Supporting Information

Title: Hydroxylated fullerene: A stellar nanomedicine to treat lumbar radiculopathy via antagonizing TNF-α induced ion channel activation, calcium signaling and neuropeptides production

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Number of pages: 6
Number of figures: 2

TNF-α induced phosphorylation of ERK and AKT of DRG in a time-dependent manner

Two days after DRG *in vitro* culture, fresh growth media composed of F-12 medium, 100 U/mL penicillin and 100 μg/mL streptomycin was supplemented with tumor necrosis factor alpha (TNF-α) (Cell Signaling Technology, Danvers, MA, 25 ng/mL) and allowed TNF-α treatment for various time points (0, 15 min, 30 min, 5 h and 24 h). At the end of incubation, culture media was collected and DRGs were briefly rinsed with 1×PBS and lysed with RIPA buffer containing 1×protease inhibitor cocktail and PMSF (Sigma-Aldrich). DRG lysates were then subjected to SDS-PAGE and western blotting analysis accordingly our published protocol.[1-2] In brief, equal amounts of protein (~ 20 μg per sample) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After incubation with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 1 h at room temperature, the membranes were incubated with mouse phospho-ERK1/2 (1:1000) (cell signaling, Danvers, Massachusetts), rabbit total ERK 1/2 (1:1000) (cell signaling), rabbit phosphor-Akt1/2/3 (1:1000) (Santa Cruz Biotechnology, Dallas, TX), rabbit total Akt1/2/3 (1:1000) (Santa Cruz Biotechnology), or mouse phosphor-p65 monoclonal IgG (1:1000) (Santa Cruz Biotechnology) antibodies overnight at 4 °C, followed by incubation with a goat-anti-mouse Alexa Fluo680 (1:5000) or goat-anti-rabbit Alexa Fluo800 (1:5000) (ThermoFisher Scientific) for 1 h at room temperature. Membranes were washed, scanned and analyzed with Odyssey Infrared Imaging System (LI-COR Biosciences). Experiment were performed in duplicated samples. As shown in the representative western blots in **Fig. S1**, TNF-α induced the most abundant phosphorylation of both AKT and ERK at 5 h post treatment, this time point was therefore selected to study fullerol's effect on these signaling pathways in the context of DRG and TNF-α induced neuroinflammation, which was consistent with our prior publication.[3]

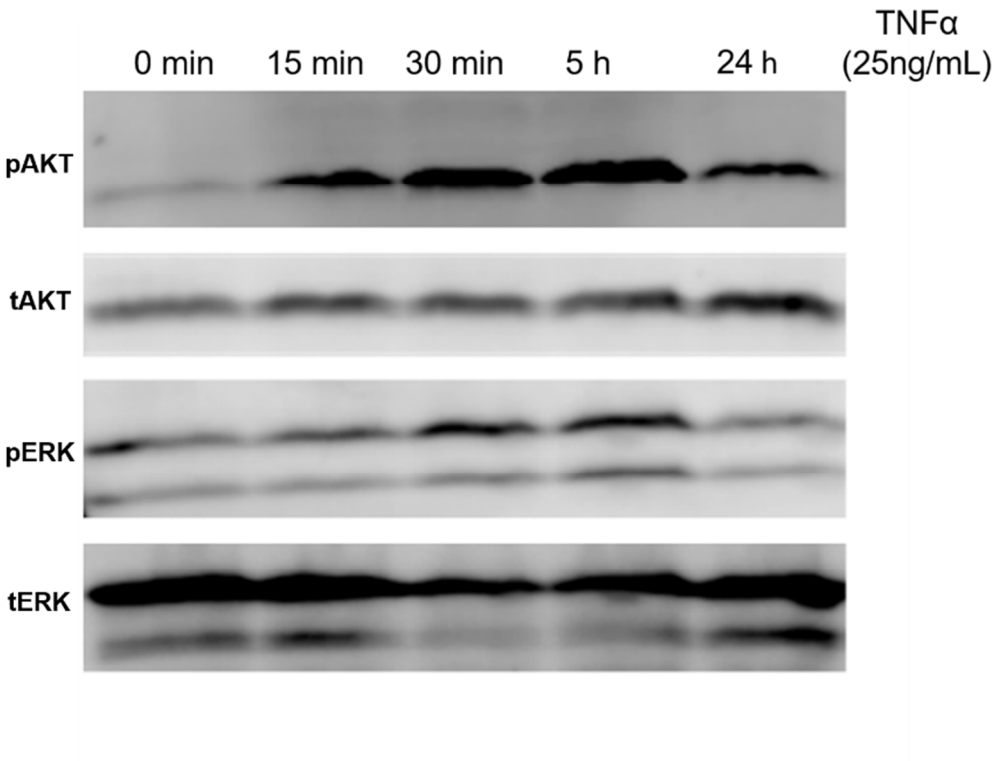

Figure S1. Western blotting of time-dependent effect of TNF- α treatment on DRG explant. For each group, mouse DRG explants (*n*=7–10 per group) were homogenized with lysis buffer in individual micro-homogenizer after TNF-α (25 ng/mL) treatment for various time points (0, 15 min, 30 min, 5 h, 24 h).

Fullerol suppressed LPS-induced phosphorylation of AKT and ERK in Raw 264.7 cells

Raw 264.7 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco, Grand Island, NY) supplied with 10% fetal bovine serum (Gibco), 1% penicillin (100 μg/mL), and 1% streptomycin (100 μg/mL) (Gibco) and maintained in 5% CO_2 at 37 °C. Raw 264.7 cells were seeded onto 24-well plates at a density of 5×10^5 cells/mL and cultured for 1-2 days until confluency of 90%. Western blot assay was employed for the investigation of related protein levels as the method described in our previous publication.[3] After pre-incubation with fullerol (0, 1, or 10 μM) for 20 h and LPS (100 ng/mL) stimulation for 0.5 h in serum-free DMEM, cells were washed with 1×PBS and lysed with RIPA buffer containing 1×protease inhibitor cocktail and PMSF (Sigma-Aldrich). Equal amounts of protein (~ 20 μg per sample) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After incubation with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 1 h at room temperature, the membranes were incubated with mouse phospho-ERK1/2 (1:1000) (cell signaling, Danvers, Massachusetts), rabbit total ERK 1/2 (1:1000) (cell signaling), rabbit phosphor-Akt1/2/3 (1:1000) (Santa Cruz Biotechnology, Dallas, TX), rabbit total Akt1/2/3 (1:1000) (Santa Cruz Biotechnology), or mouse phosphor-p65 monoclonal IgG (1:1000) (Santa Cruz Biotechnology) antibodies overnight at 4°C, followed by incubation with a goat-anti-mouse Alexa Fluo680 (1:5000) or goat-anti-rabbit Alexa Fluo800 (1:5000) (ThermoFisher Scientific) for 1 h at room temperature. Membranes were washed, scanned and analyzed with Odyssey Infrared Imaging System (LI-COR Biosciences). Experiment were performed in duplicated samples each time. Results from three independent experiments were analyzed (*n*=3). As shown in **Fig. S2**, fullerol at 10 μM significantly decreased both basal expression and LPS induced pATK/tAKT and pERK/tERK.

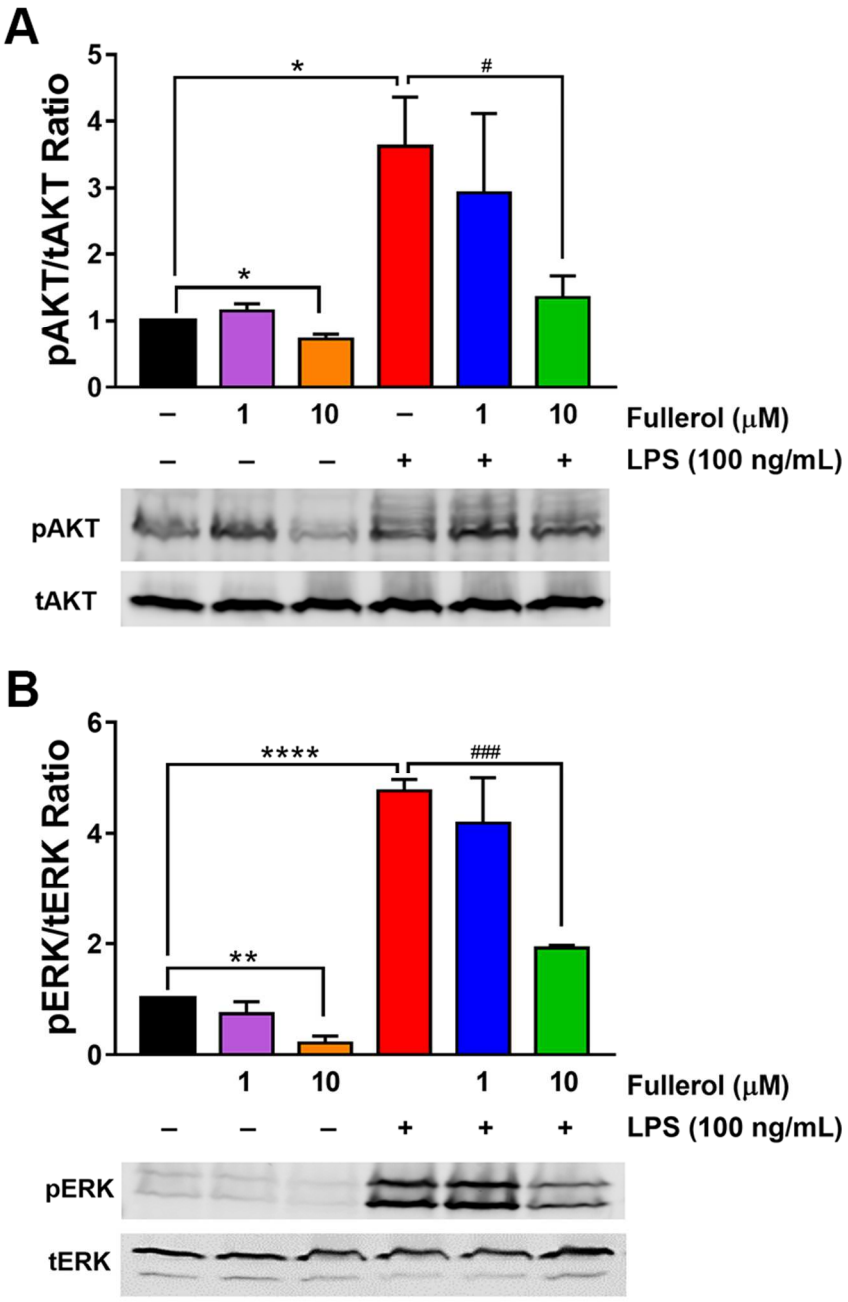

Figure S2. Fullerol attenuated LPS induced phosphorylation of AKT and ERK in a dose-dependent manner in Raw 264.7 cells indicated by western blotting. (A). Representative western blots and quantitative analysis of phosphor-AKT (pAKT) and total AKT (tAKT) suggested that fullerol (at 10 μM) abolished the basal pAKT/tAKT expression while significantly attenuated LPS-induced phosphorylation level of AKT. (B) Representative western blots and quantitative analysis of phosphor-ERK (pERK) and total ERK (tERK) illustrated similar effect of fullerol (at 10 μM) for decreasing both basal level and LPS elicited ERK phosphorylation. *p<0.05, **p<0.01 ****p<0.0001 *vs.* Control cells; $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 *vs.* LPS treated cells.

References

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