

# Dexamethasone induces the expression of LRRK2 and $\alpha$ -synuclein, two genes that when mutated cause Parkinson's disease in an autosomal dominant manner

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**LRRK2** (leucine-rich repeat kinase 2) has been identified as a gene corresponding to PARK8, an autosomal-dominant gene for familial Parkinson's disease (PD). LRRK2 pathogenic-specific mutants induce neurotoxicity and shorten neurites. To elucidate the mechanism underlying LRRK2 expression, we constructed the LRRK2-promoter-luciferase reporter and used it for promoter analysis. We found that the glucocorticoid receptor (GR) transactivated LRRK2 in a ligand-dependent manner. Using quantitative RT-PCR and Western analysis, we further showed that treatment with dexamethasone, a synthetic GR ligand, induced LRRK2 expression at both the transcriptional and translational levels, in dopaminergic MN9D cells. Dexamethasone treatment also increased expression of  $\alpha$ -synuclein, another PD causative gene, and enhanced transactivation of the  $\alpha$ -synuclein promoter-luciferase reporter. In addition, dexamethasone treatment to MN9D cells weakly induced cytotoxicity based on an LDH assay. Because glucocorticoid hormones are secreted in response to stress, our data suggest that stress might be a related factor in the pathogenesis of PD. [BMB Reports 2013; 46(9): 454-459]

## INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease characterized primarily by movement-related symptoms such as tremors, rigidity and bradykinesia. In genetic analyses per-

formed over the last two decades using samples obtained from familial PD patients, researchers have mapped at least 18 PD-associated (PARK) loci encoding proteins that, when mutated, cause PD (1). The LRRK2 (leucine-rich repeat kinase 2) gene encodes PARK8, a locus that has been linked to autosomal-dominant PD (2, 3). LRRK2 protein exhibits several interesting features. First, LRRK2 is a large protein containing active GTPase and kinase domains-two enzymatic functionalities important in signal transduction (2-5). Second, pathogenic LRRK2 mutations observed in familial PD cases have also been observed in 1-2% of sporadic PD cases, suggesting its critical role in the pathogenesis of PD (6). Finally, over-expression of wild type (WT) LRRK2 increases cytotoxicity, promotes protein aggregation and enhances the levels of intracellular reactive oxygen species (ROS), albeit to a lesser extent than pathogenic LRRK2 mutants, such as G2019S (7, 8). Notably, both WT and pathogenic LRRK2 mutants when over-expressed induce a similar decrease in the rate of endocytosis of synaptic vesicles (9). Collectively, these results led us to hypothesize that over-expression of WT LRRK2 at sufficiently high levels may trigger PD pathogenesis. This hypothesis is supported by a report showing that over-expression of WT  $\alpha$ -synuclein, encoded by another autosomal dominant PD gene (SNCA), causes PD or PD-like phenotypes. For example, duplications or triplications of the WT gene for  $\alpha$ -synuclein have been identified as mutations that correspond to PARK4 (10) and over-expression of WT  $\alpha$ -synuclein by transient transfection in yeast results in cytotoxicity (11).

To identify transcription factors that regulate LRRK2 expression, we initially selected several nuclear receptors whose functions in PD or neurogenesis have been well-established (12), including Nurr1 [NR4A2, (13)], LXR $\beta$  [Live X receptor, NR1H2, (14)], RAR $\beta$  [retinoic acid receptor  $\beta$ , NR1B2, (15)] and GR [glucocorticoid receptor, NR3C1, (7)]. We found that GR transactivated LRRK2 expression in a ligand-dependent manner. Interestingly, GR also transactivated  $\alpha$ -synuclein transcription in a ligand-dependent manner, suggesting a potential cumulative effect of glucocorticoid stress hormone on PD. These results imply that stress may be one of several factors to

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function in PD pathogenesis.

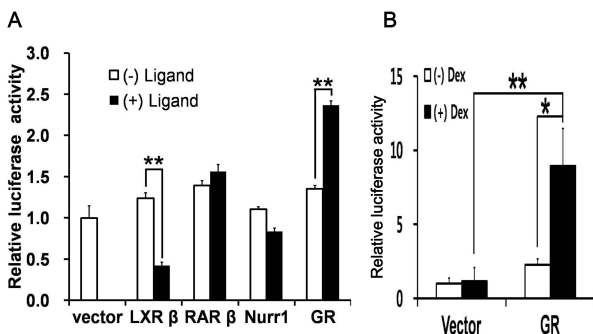
## RESULTS

### The dexamethasone induces expression of LRRK2

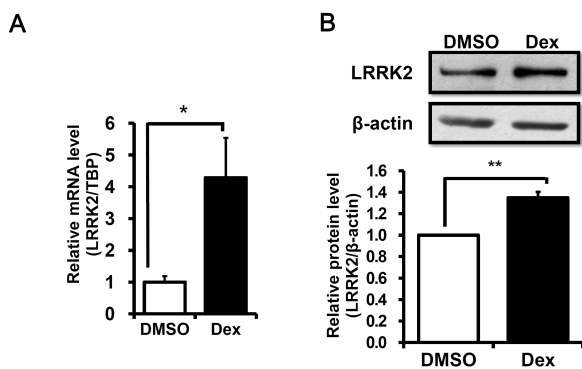
To elucidate LRRK2 induction mechanisms that might be related to the etiology of PD, we sought to identify transcription factors that regulate LRRK2 expression. We chose to test Nurr1,

LXR $\beta$ , RAR $\beta$  and GR, the nuclear receptors which play critical roles in PD or neurogenesis (7, 13-15). Each of these nuclear receptors was tested for its ability to regulate *LRRK2* transcription by co-transfecting dopaminergic MN9D cells with the corresponding expression plasmids and *LRRK2* pro-luc reporter plasmids. Subsequent luciferase assays showed that GR transactivates the *LRRK2* promoter, driving an increase in luciferase expression whereas LXR $\beta$  represses it: the actions of both nuclear receptors were dependent on their ligands, dexamethasone and T0901317 for GR and LXR $\beta$ , respectively (Fig. 1A). We decided to focus on GR in this study and used dexamethasone, instead of glucocorticoid, as the GR activator since it is common to use dexamethasone as a potent, synthetic, specific ligand for GR at 0.1-5  $\mu$ M depending on the experimental purpose. In control cells transfected with empty vector, dexamethasone treatment alone was unable to activate the reporter gene (Fig. 1B); confirming that *LRRK2* promoter-dependent transactivation of luciferase was mediated by GR.

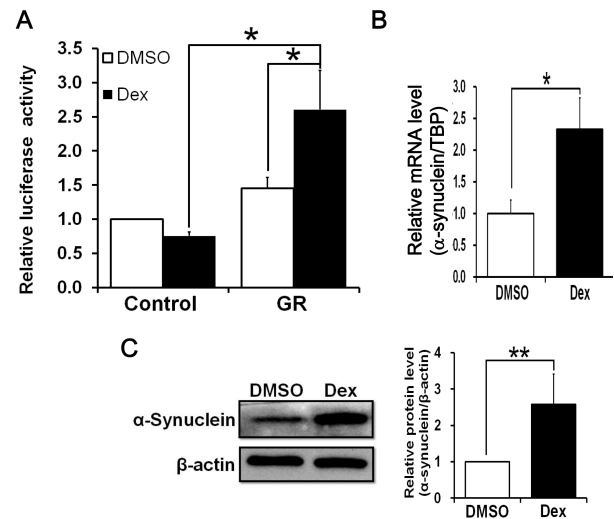
To further confirm the GR-dependent induction of *LRRK2*, we treated MN9D cells with dexamethasone and measured *LRRK2* mRNA and protein levels by qRT-PCR and Western blotting, respectively. The qRT-PCR analyses revealed that *LRRK2* mRNA levels [normalized to those of *TBP*, (16)] increased approximately 4-fold ( $P < 0.05$ ) in cells treated with



**Fig. 1.** GR transactivates the *LRRK2* promoter. (A) MN9D murine dopaminergic cells were transiently co-transfected with expression plasmids for GR, LXR $\beta$ , RAR $\beta$  or Nurr1, and with *LRRK2* pro-luc reporter plasmids. Specific ligand (dexamethasone for GR, T0901317 for LXR $\beta$  or all-trans retinoic acid for RAR $\beta$ ) for each receptor was added at 2.5  $\mu$ M 6 hours after transfection and cells were harvested for luciferase assays after incubating for an additional 42 hours. (B) MN9D cells were transiently co-transfected with *LRRK2*-luc reporter plasmid and a GR expression plasmid or empty vector. Dexamethasone (1  $\mu$ M) was added as a ligand. Experiments were repeated three times in triplicate and data are expressed relative to the vector control values and are presented as means  $\pm$  SEMs (\* $P < 0.05$  and \*\* $P < 0.01$ ).



**Fig. 2.** Dexamethasone induces LRRK2 expression at both transcriptional and translational levels. MN9D cells were treated with dexamethasone (1  $\mu$ M) for 24 hours, and total RNA or total cell lysates were prepared for qRT-PCR (A) or Western blot analysis (B), respectively. Two qRT-PCR and three western blot analyses were performed with separately prepared samples. A representative image is shown at the top and a bar graph derived from analysis of repeated experiments is shown at the bottom (\* $P < 0.05$  and \*\* $P < 0.01$ ).



**Fig. 3.** Dexamethasone transactivates the  $\alpha$ -synuclein promoter and induces  $\alpha$ -synuclein expression. (A) GR increased the transcriptional activity of  $\alpha$ -synuclein promoter-luciferase reporter, syn-luc. The experiment was carried out as described in Fig. 1 except using the luciferase reporter under the control of the  $\alpha$ -synuclein promoter. (B, C) MN9D cells were treated with dexamethasone (1  $\mu$ M) for 24 hours, and total RNA or total cell lysates were prepared for qRT-PCR (B) or Western blot analysis (C), respectively. Two qRT-PCR and three western blot analyses were performed with separately prepared samples. A representative image of the Western blot is shown at the left (C) and a bar graph derived from analysis of repeated experiments is shown at the right (\* $P < 0.05$  and \*\* $P < 0.01$ ).

dexamethasone compared to the untreated cells (Fig. 2A). Dexamethasone treatment also induced a modest increase (1.3-fold,  $P < 0.01$ ) in LRRK2 protein levels (Fig. 2B).

### The dexamethasone induces expression of $\alpha$ -synuclein

LRRK2 is a causative gene for the autosomal dominant inheritance of familial PD. As with LRRK2, mutations in the SNCA gene, which encodes  $\alpha$ -synuclein, cause familial PD in an autosomal dominant manner (17). Moreover, over-expression of WT  $\alpha$ -synuclein by gene duplication or triplication also causes familial PD (10, 18).  $\alpha$ -Synuclein is also a major component of the Lewy body, a pathological marker of PD. Thus, we postulated that dexamethasone might also induce expression of  $\alpha$ -synuclein in a similar manner to LRRK2 and performed the parallel experiments. We first tested whether GR transactivated syn-luc reporter plasmids containing the luciferase gene under the control of the  $\alpha$ -synuclein promoter. Just as GR transactivated the LRRK2 promoter, it also transactivated the  $\alpha$ -synuclein promoter in a ligand-dependent manner, increasing the luciferase activity by approximately 2.5-fold ( $P < 0.05$ , Fig. 3A). Both RT-PCR and Western blot analyses demonstrated that dexamethasone treatment of MN9D cells increased both  $\alpha$ -synuclein mRNA and protein levels by about 2.5-fold ( $P < 0.05$ , Fig. 3B,  $P < 0.01$ , Fig. 3C).

We also tested whether dexamethasone could induce expression of LRRK2 and  $\alpha$ -synuclein in primary rat neuronal cells. 1  $\mu$ M of dexamethasone treatment induced both LRRK2 and  $\alpha$ -synuclein expression in primary rat hippocampal neuronal cells approximately 2-fold at translational level (Fig. 4A and B).

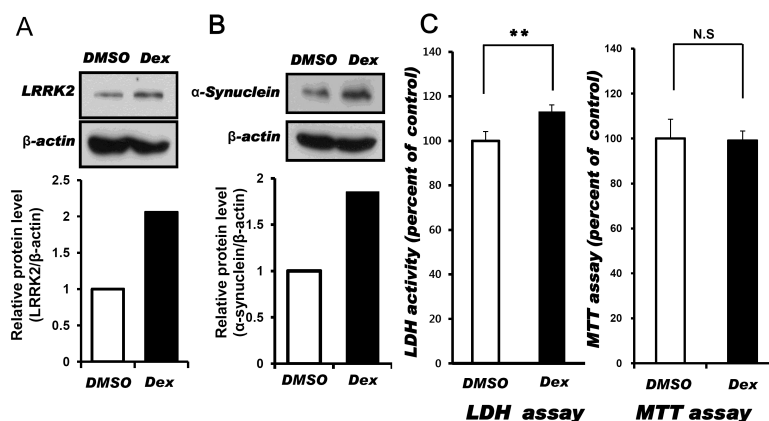
### The dexamethasone treatment causes cytotoxicity in MN9D cells

To test whether induction of LRRK2 and  $\alpha$ -synuclein by dexamethasone treatment affect cytotoxicity, we employed LDH assay which measures membrane integrity. Treatment of MN9D cells with dexamethasone slightly increased LDH activity by about 10% (Fig. 4C), which was statistically significant ( $P < 0.01$ ) and reproducibly observed. However, we could not observe any significant difference in cell viability in MTT assay (Fig. 4C) which measured mitochondrial enzyme activity. We tested primary neuron hippocampal cultures and obtained the similar results that dexamethasone treatment exhibited weak cytotoxicity by LDH assay, but no cytotoxicity by MTT assay (data not shown).

Altogether, these data provided the first evidences that glucocorticoid, stress hormone, induces LRRK2 and  $\alpha$ -synuclein expression and suggested that stress might be one of factors for PD pathogenesis.

## DISCUSSION

Glucocorticoids are secreted into the bloodstreams in response to stress, and serve to restore homeostasis by binding to GR and transcriptionally activating various GR target genes. Therefore, glucocorticoids are also referred to as stress hormones. Several studies have indicated that stress and/or cortisol, glucocorticoid in humans, are associated with PD. One study reported that former-Far-East prisoners of war developed PD many years after their release at much higher rates than control groups, suggesting that stress could facilitate the onset of PD (19). It has also been reported that cortisol con-



**Fig. 4.** (A, B) The dexamethasone treatment induces both LRRK2 and  $\alpha$ -synuclein protein expression in primary cultures. E18 rat primary hippocampal neuronal cells were prepared and incubated for 5 days. After further incubation for one day in medium containing charcoal-stripped serum, the cells were then treated with dexamethasone at 5  $\mu$ M. Total cell lysates were prepared after 24 hours and western blot analysis was performed with LRRK2 antibody (A). For  $\alpha$ -synuclein western blot, the dexamethasone treatment was done for 48 hours to maximize induction of  $\alpha$ -synuclein (B). Both membranes were stripped and subjected to western blot analysis with  $\beta$ -actin antibody to normalize loading amount. Western blot is shown at the top and a bar graph at the bottom. (C) Treatment of MN9D cells with dexamethasone induces weaker cytotoxicity by the LDH assay, but not by the MTT assay. MN9D cells were incubated with stripped serum for a day and treated with 1  $\mu$ M dexamethasone for a day. Activities of LDH in medium and cell pellet were measured and secreted amounts of LDH were calculated. The experiments were repeated three times in triplicate and each value is expressed relative to the vector control value and presented as mean  $\pm$  SEM (\*\* $P < 0.01$ ). N.S.: not significant.

centrations are higher in Parkinsonism (20) and that stress accelerates neural degeneration and amplifies motor symptoms in rat PD models (7). In fact, stress has been hypothesized to induce PD (21). Interestingly, administration of levodopa, a therapeutic used for PD patients, reduces cortisol secretion in PD patients (22, 23). Our data indicated that treatment of the dopaminergic MN9D cell line with dexamethasone trans-activated two major autosomal dominant familial PD genes, *LRRK2* and  $\alpha$ -synuclein (Fig. 2, 3), which might suggest a link between an increased glucocorticoid level and the pathogenesis of PD and a putative mechanism to link stress and PD, although the present data were obtained only at cell culture level.

To decide whether GR directly bind to the promoters of both genes, we searched for the conserved GR response element (GRE), GGTACAnnTGTCT (24) in both *LRRK2* and  $\alpha$ -synuclein promoters by deletion study and/or bioinformatic methods, but could not find any conserved canonical GRE (Supplementary Fig. 1).

Dexamethasone treatment weakly increased membrane permeability based on an LDH assay, indicating its cytotoxicity (Fig. 4C). However, an MTT assay which measures mitochondrial enzyme activity did not show any change of cell viability after dexamethasone treatment (Fig. 4C). Our repeated experiments exhibited similar results. Although one needs caution to interpret the data, these data suggest that the weak cytotoxicity observed is the LDH assay-specific. At present, reasons for the difference between two assays are not clear. A possible explanation is that  $\alpha$ -synuclein protofibrils increase membrane permeability (25), which might explain the LDH assay-specific cytotoxicity by dexamethasone treatment. In other words,  $\alpha$ -synuclein protein increased by dexamethasone treatment might form protofibrils which increase membrane permeability. Otherwise, it is possible the discrepancy of the cell viability was simply caused by the sensitivity difference of the assay methods which sometimes was observed based on specific stimuli (26). However, we could not exclude a possibility that the cytotoxicity observed was due to unknown reasons, not to an increase of *LRRK2* and  $\alpha$ -synuclein proteins.

In our *LRRK2* reporter assay (Fig. 1A), we reproducibly observed repression of the *LRRK2* promoter by LXR $\beta$ . LXR $\beta$  protected dopaminergic neurons in a mouse model of Parkinson disease (27) and suppression of *LRRK2* by LXR $\beta$  might be one of the reasons for this phenomenon.

In summary, dexamethasone, a potent synthetic glucocorticoid that acts through GR, induces the expression of both *LRRK2* and  $\alpha$ -synuclein, which when mutated, are known to cause PD in an autosomal-dominant manner. Our data provide the first evidence that glucocorticoid stress hormones induce *LRRK2* and  $\alpha$ -synuclein expression. A study to investigate *LRRK2* and  $\alpha$ -synuclein protein levels in stressed animals may be required to further demonstrate this point.

## MATERIALS AND METHODS

### Construction of *LRRK2* promoter-luciferase reporter constructs

The *LRRK2* promoter region (from 142148 to 144232 of the GeneBank clone AC079630) was amplified from a *Homo sapiens* 12 BAC RP11-476D10 template (Roswell Park Cancer Institute, Human BAC Library) by polymerase chain reaction (PCR) using the primer pair 5'-CTCGAGGAATGAATTGTGGA AATTAGGAGAC-3' (forward) and 5'-CTAGCCATGGTGGCAC CTGCTTCC-3' (reverse); The underlined letters indicate restriction enzyme sites and lower-case letters denote the translation initiation codon of *LRRK2*. The resulting PCR product contained the *LRRK2* promoter region from -1961 to +125 (142148 to 144233 in NCBI accession number AC079630) and included the translation-initiation codon at +123 to +125 (+1 corresponds to the transcription starting point.). The PCR product was digested with *Xho*I and *Nco*I, and the digested fragment was cloned into the pGL3-Basic vector (Promega, WI, Madison, USA) digested with the same set of restriction enzymes. The resulting reporter plasmid, *LRRK2* pro-luc, contained the luciferase gene under the control of the *LRRK2* promoter.

Another reporter containing the  $\alpha$ -synuclein promoter fused to the luciferase gene, syn-luc, was constructed by the cloning of a DNA fragment corresponding to  $\alpha$ -synuclein promoter into pGL3-Basic reporter (Promega, WI, USA). The  $\alpha$ -synuclein promoter fragment (the region from 3578 to 6329 in NCBI accession number NG\_011851) was cut out of  $\alpha$ -synuclein promoter-CAT reporter plasmid which was obtained from Dr. E. Jun (UMDNJ, USA).

### Cell culture, transfection and luciferase assay

The MN9D murine dopaminergic cell line was obtained from Dr. Oh Y. (Yunsei Univ. Korea) and cultured in Dulbecco's Modified Eagle Medium (DMEM, WeLGene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, WeLGene), sodium bicarbonate (3.7 g/L), antibiotic/antimycotic solution (1%, WeLGene) and HEPES (5.9 g/L) at 37°C in a humidified CO<sub>2</sub> incubator. The cells were seeded at 5 × 10<sup>4</sup> cells/well in a 48 well plate, and incubated for 1 day before transfecting with DNA mixtures consisting of 100 ng of *LRRK2* pro-luc or syn-luc reporter, 100 ng of plasmids expressing the indicated nuclear receptors, and 10 ng of pRL-CMV (Promega) using Lipofectamine 2000 (Invitrogen, CA, USA) and OptiMEM (Invitrogen) as recommended by the manufacturer. To express the indicated nuclear receptors, mammalian expression vector containing the full length of each receptor gene was used. Inclusion of pRL-CMV was intended to normalize for differences in transfection efficiencies among transfected samples. The indicated ligands, all of which were obtained from Sigma (Saint Louis, Mo, USA), were treated at 2.5 or 1  $\mu$ M after 6 hours of transfection. Then, cells were incubated for an additional 42 hours and harvested for luciferase assay which was performed using a Dual Luciferase Assay kit (Promega). To

eliminate potential effects of ligands that may be present in normal FBS, cells were pre-incubated with medium containing charcoal-stripped FBS for a day before transient transfection.

E-18 primary rat hippocampal and cortical neurons were prepared and cultured as previously described (9, 28). At 5 days *in vitro*, the culture medium was replaced with medium containing charcoal-stripped serum. After incubation for a day, 5  $\mu$ M of dexamethasone or the same volume of DMSO was treated to the cells for indicated times and cells were harvested for western analysis.

### qRT-PCR and Western blot analysis

For quantitative reverse transcription-polymerase chain reactions (qRT-PCR), total RNA was isolated from the corresponding cells and target mRNAs were amplified by RT-PCR using the following specific primer sets; *LRRK2*: 5'-AGGAGCTGCCCCCTGAAGACA-3' (forward) and 5'-TGTGCCACCC TCCCATGT-3' (reverse);  $\alpha$ -synuclein: 5'-AGAAGACCAAAGAGCAAGTGACA-3' (forward) and 5'-GACTGGGCACATTGGAAGTGAAG-3' (reverse). For quantitation purpose, a TATA-box binding protein (TBP) specific primer set 5'-CAGCCTTCACCTTATGCTC-3' (forward) and: 5'-TTGCTGCTGCTGCTT TGTT-3' (reverse) was included in each PCR reaction as an internal control. The PCR amplification of *LRRK2*,  $\alpha$ -synuclein and *TBP* mRNAs were carried out in an iCycler (Bio-Rad, MA USA) using SYBR green supermix (Roche, Mannheim, Germany) under the following cycling conditions: 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds followed by 95°C for 1 minute and 55 °C for 10 minutes.

For Western blotting, cells were harvested and lysed with lysis buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Triton, 5% Glycerol, 1 mM EDTA], and proteins in lysates were resolved by SDS-PAGE and transferred to PVDF membranes. Specific proteins in membranes were detected by Western analysis using anti-*LRRK2* (29) or anti- $\alpha$ -synuclein (sc-7011, Santa Cruz Biotechnologies, CA, USA) antibodies. Anti- $\beta$ -actin (A5441, Sigma Co.) was used as a loading control. The membrane was incubated with proper horseradish peroxidase-conjugated secondary antibodies and primary antibodies were detected with ECL prime chemiluminescence reagent (GE Healthcare Bio-Sciences). Western blot images were analyzed using the CS Analyzer software v2.0 (ATTO Co, Japan), and band densities were expressed relative to control values.

### Cytotoxic assay

To test whether dexamethasone treatment induced cytotoxicity, MN9D cells ( $2.5 \times 10^5$  cell/well, 12well plate) were cultured in media containing charcoal-stripped FBS for a day and treated with 1  $\mu$ M dexamethasone for another day. To measure cytotoxicity, lactate dehydrogenase (LDH) assay was employed. Amounts of LDH in media and lysate of cell pellet were measured by LDH assay kit (Sigma-Aldrich, Saint Louis Mo, USA) as recommended by the manufacturer. Relative amounts

of LDH secreted to the medium was calculated as LDH present in media/(LDH present in media + LDH present in cell pellet) and compared to that of the control group which was treated with DMSO. An MTT assay was performed as described (30).

### Statistical analysis

For all experiments, data were expressed relative to control values and presented as means  $\pm$  standard errors of the mean (SEMs). P values were calculated using Student's t-test and P value less than 0.05 was considered to be statistically significant.

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