockdown (Figure 6B).

3.9 Effect of MBG on Apoptotic Signaling

Apoptosis was evaluated by FACS analysis. MBG (1, 10 or 100 nM) significantly induced apoptotic signaling compared to DMSO (vehicle)-treated cells, whereas 0.1 nM MBG had no effect ($p<0.05$) (Figure 7A). There was a significantly lower percentage (~60%) of healthy cells in the MBG 1, 10 and 100 nM-treated cells compared to basal and to 0.1 nM treated cells (~

90%) (Figure 7A) ($p<0.05$). The effect of MBG on the apoptotic signaling in CTB cells was prevented by Gadd45a knockdown (Figure 7B).

3.10 Effect of MBG on p38 Phosphorylation in CTB Cells

MBG (1, 10 and 100 nM) caused a significant increase in the ratio of phosphorylated p38 to total p38 in CTB cells compared to basal and to 0.1 nM treated cells (Figure 8A) ($p<0.05$). MBG had no effect on total p38 expression demonstrating that the changes in the ratio were due to increased phosphorylation. The RNAi-mediated inhibition of Gadd45a expression abolished the MBG-induced upregulation of p38 phosphorylation (Figure 8B).

3.11 Effect of MBG on sFlt-1 Secretion in CTB Cells

The application of MBG ≥1 nM significantly increased the secretion of sFlt-1 in the culture medium of CTB cells (Figure 9A). The RNAi-mediated inhibition of Gadd45a prevented this effect (Figure 9B).

3.12 Effect of MBG on sFlt-1 (VGFR-1) Receptor Expression in CTB Cells

MBG (1, 10 and 100 nM) caused a significant increase in the VEGFR-1 expression in CTB cells compared to basal and to 0.1 nM treated cells (Figure 10A) ($p<0.05$). The RNAi-mediated inhibition of Gadd45a expression abolished the MBG-induced upregulation of VEGFR-1 in CTB cells (Figure 10B).

3.13 Effect of MBG on 8-IP Secretion in CTB Cells

The administration of MBG ≥1 nM significantly increased the secretion of 8-isoprostane in CTB cells (Figure 11A). The effect was abolished in the Gadd45a knockdown CTB cells (Figure 11B).

3.14 Effect of MBG on IL-6 Secretion in CTB Cells

The administration of 1, 10 and 100 nM MBG induced the secretion of IL-6 in the culture medium of CTB cells compared to DMSO-treated and to 0.1 nM treated cells (Figure 12A). The RNAi-mediated inhibition of Gadd45a expression abolished this effect in CTB cells (Figure 12B).

4. Discussion

We have proposed that excessive volume expansion in pregnancy [41] serves as the initial stimulus for the increased levels of circulating MBG very early in pregnancy [7, 42]. The addition of MBG to CTBs in cell culture causes cytotrophoblast dysfunction [14-16]. Assuming that this situation also obtains *in vivo*, defective placentation would supervene. The syndrome of PE is thought to result from inadequate "placentation" related to a failure of the CTB cells to adequately remodel the vasculature of the uterus in a significant segment of the PE population [52]. This results in hypoperfusion of the maternal-fetal unit, and is thought to result in oxidative stress and endothelial dysfunction, which are responsible for the development of the syndrome [53]. The resultant hypoxia and ischemia would lead to a continued elevation in MBG levels in preeclamptic patients. Placental stresses during PE cause the induction of Gadd45a and the activation of its downstream effector p38, which in turn induces the secretion of sFlt-1 [41]. Therefore, we investigated the consequences of MBG-induced Gadd45a stress signaling in our rat model of PE. This study provides novel data suggesting that Gadd45a expression, apoptotic signaling, and sFlt-1 receptor expression are upregulated in the placental tissues of our rat model (Figures 1-4). MBG was found to be involved in the causation of these alterations, and RBG prevented these changes. The administration of MBG to pregnant rats reproduces the syndrome of PE, which includes hypertension and proteinuria [20, 54]. We have reported that RBG corrects hypertension in the rat model of PE [20]

and, when administered early in pregnancy, completely prevents all of the manifestations of the PE syndrome [22]. Apoptosis has been linked to the clinical manifestations of PE [55, 56]. Increased apoptosis has been observed in extravillous trophoblast cells of placentas from pregnancies complicated by PE and IUGR [57, 58].

Important progress in our understanding of the pathogenesis of PE resulted from the finding of an increase in circulating antiangiogenic factors such as sFlt-1 [25], which is barely detectable in normal pregnancies. In an animal model, the administration of sFlt-1 elicited many of the typical pathologic changes associated with preeclamptic pregnancies [25]. Xiong et al, showed that induction of Gadd45a expression was required upstream of hypoxia-mediated sFlt-1 induction [41]. Our data are in agreement with the latter study. Furthermore, the data in this communication indicate the involvement of MBG in the Gadd45a stress signaling cascade. Moreover, we have demonstrated that RBG prevents Gadd45a stress signaling. Recently, it has been suggested that autoantibodies from women with PE induce sFlt-1 production via angiotensin receptor activation and calcineurin signaling [59]. Most recently, studies in the authors' laboratory demonstrated that the administration of MBG produced an upregulation of the AT_1 receptor in the placental tissue of this rat model of PE [60]. The administration of RBG, an antagonist of MBG, prevented these changes in the AT_1 receptor [60]. Whether Gadd45a interferes with the MBG-induced AT_1 receptor-signaling cascade remains to be investigated.

To determine the cellular mechanisms of Gadd45 signaling, we examined the effect of MBG on the Gadd45a stress signaling cascade in CTB cells. We found that Gadd45a expression was upregulated in MBG-treated CTB cells (Figure 5). The MGB-induced cell cycle arrest in CTB cells was prevented

by the siRNA knockdown of Gadd45a (Figures 6A & 6B). Gadd45a is an upstream activator of cell cycle arrest. Inhibiting endogenous expression of Gadd45 genes in human cells by antisense Gadd45 constructs was found to impair cell cycle arrest following exposure to UV radiation [61, 62]. In addition, microinjection of a Gadd45a expression vector into primary human fibroblasts arrested the cell cycle [62]. Similarly, in the studies presented in this communication, MBG-induced apoptotic signaling in CTB cells was abolished by the knockdown of Gadd45a (Figures 7A & 7B). Ample evidence exists that Gadd45 proteins have a pro-apoptotic function [36, 63, 64]. An ectopic expression of Gadd45 proteins was shown to induce apoptosis in HeLa cells [36] as well as enhance stress-mediated apoptosis in both M1 leukemia and H1299 lung carcinoma cells [63]. This induction of apoptosis was shown to be dependent upon the interaction of Gadd45 proteins with MEKK4, an upstream activator of the stress-induced p38/Jnk kinases [36]. Furthermore, Gadd45a has been implicated in apoptosis of UV-irradiated keratinocytes [64].

As we have previously reported [14], MBG significantly increased the phosphorylation of p38 in CTB cells. The RNAi-mediated inhibition of Gadd45a expression abolished the MBG-induced upregulation of p38 (Figures 8A & 8B). The stimulated Jnk and p38 pathways are known to regulate cell proliferation, invasion, survival, migration, growth arrest and apoptosis [65, 66].

The knockdown of Gadd45a prevented the MBG-induced induction of sFlt-1 secretion, sFlt-1 receptor expression, 8-IP and IL-6 secretion in CTB cells (Figures 9A & 9B; 10A & 10B; 11A & 11B; 12A & 12B). Xiong et al, demonstrated that the induction of Gadd45 expression and its activation of p38 were required upstream of sFlt-1 induction [41]. In a previous study, we have shown that p38 inhibition prevented the secretion of IL-6 by CTB cells in response to MBG [14].

The inflammatory cytokine IL-6 has been reported to be increased in the serum of severe cases of PE [67], which, in turn, also causes increased secretion of sFlt-1 mediated via Gadd45a-p38 signaling. It has been suggested that Gadd45a-p38 signaling may also function to promote the development of a preeclamptic state by increasing the levels of IL-6 [41]. Thus, strategies to diminish the presence of this cytokine in the inflammatory cascade, or to combat the production of other cytokines (e.g. with IL-10) or with anti-inflammatory agents [68] may represent another avenue in the approach to the treatment of this disorder. The action of MBG may occur upstream of the Gadd45-p38-IL6-mediated stress signaling in the pathogenesis of PE. It seems likely that intervention in the Gaad45a stress signaling pathway might represent a preventive or therapeutic stratagem in PE. Further work using more elaborate *in vitro* (i.e., placental explants) and *in vivo* (i.e., Gadd45a null mice) models will be needed to understand the role of Gadd45a stress signaling in the etiology of PE.

Figure 13 depicts a proposed model of the involvement of elevated levels of MBG in Gadd45a-mediated stress signaling in PE. An increase in the circulating level of MBG is suspected to be related to initial excessive volume expansion early in pregnancy [42]. In this postulated sequence of events, MBG causes the induction of Gadd45a stress signaling, which in turn activates p38 and apoptotic signaling [14]. Consequently, secretion of sFlt-1 is induced [25]. All of these abnormalities culminate in the production of endothelial cell dysfunction [69] and oxidative stress [12]. These baleful actions of MBG can be prevented by the *in vivo* administration of RBG. This action of MBG, which is mediated by p38, and involves the activation of apoptosis, can be prevented *in vitro* by the addition of a p38 inhibitor [14]. The data of the current study as well as previous reports suggest that the employment of Gadd45a or p38 inhibition may represent an important therapeutic strategy in PE.

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Table 1

Blood pressure, urinary protein excretion, creatinine clearance, hematocrit values, and number of pups in the four groups of animals

Animal group	**NP (n =8)**	**PDS (n = 9)**	**NPM (n =8)**	**PDSR (n=8)**
Baseline BP (mmHg)	108 ± 6	106 ± 4	107 ± 4	105 ± 3
Final BP (mmHg)	91 ± 5†*	145 ± 3†**	133 ± 7†**	88 ± 5†*
Urinary Protein Excretion (mg/24hr)	2.5 ± 0.7*	5.1 ± 0.5 **	4.7 ± 0.5 **	2.4 ± 0.9*
Creatinine Clearance (ml/min)	0.82 ± 0.28	1.04 ± 0.45	1.14 ± 0.37	0.96 ± 0.28
Hematocrit	0.35 ± 0.03 *	0.39 ± 0.03 **	0.39 ± 0.03 **	0.34 ± 0.03*
Number of Pups	14.9 ± 1.8*	10.8 ± 1.3**	12.1 ± 1.9**	14.6 ± 1.2*
% Malformed Pups	0%*	18%**	6%**	0%*

Values are mean ± SE; n = number of rats. NP = normal pregnant animals; PDS = pregnant animals receiving DOCA and saline; NPM= normal pregnant rats injected with MBG; PDSR= PDS rats injected with RBG.

There were no differences in baseline BP between the groups.

† Statistically significant difference between baseline BP and final BP, $p < 0.05$

*NP and PDSR groups are different from PDS and NPM, $p < 0.05$

** PDS and NPM groups are different from NP and PDSR, $p < 0.05$

Figure Legends

Fig. 1. Western Blot analysis was performed to evaluate the expression of Gadd45a in the placental tissues from the four groups of rats: normal pregnant (NP, *n* = 8); pregnant rats which received weekly injections of desoxycorticosterone acetate and 0.9% saline as their drinking water (PDS, *n* = 9); normal pregnant rats injected with MBG (NPM, n = 8); and PDS rats injected with RBG, (PDSR, n = 8). The data were expressed as the fold-change from NP utilizing densitometry. The Gadd45a protein expression was significantly higher in the PDS and NPM groups compared to the NP and PDSR animals ($^{*}p<0.001$, n=5). The data are presented as the mean ± SEM.

Fig. 2. Activation of caspases 3 and 8 activities in the placental tissues of the rat model of PE. Caspase-3 and 8-activities were significantly higher in the PDS and NPM groups compared to the NP and PDSR animals ($^{*}p<0.002$, n=5). The data are presented as the mean ± SEM.

Fig. 3. sFlt-1 concentrations in the placental tissues from the four groups of rats were assayed utilizing a commercially available kit. sFlt-1 concentrations are expressed as ng/g tissue. SFlt-1 was significantly higher in the PDS and NPM groups compared to the NP and PDSR animals ($^{*}p<0.05$, n=5). The data are presented as the mean ± SEM.

Fig. 4. Western blot analysis was performed to evaluate the expression of VEGFR-1 in the placental tissues from the four groups of rats. The data are expressed as the fold-change over NP utilizing densitometry. Gadd45a protein expression was significantly higher in the PDS and NPM groups compared to the NP and PDSR animals ($^{*}p<0.001$, n=5). The data are presented as the mean ± SEM.

Fig. 5. Gadd45a protein was activated in the MBG-treated CTB cells. (A) The western blot data were analyzed utilizing densitometry. Gadd45a protein expression was significantly increased in 1, 10 and 100 nM MBG- treated cells when compared to the DMSO (vehicle)-treated cells ($^{*}p<0.001$, n=5, 8 replicates each). The 0.1 nM MBG treatment had no effect. (B) The RNAi-mediated inhibition of Gadd45a expression.

Fig. 6. (A) Cell cycle progression in CTB cells was arrested by MBG. The MBG-induced effect on cell cycle arrest was determined by fluorescence-activated cell sorting (FACS) analysis. G0/G1 is the resting/early interphase, S represents synthetic (mid-interphase) and G2/M is the late interphase/mitotic phase. MBG (1, 10 or 100 nM) significantly arrested progression of the cell cycle compared to DMSO (vehicle)-treated cells, whereas 0.1 nM MBG had no effect. There was a significantly higher percentage (~80%) of cells in the G0/G1 phase in the MBG 1, 10 and 100 nM-treated cells compared to basal and to 0.1 nM treated cells (~ 55%) ($^{*}p<0.05$, n=5, 8 replicates each). (B) The MBG-induced cell cycle arrest was attenuated by siRNA knockdown of Gadd45a. Results are presented as the mean ± SEM (n = 5, 8 replicates each).

Fig. 7. (A) MBG ≥1nM significantly induced apoptotic signaling compared to DMSO (vehicle)-treated cells, whereas 0.1 nM MBG had no effect. There was a significantly lower percentage

(~60%) of healthy cells in the MBG ≥1nM nM-treated cells compared to basal and to 0.1 nM treated cells (~ 90%). (B) The MBG -induced apoptotic signaling in CTB cells was abolished by siRNA knockdown of Gadd45a. Results are presented as the mean ± SEM (n = 5, 8 replicates each).

Fig. 8. (A) Increased p38 phosphorylation by MBG. CTB cells were treated with 0.1, 1, 10 or 100 nM MBG and p38 phosphorylation was measured by Western blotting. p38 phosphorylation was increased significantly in ≥1nM MBG-treated cells when compared to DMSO (vehicle)-treated cells ($^{*}p<0.003$, n=5). The results are expressed as the ratio of phosphorylated-p38 (p-p38) to the total-p38 (t-p38) in MBG-treated and DMSO-treated cells and to 0.1 nM treated cells. (B) The RNAi- mediated inhibition of Gadd45a expression prevented the MBG-induced upregulation of p38 phosphorylation. Results are presented as the mean ± SEM.

Fig. 9. (A) MBG induced sFlt-1 secretion. CTB cells were treated with 0.1, 1, 10 or 100 nM MBG and sFlt-1 concentrations were measured in the cell culture media by a commercially available EIA kit. sFlt-1 concentration was increased significantly in ≥1nM MBG-treated cells when compared to DMSO (vehicle)-treated cells and to 0.1 nM treated cells ($^{*}p<0.05$, n=5, 8 replicates each). (B) The RNAi- mediated inhibition of Gadd45a expression prevented the MBG-induced upregulation of sFlt-1. Results are presented as the mean ± SEM (n = 5, 8 replicates each).

Fig. 10. (A) Effect of MBG on VEGFR-1 expression. CTB cells were treated with 0.1, 1, 10 or 100 nM MBG and VEGFR-1 was measured by Western blotting. VEGFR-1 expression was increased significantly in ≥1nM MBG-treated cells when compared to DMSO (vehicle)-treated cells and to 0.1 nM treated cells ($^{*}p<0.003$, n=5). (B) The RNAi-mediated inhibition of Gadd45a expression prevented the MBG-induced upregulation of VEGFR-1 expression. Results are presented as the mean ± SEM.

Fig. 11. (A) Effect of MBG on 8-IP secretion. CTB cells were treated with DMSO, 0.1, 1, 10 or 100 nM MBG and 8-IP was measured in the cell media by EIA. MBG ≥1nM significantly increased the secretion of 8-IP compared to DMSO (vehicle)-treated cells and to 0.1 nM treated cells ($^{*}p<0.05$, n = 5, 8 replicates each). (B) The MBG-induced upregulation of 8-IP secretion by CTB cells was eliminated by siRNA knockdown of Gadd45a. Results are presented as the mean ± SEM (n = 5, 8 replicates each).

Fig. 12. (A) Effect of MBG on IL-6 secretion. CTB cells were treated with DMSO, 0.1, 1, 10 or 100 nM MBG. IL-6 was measured by a commercially available EIA kit. MBG concentrations of ≥1nM significantly increased the secretion of 8-IP compared to DMSO (vehicle)-treated cells and to 0.1 nM treated cells ($^{*}p<0.05$, n = 5, 8 replicates each). (B) The RNAi-mediated inhibition of Gadd45a expression prevented the MBG-induced secretion of IL-6 by the CTB cells. Results are presented as the mean ± SEM (n = 5, 8 replicates each).

Fig. 13. Working model for MBG-induced Gaad45a signaling in the pathogenesis of PE. Excessive volume expansion in pregnancy causes an increase in the circulating levels of MBG. MBG causes the induction of Gadd45a stress signaling which in turn activates p38 phosphorylation, IL-6 secretion, apoptotic signaling and suppresses cell cycle progression.

Consequently, this stress signaling accelerates the secretion of sFlt-1. These events result in oxidative stress and, concomitantly, endothelial dysfunction.
The administration of RBG, a specific antagonist of MBG, early in pregnancy, prevents the development of this syndrome and its phenotypic characteristics. The RNAi-mediated inhibition of Gadd45a expression prevented the MBG-induced CTB cell stress signaling.

Figure 1

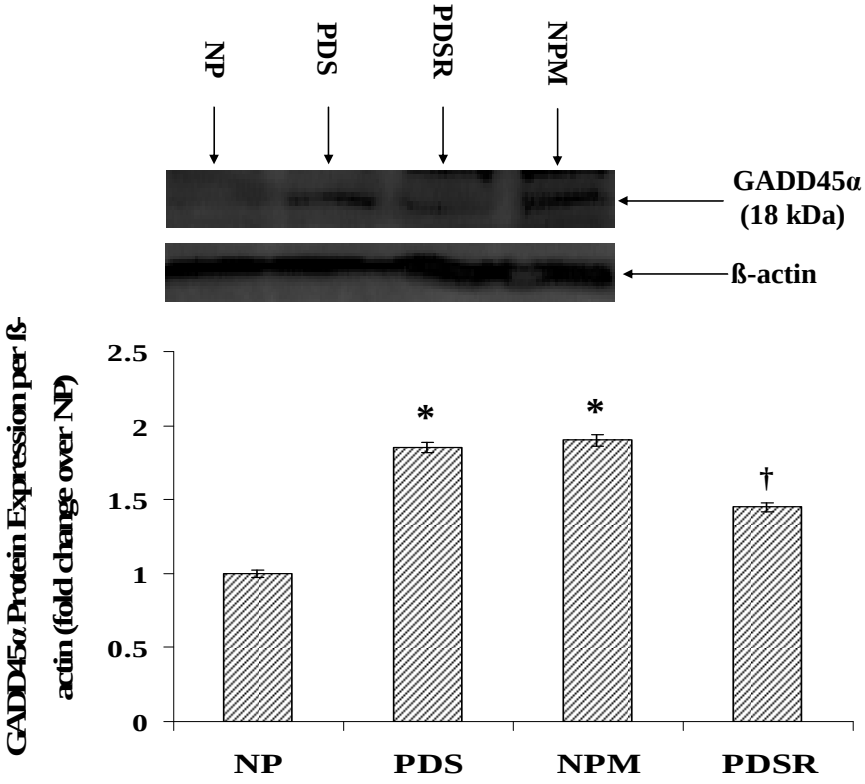


Figure 2

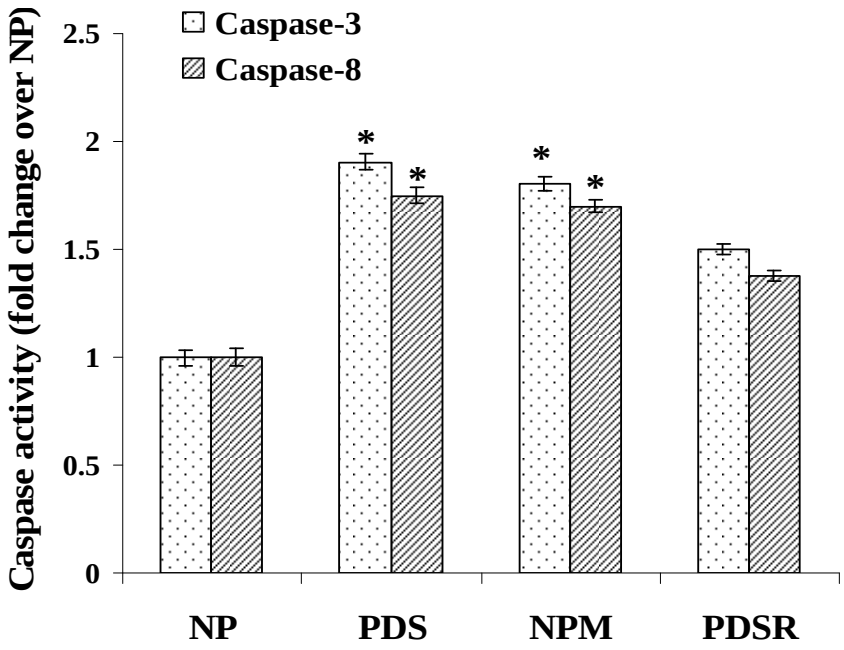


Figure 3

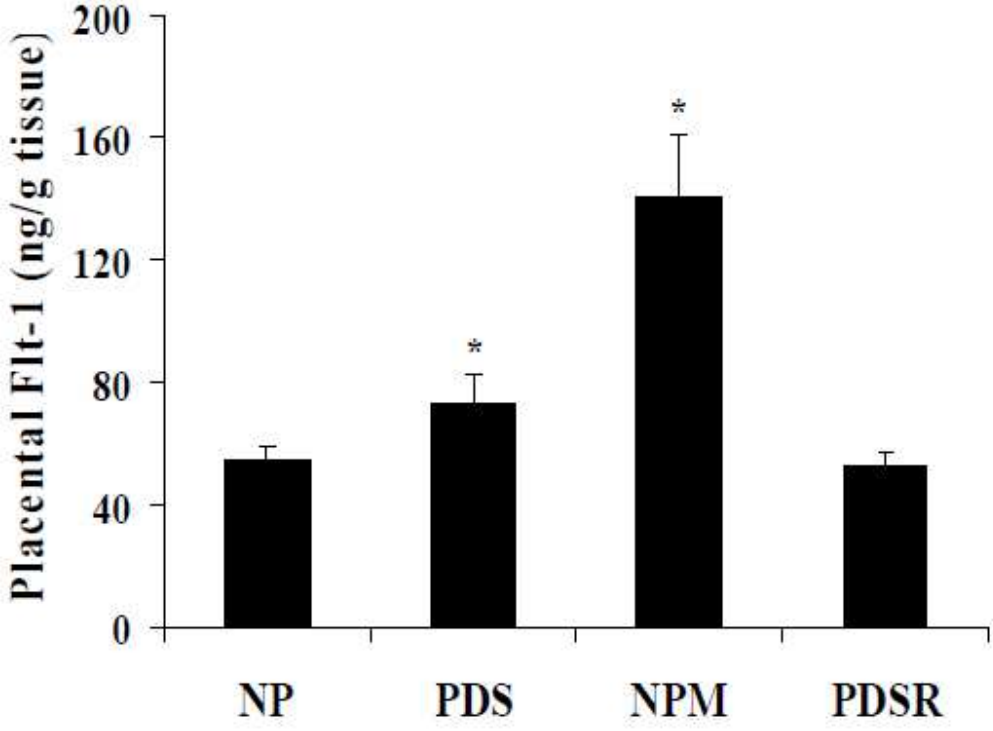


Figure 4

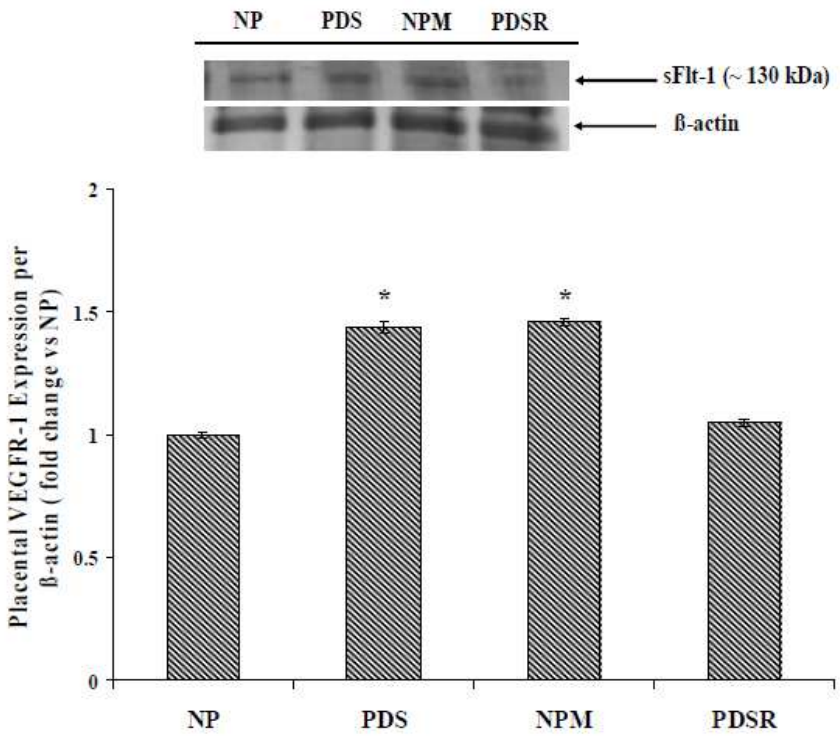
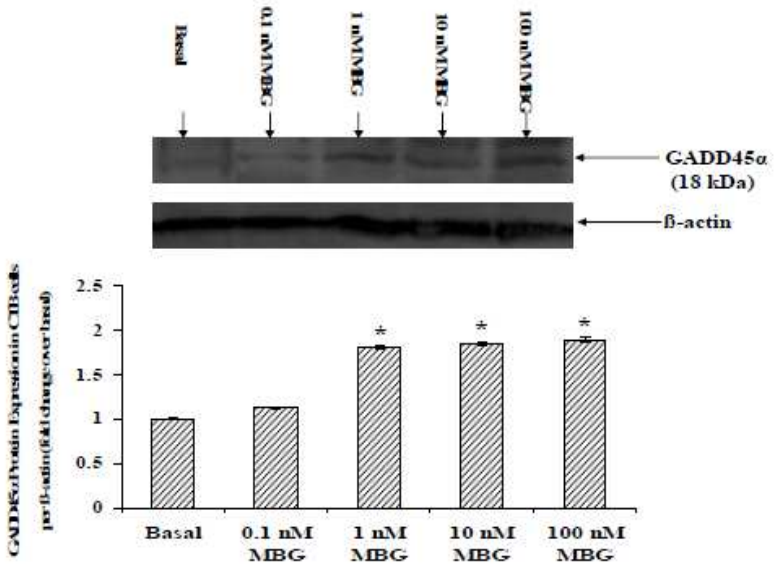


Figure 5

A.



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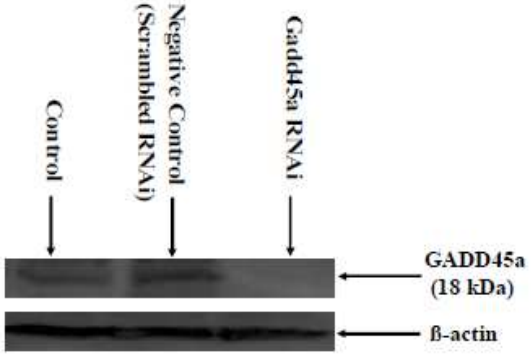
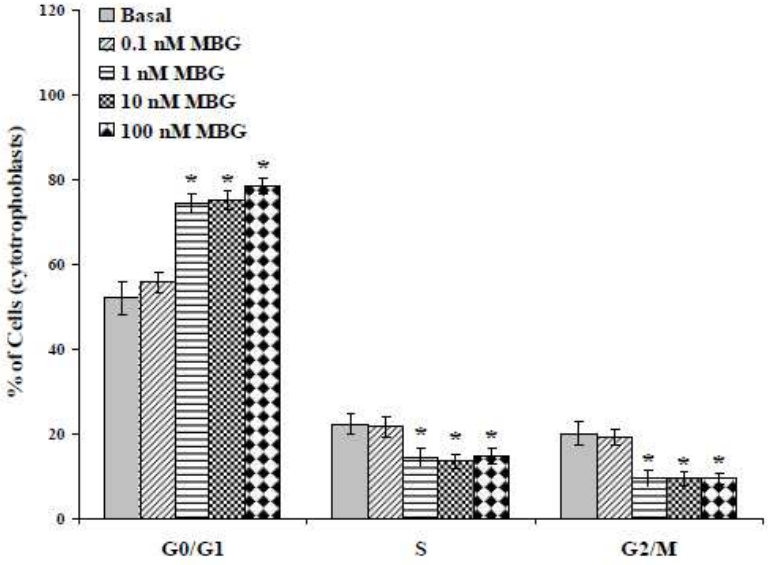


Figure 6

A.



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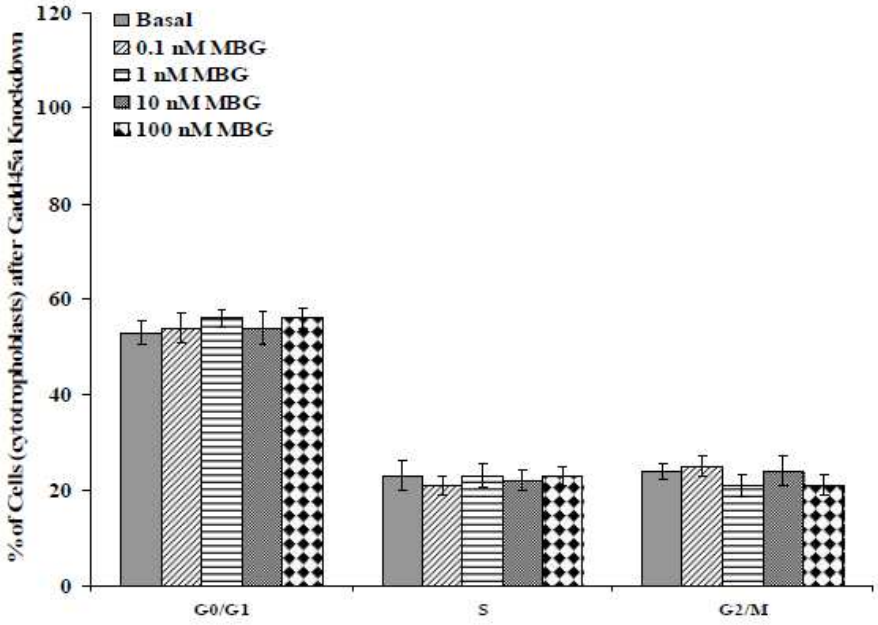
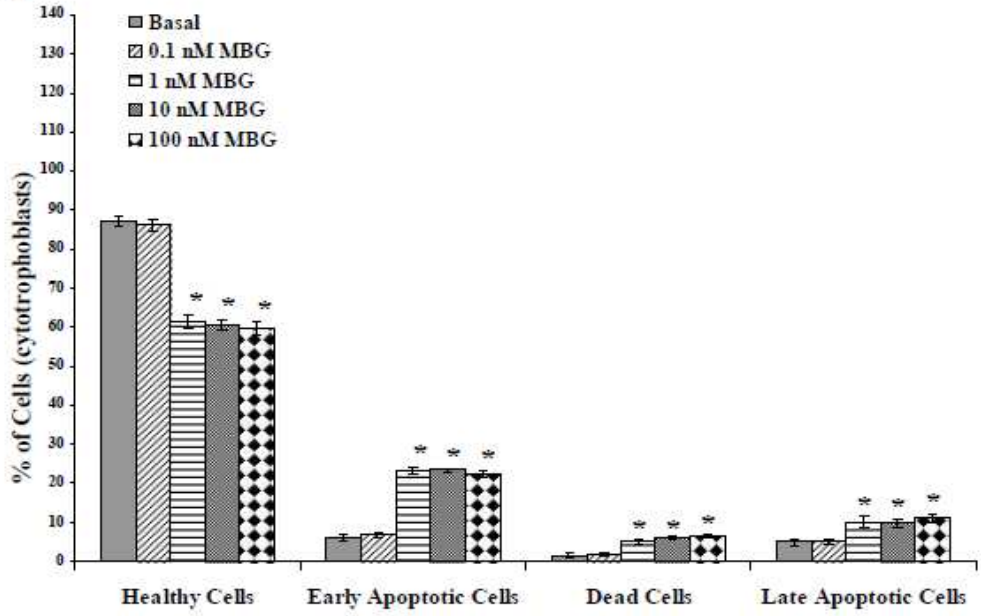


Figure 7

A.



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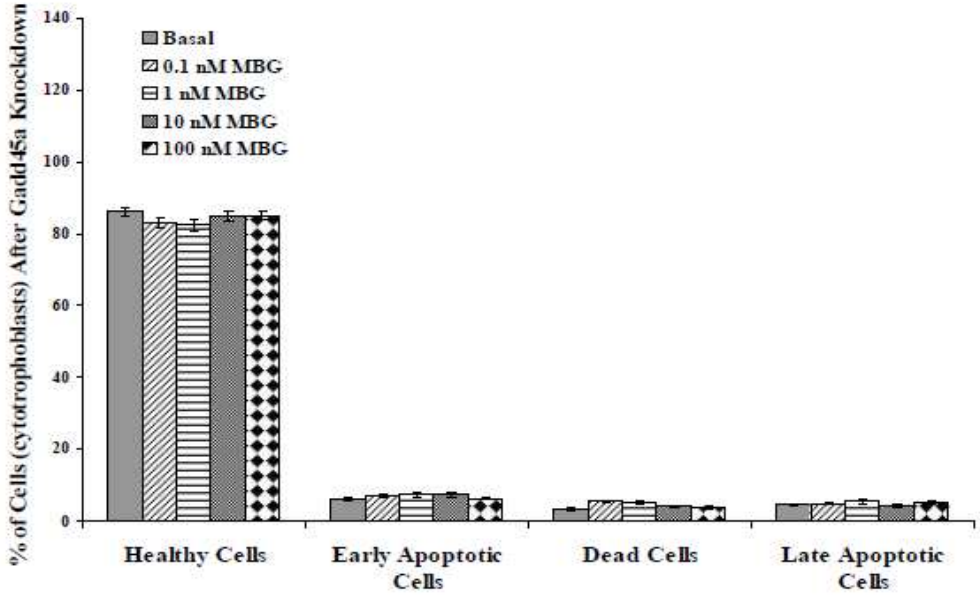
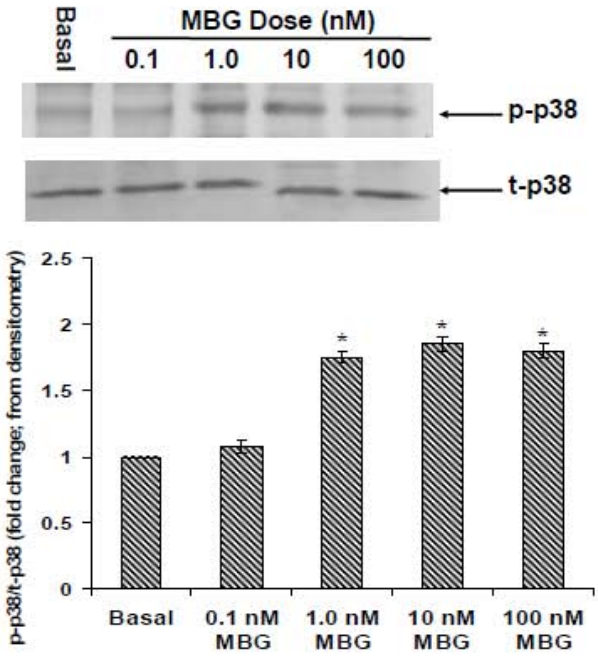


Figure 8

A.



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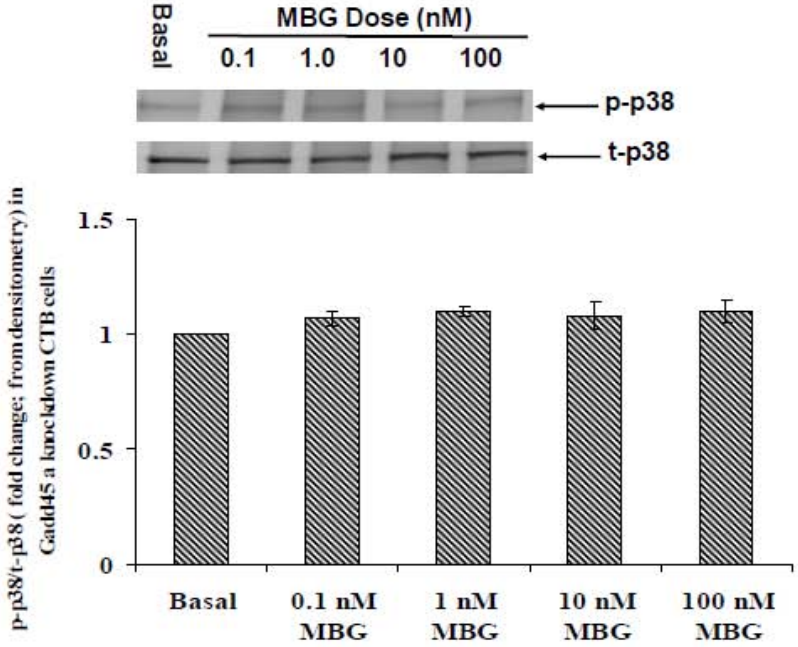
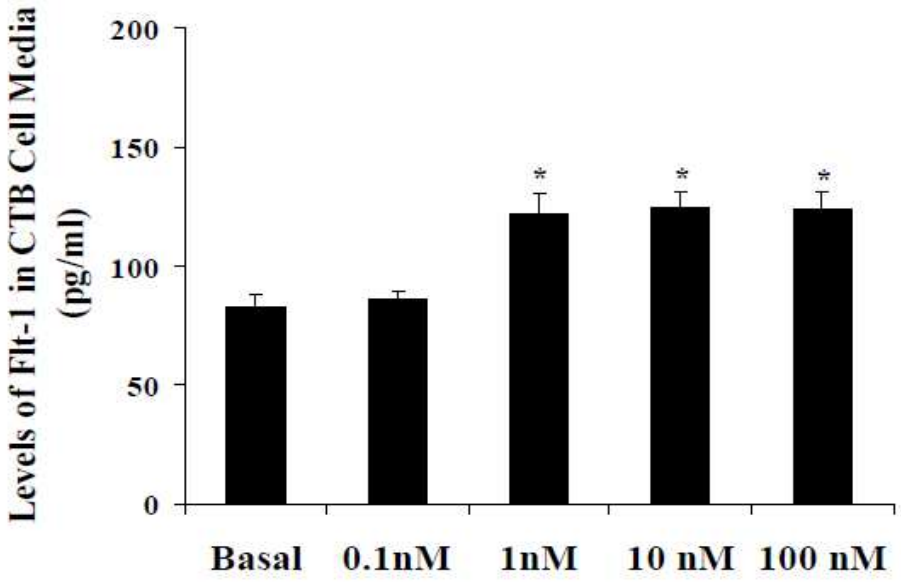


Figure 9

A.



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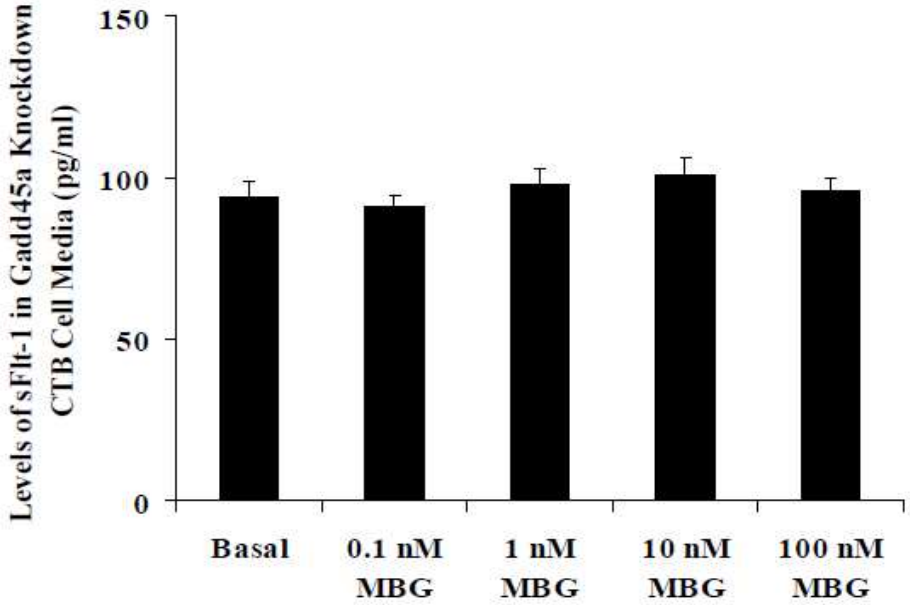
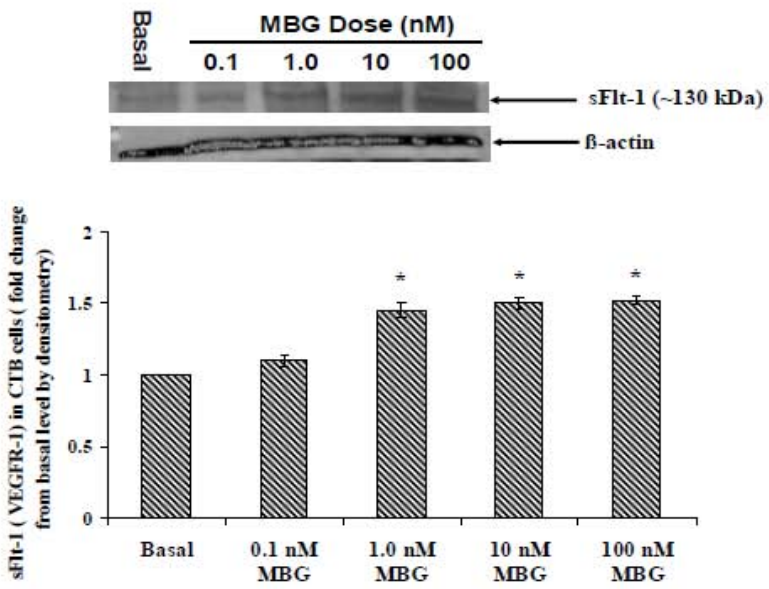


Figure 10

A.



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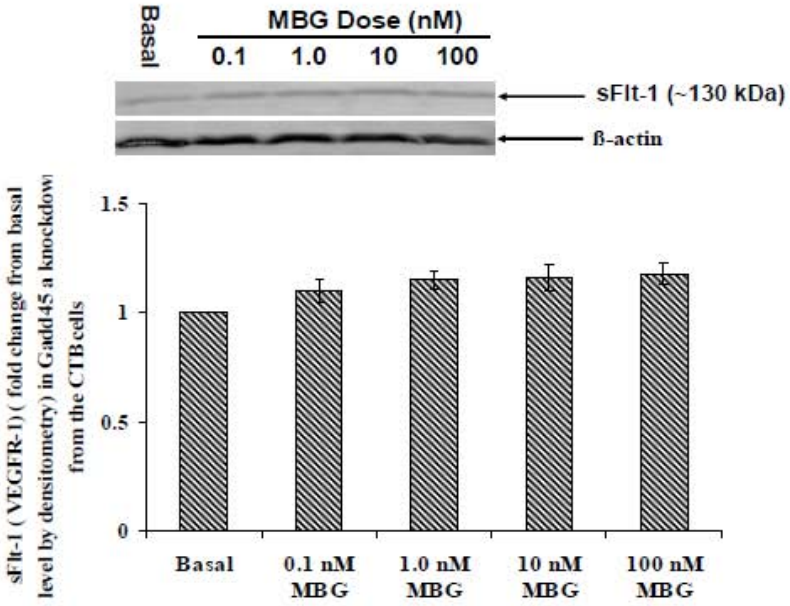
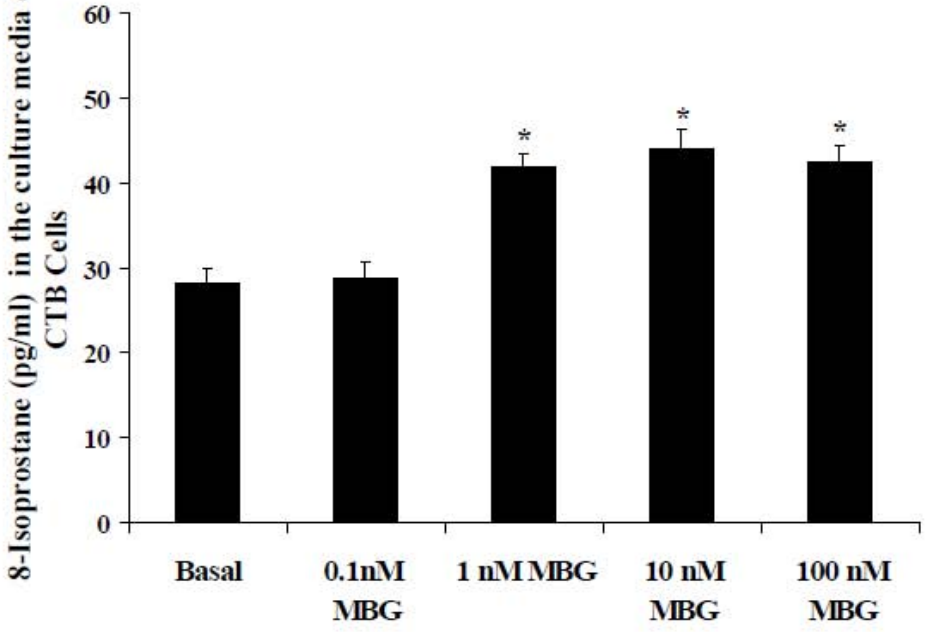


Figure 11

A.



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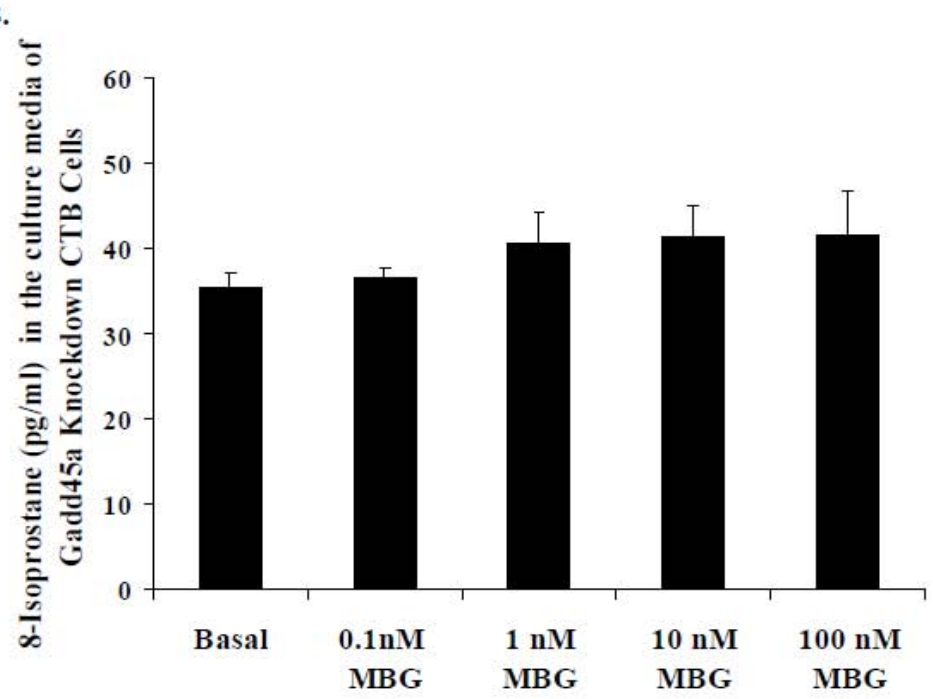
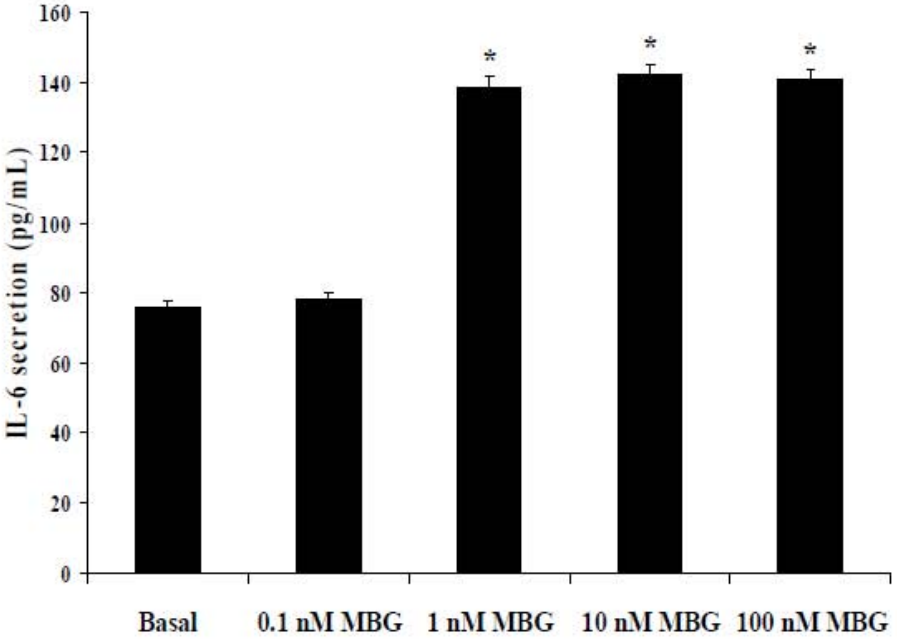


Figure 12

A.



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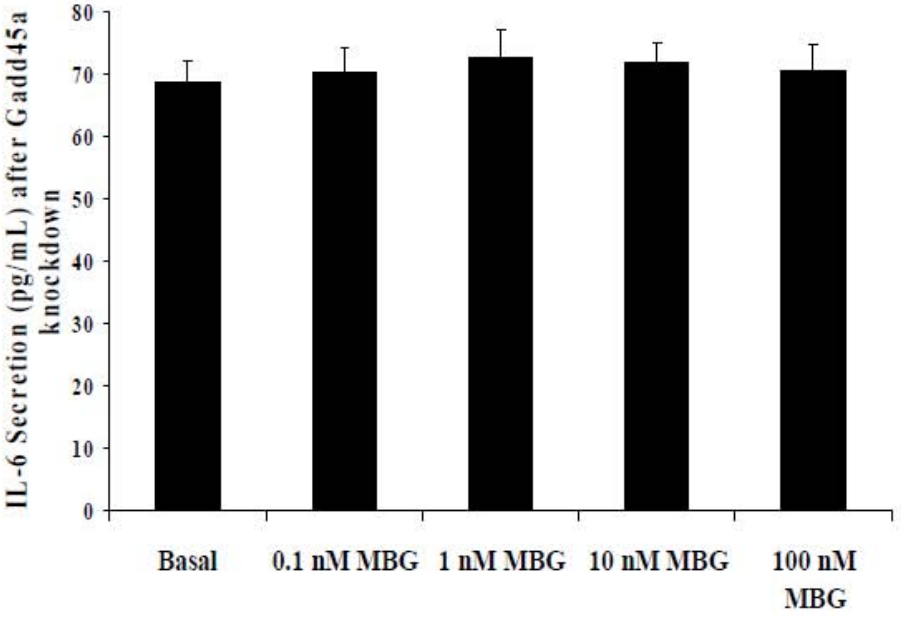
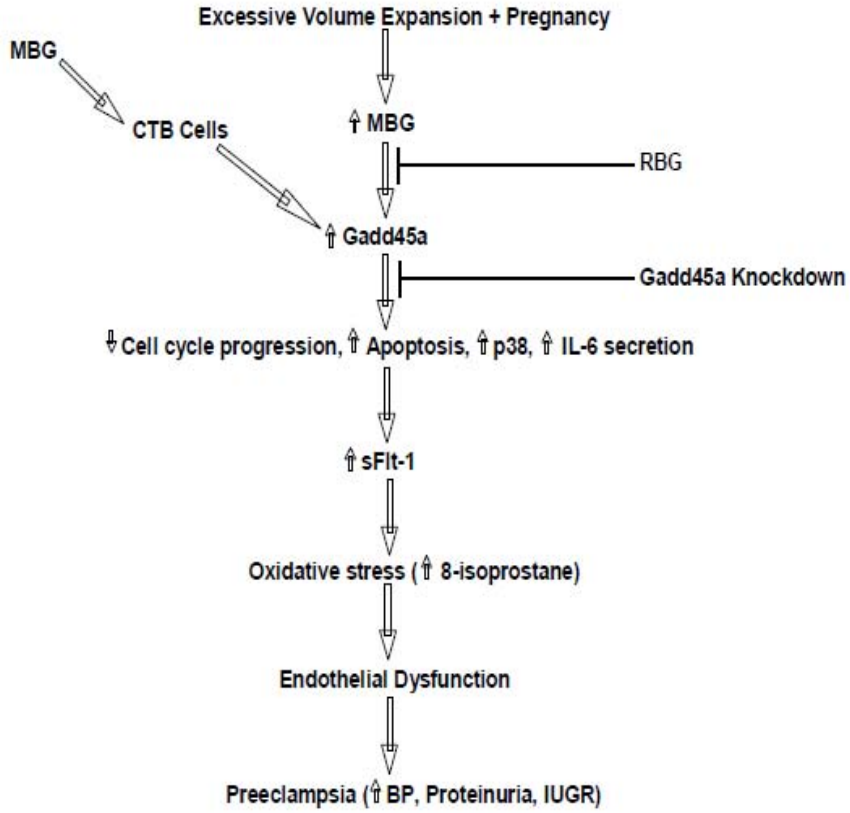


Figure 13



Highlights

- Placental Gadd45a expression was significantly higher in a rat model of preeclampsia.
- Caspases 3 and 8 activities and sFlt-1 receptor expression were higher in the rat model.
- MBG is involved in the alteration in Gadd45a stress- signaling both in vivo and in vitro.
- RBG prevents these changes when administered in vivo.