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ORIGINAL ARTICLE

The beneficial effect of salubrinal on neuroinflammation and neuronal loss in intranigral LPS-induced hemi-Parkinson disease model in rats

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ABSTRACT

Objective: Endoplasmic reticulum stress (ERS) and neuroinflammation are triggers for neurodegenerative disorders. Salubrinal is a selective inhibitor of protein phosphatase 1 (PP1) complex involving dephosphorylation of phosphorylated eukaryotic initiation factor-2α (eIF2α), the key crucial pathway in the ERS. Therefore, this study assessed the effects of inhibition of the ERS with salubrinal in the intranigral hemi-Parkinson disease (PD) model.

Materials and methods: Animals were treated with salubrinal for one week after the PD model was created by intranigral lipopolysaccharide (LPS) administration. Apomorphine-induced rotation, rotarod, cylinder, and pole tests were performed to evaluate behavioral changes. Proinflammatory cytokines and the expression level of the dual specificity protein phosphatase 2 (DUSP2), PP1, and p-eIF2α were evaluated. Nigral expression of inducible nitric oxide synthase (iNOS), nuclear factor kappaB (Nf-κB), and cyclooxygenase (COX)-2 was determined. Finally, tyrosine hydroxylase and caspase-3/ caspase-9 expressions were assessed by immunohistochemistry.

Results: Salubrinal reduced the motor impairments and dopamine-related behavioral deficiencies caused by the LPS. Salubrinal attenuated the LPS-induced increased levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and salubrinal rescued the loss of TH expression and dopamine levels and prevented the caspase-3/9 increase in the substantial nigra (SN). LPS potently increased iNOS, Nf-κB, and COX-2 expression, but this effect was reduced after salubrinal treatment. Additionally, salubrinal attenuated the LPS-induced PP1 and DUSP2 increase.

Conclusion: Our results reveal that salubrinal is attenuating several inflammatory mediators and thereby decreased the inflammatory effects of LPS in the neurons of the SN. Together this results in increased cellular survival and maintained integrity of SN. Taken together our data show the beneficial effects of inhibition of ERS to restrict neuroinflammatory progression and neuronal loss in a PD model.

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Endoplasmic reticulum stress; salubrinal; Parkinson's disease; LPS

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder around the globe [1]. Although the underlying pathological mechanisms of this debilitating disorder are still not well understood, accumulating data suggest that neuroinflammation plays a crucial role [2]. Neuroinflammation also has been linked to several other neurodegenerative disorders, including Alzheimer disease, and has frequently been associated with the progressive and treatment unresponsive behavior of these diseases [3]. In the last decade several studies have demonstrated that chronic neuroinflammation exacerbates dopaminergic neuronal loss in PD [4]. Moreover, microglial activation and neuroinflammation are sequential events in PD, and several studies using animal models have established their direct relation with disease progression [5]. Therefore, amelioration of neuroinflammation potentially can be used for the treatment of PD.

Anti-inflammatory modalities gained significant attention, especially after the neuroprotective actions of the nonaspirin, nonsteroidal anti-inflammatory drugs in PD were revealed [6]. Although, several anti-inflammatory drugs, such as dexamethasone and acetylsalicylic acid showed neuroprotective actions on PD models, only a few entered the clinical phase [7,8]. Surprisingly, later studies showed that some of the identified compounds increased the risk for PD instead of having a protective effect [7]. To completely understand this discrepancy and the mechanistic action of anti-inflammatory drugs it will be important to unravel the underlying molecular pathways that induce neuroinflammation and subsequently PD.

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Endoplasmic reticulum stress (ERS) results in impaired protein folding and accumulation of misfolded proteins. This process is detrimental for neuronal survival [9], most likely *via* neuroinflammation and microglial activation [10,11]. In toxin-induced PD-models several ER-related genes show increased expression, including human ubiquitin ligase HRD1, protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [12]. Furthermore, *in vivo* toxin-based studies and postmortem analysis showed that phosphorylated eukaryotic initiation factor-2α (eIF2α), which is an accepted marker for the ERS, was significantly increased in the PD group compared with the control [13]. This suggest that ERS plays a role in the progression of PD. Increased ERS also has a vital role in protein synthesis, proteostasis, which is fundamental for neuroinflammation. Particularly, proinflammatory cytokines and factors that regulate inflammatory response and cellular survival in neuroinflammation are directly related to PD-related neuronal death.

Salubrinal is a novel inhibitor of cellular stress and known to inhibit protein phosphatase 1 (PP1), which decreases global translation, and is reducing ERS and ultimately promotes cell survival [14]. *In vitro* cell studies with rotenone and paraquat revealed that treatment with the caspase inhibitor z-VAD or salubrinal reduced ERS and increased cellular viability [15,16]. Because of the protective effects of salubrinal against protein toxicity, stroke, excitotoxicity, and traumatic brain injury, it might also provide protection against PD. To this end we aimed to investigate possible effects of salubrinal on lipopolysaccharide (LPS)-induced PD within the context of neuroinflammation. Our data show that salubrinal protect against LPS-induced motor impairment and cellular death. Additionally, we show that salubrinal prevented LPS-induced inflammation in the substantia nigra (SN), most likely *via* modulation of dual specificity protein phosphatase 2 (DUSP2). Taken together our data show the beneficial effects of inhibition of ERS to restrict neuroinflammatory progression and neuronal loss in a PD-model.

Materials and methods

Animals

Forty male Wistar albino rats (240–260 g), which were obtained from the animal vivarium of Ondokuz Mayı s University Animal Facility, were used in this study. All experiments were approved by the Animal Ethics Committee of Ondokuz Mayıs University (HADYEK 2019_36) and the experiments were performed with all preventive efforts to reduce animal suffering. The obtained experimental results were reported according to the ARRIVE guideline [17]. Animals were maintained in separate cages and weighed weekly under standard conditions (22 ± 2 °C, 55 ± 2% humidity, and 12/12-h day and night cycle). The number of animals were determined based on power analysis performed with the G-power. The results reveal that to obtain effects with 95% power, as determined by one-way analysis of variance between means with alpha at 0.05, a minimum of 40 animal were required for our study.

Chemicals

LPS (O55:B5), salubrinal, dimethylsulfoxide (DMSO), and apomorphine hydrochloride were purchased from Sigma-Aldrich In. (St. Louis, MO). LPS was dissolved in phosphate-buffered saline (PBS; pH 7.4) and stored at 4 °C until use. Salubrinal was dissolved in DMSO for stock concentration (100X) then diluted with physiological saline to the final concentration (1 mg/kg). Apomorphine hydrochloride was freshly prepared in PBS solution on the day of the injection. The dose of salubrinal was determined based on previous studies that have reported neuroprotective actions of salubrinal [18–20].

Intranigral LPS injection and drug treatments

Animals were anesthetized with ketamine hydrochloride (80 mg/kg, intraperitoneal [i.p.]) and xylazine hydrochloride (10 mg/kg, i.p.), and then placed in a stereotaxic apparatus (World Precision Instruments, US). The skull of the animals was shaved and minimally incised to reduce the risk for infection. After antisepsis, one burr hole was drilled for intranigral injection according to the rat brain atlas [21]. LPS (5 μg/2 μL) was injected with a 28 G Hamilton syringe using the following coordinates: AP, +3.2 mm from the interaural midpoint; ML, +2.0 mm from the intraparietal suture; and DV, −6.5 mm from the dura mater. LPS injection was performed on the same side for each animal. The salubrinal and control groups were injected with only PBS solution (1 μL) into the same coordinates. Salubrinal (1 mg/kg, i.p.) administration to the salubrinal + LPS group started 2 hours before the LPS injection and continued for seven days.

Apomorphine induced rotation test

An apomorphine induced rotation test was used for the evaluation of dopamine deficient behavior in hemi-Parkinsonian rats [22]. Because apomorphine stimulates sensitized dopaminergic neurons, the number of turns was correlated with dopaminergic neuronal damage. The apomorphine-induced rotation test was performed 24 hours after the last drug administration. Animals were placed in an open plexiglass apparatus and after apomorphine (1 mg/kg, subcutaneous) administration the number of the turns was recorded for 30 min.

Behavioral tests

After the apomorphine washout period had passed, animals were tested with rotarod, cylinder, and pole tests. Locomotor activity, motor balance, and skills were evaluated with the rotarod test [23]. Animals were reversely placed on a rotating platform, and latency of the falling was recorded. Before the experiments, the rats were pretrained for 2 days to assure stable performance. On the test day, the rats were placed on an accelerating platform and the falling latencies were recorded. For the cylinder test, the rats were placed in an open plexiglass cylinder (30 × 20 cm) apparatus, and the number of the rearing were recorded. Regularly, rats that are

placed in a cylinder will engage in exploratory behavior, including rearing. The times that the animals raise their forelimbs above shoulder level and contact the cylinder wall with each hindlimb were recorded during a 5 min period. The percentage of the use of contralateral limb was calculated according to the total touches [23]. Bradykinesia was assessed with the pole test. The animals were placed on the top of a 1 m long perpendicularly placed metal pole, and the descending time from the pole was recorded [24].

Biochemical analysis

Following the behavioral tests, all animals were transcardially perfused with a heparinized-PBS solution and decapitated. Brain tissue was carefully removed, and the SN was isolated. Samples were homogenized in ice-cold PBS with a glass homogenizer and centrifuged for 5 min. The protein concentration of the samples was measured with Lowry's method, and samples were stored at −80 °C until analysis [25]. Dopamine (Dopamine assay kit #201-11-0220, Sunred Co., Wuhan, China), interleukin (IL)-1β (IL-1β assay kit #201-11-0120, Sunred Co., Wuhan, China), IL-6 (IL-6 assay kit #201-11-0136, Sunred Co.), and tumor necrosis factor (TNF)-α (TNF-α assay kit #201-11-0765, Sunred Co.) levels in the samples were determined with commercially available enzyme-linked immunosorbent analysis (ELISA) kits strictly following the manufacturer's instructions. The amount of dopamine, IL-1β, IL-6, and TNF-α were calculated according to the standard curve obtained with the solutions that were included in the kits.

Western blot analysis

After decapitation and following the same procedure used for the biochemical analysis, the SN was isolated. Subsequently the samples were homogenized with chilled radioimmunoprecipitation assay (Tris-hydrochloride, sodium chloride, sodium dodecyl sulphate [SDS], sodium deoxycholate, ethylenediaminetetraacetic acid, proteinase inhibitor cocktail, radioimmunoprecipitation assay) buffer in a glass homogenizer. The protein concentration was determined, and an equal amount of the samples was loaded in 4–20% SDS-polyacrylamide gel electrophoresis gels. Following separation, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked for 1 h with 5% bowene serum albumin solution and then washed three times with 0.1% Tris buffered saline-Tween 20 (TBS-T). The membranes were incubated with primary antibodies (inducible nitric oxide synthase (iNOS)#13120, β-actin#8457, nuclear factor kappaB (NF-κB)#3034, PP1#2582 cell signaling, TH#E-AB-70077, and dual specificity protein phosphatase 2 (DUSP2)#E-AB-DK1765 Elabscience, eIF2S1 alpha#GTX112919, and eIF2 alpha#GTX50300 Genetex) at 4 °C overnight. After three washes with TBS-T, the membranes were incubated with secondary antibody (Bio-rad, rabbit anti-goat IgG (H + L)-horse radish peroxidase (HRP) conjugate #1721034) for 2 h. The proteins were visualized using an ECL kit. The intensities were measured using ImageJ (NIH, US) and compared with the internal standard β-actin.

Immunohistochemistry analysis

For the immunoperoxidase method, Streptavidin-biotin peroxidase complex was applied to five different series of sections on polylysine slides [26]. Selected sections were stained to demonstrate the presence of caspase-3 (anticaspase-3 antibody, ab405, Abcam-Cambridge, UK); caspase-9 (anti-caspase-9 antibody, ab52298; Abcam-Cambridge, UK), cyclooxygenase-2(COX-2; D-5 antibody, sc-514489; Santa Cruz, TX); using the streptavidin-biotin peroxidase technique, according to the manufacturer's instructions. The primary antibodies were used as 1/100 dilution. The ultravision detection system antipolyvalent, HRP/ 3,3′-diaminobenzidine (ready-to-use; TP-015-HD) was used as the secondary antibody, and 3,3′-diaminobenzidine was used as the chromogen (Thermo scientific, Cheshire, UK). The primary antibody was omitted in negative controls. All examinations were performed by a specialized pathologist blinded to the sample treatments. All brain samples, especially SN sections were analyzed for immunopositivity, and a semiquantitative analysis was carried out as detailed later. Samples were analyzed by examining five different sections in each sample. They were scored from 0–3, according to the intensity of staining (0, absence of staining; 1, slight; 2, medium; and 3, marked). After the routine microscopic examination, computer-assisted histomorphometric measurements and immunohistochemical scoring were obtained using an automated image analysis system (Olympus CX41, Olympus Corporation, Tokyo, Japan). The Database Manual CellSens Life Science Imaging Software System (Olympus Corporation) was used for evaluation of the lesioned area.

Statistical analysis

All data were analyzed with SPSS (v21.0, IL) and were expressed as the mean ± SD. After determining the normality of data, Kruskal-Wallis, and one-way analysis of variance tests were performed. Tukey's and Bonferroni tests were used for post hoc analysis, and p values less than.05 were considered statistically significant.

Results

Salubrinal alleviates LPS-induced dopaminergic desensitization, motor impairment, bradykinesia, and forelimb akinesia

LPS injection caused significant rotational behavior (165 ± 24.7) within the 30 min period measurement of the apomorphine-induced rotation test (Figure 1). Salubrinal significantly reduced the rotation numbers (68.8 ± 6.91) seen in the LPS group ($F(3,20)=218$, $p < .001$, Figure 1). Motor coordination was evaluated with the rotarod test (Figure 2(A)). LPS injection impaired the motor balance and decreased the falling latency (41.0 ± 7.82; Figure 2(A)) compared with the control group. The LPS + salubrinal group showed improved rotarod performance compared to the LPS group (144.5 ± 7.34, $F(3,40)=164$; $p = .006$; Figure 2(A)), indicating that salubrinal administration partly rescues the LPS-induced

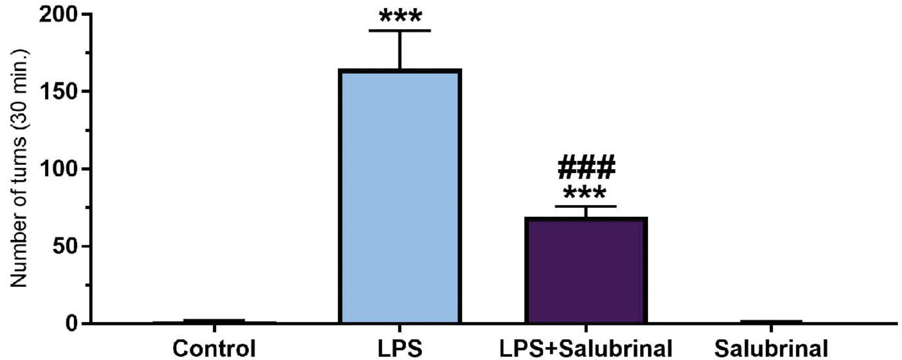


Figure 1. Apomorphine-induced rotation test results of all experimental groups. LPS caused significant increase in the turning numbers. Salubrinal suppressed that increase. All data are expressed as mean ± SD. $^{***}p < .001$ versus control group; $^{###}p < .001$ versus LPS group.

Figure 2. Behavioral tests results are given as (A) rotarod, (B) cylinder test, and (C) pole test for all experimental groups. LPS significantly deteriorated motor coordination and caused forelimb akinesia (A, B). Salubrinal significantly extended the falling latency and attenuated forelimb akinesia (A, B). Additionally, LPS caused bradykinesia, which was inhibited by salubrinal treatment (C). All data are shown as mean ± SD. $^{***}p < .001$ versus control group; $^{###}p < .001$ and $^{##}p < .01$ versus LPS group.

motor impairment. Additionally, salubrinal partially prevented (7.83 ± 0.983; $F(3,20)=83.2$; $p = .02$; Figure 2(B)) the LPS induced decreased use of contralateral paw (4 ± 0.894; $p < .001$, Figure 2(B)) in LPS + salubrinal group. Furthermore, a pole test showed that LPS injection caused significant bradykinesia and increased the descending latency (15.1 ± 1.83; $p < .001$; Figure 2(C)). Similar to the other tested behavioral parameters, salubrinal also significantly decreased the prolonged descending latency (11.4 ± 0.991; $F(3,20)=151$; $p < .001$; Figure 2(C)).

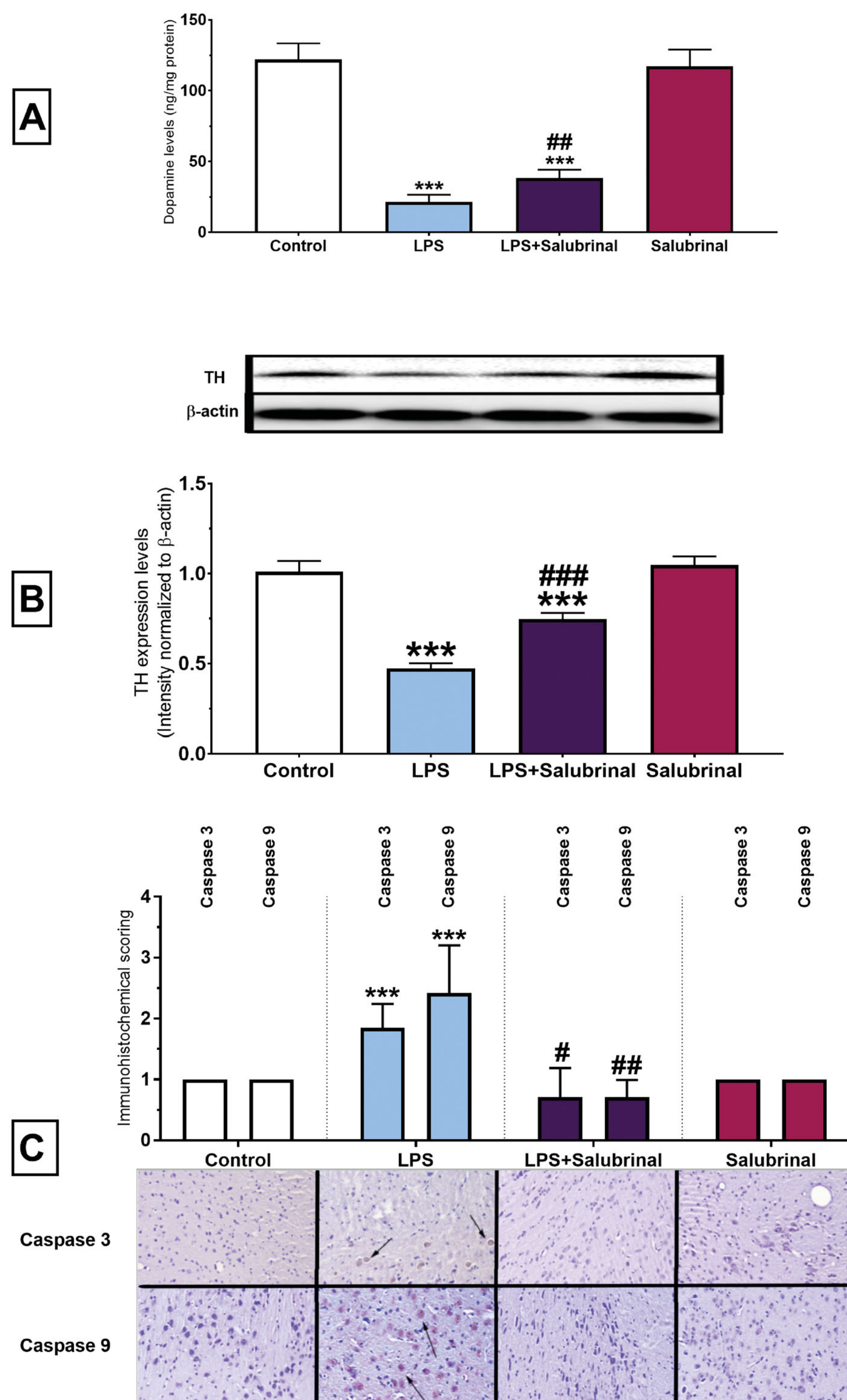


Figure 3. Dopamine levels in in the SN of all experimental groups. LPS significantly decreased total dopamine levels. (A) Salubrinal significantly prevented that decrease. TH immunoreactivity evaluated by immunoblotting. (B) LPS significantly caused a decrease in TH expression, whereas salubrinal significantly inhibited that decline. (C) Caspase-3 and caspase-9 immunoreactivity were evaluated by immunohistochemistry. LPS increased the caspase-3 and caspase-9 histological scores. However, salubrinal significantly ameliorated caspase-3 and caspase-9 levels. All data are expressed as mean ± SD. The blot shown is representative for three independent experiments. Immunopositive neurons are indicated by black arrows, Streptavidin biotin peroxidase method, scale bars = 50μm. Panels were ordered as control, LPS, LPS + salubrinal and salubrinal. $^{***}p < .001$ versus control group; $^{\#}p < .05$ versus LPS group.

Salubrinal rescues the decrease of dopamine levels and TH immunoreactivity and attenuated the exacerbation of caspase-dependent apoptosis after LPS injection in the SN

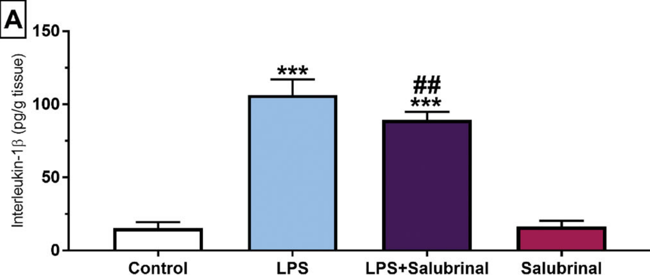
Next the dopamine levels in the SN were determined using an ELISA based assay. Our results show that LPS injection significantly decreased the dopamine levels in the SN (21.4 ± 5.28; $p < .001$; Figure 3(A)), whereas this decrease was significantly lower in the LPS + salubrinal group (38.4 ± 5.84; F(3,20)=201; $p = .019$; Figure 3(A)). Consistently, salubrinal (0.748 ± 0.0349; F(3,16)=177; $p < 0.001$, Figure 3(B)) also rescued the LPS-mediated decreased TH expression in the SN (0.422 ± 0.02; $p < .001$; Figure 3(B)). Furthermore, intranigral LPS injection caused neuronal cell death as indicated by the significant increase in the caspase-3 and caspase-9 immunoreactivity in the SN compared with the control group (1.85 ± 0.39; 2.42 ± 0.78; $p < .001$, respectively; Figure 3(C)). The salubrinal + LPS group showed significantly lower neuronal cell death compared with the LPS group (0.71 ± 0.48; 0.71 ± 0.28; $p = .45$; $p = .25$; F(3,26)=65.3; F(3,26)=10.3, respectively; Figure 3(C)).

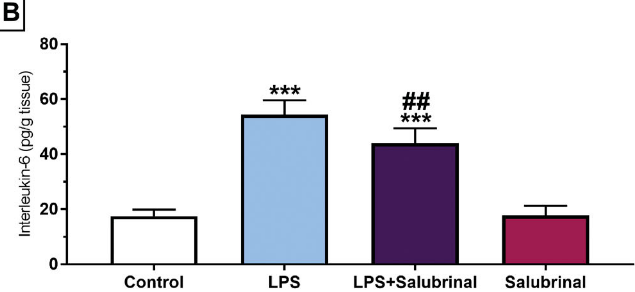
Salubrinal attenuates increased inflammatory cytokine levels and NF-κB, iNOS, and COX-2 expression

At the histopathological examination of the control and salubrinal groups normal SN histoarthitecture were observed. LPS caused degenerated neurons and gliosis in injection sites. However, salubrinal treatment decreased the histopathological findings (Supplementary Figure S1). The levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α levels were determined in the SN (Figure 4). As expected, LPS injection significantly increased the IL-1β, IL-6, and TNF- α levels (106 ± 10.6, 54.4 ± 5.24, 53.3 ± 3.96; $p < .001$, respectively; Figure 4(A–C)). The salubrinal + LPS group showed a significantly decreased inflammatory response compared with the LPS group (89.4 ± 5.52, 44.1 ± 5.33, 46.4 ± 5.31; F(3,20)=311, F(3,20)=113, F(3,20)130; $p = .001$, $p = .003$, $p = .022$, respectively; Figure 4(A C)). Additionally, NF-κB and iNOS expression was determined by immunoblotting. LPS markedly increased NF-κB and iNOS expression (1.81 ± 0.07, 0.75 ± 0.05; $p < .001$, respectively; Figure 5(A)). But in parallel with the proinflammatory cytokine levels, the salubrinal + LPS group showed a significantly reduced response compared with the control group (1.55 ± 0.08, 0.57 ± 0.04; F(3,16)=76.1, F(3,16)=73.8; $p < .001$, respectively; Figure 5(A)). Next, COX-2 immunoreactivity was investigated by immunohistochemical analysis (Figure 5(C)). The LPS group showed an increased COX-2 immunoreactivity in the SN compared with the control group, (2.14 ± 0.49; $p < .001$; Figure 5(C)), whereas salubrinal significantly lowered this LPS affect (1.14 ± 0.69; $p = .013$; F(3,12)=19.9; Figure 5(C)).

Salubrinal modulates inflammation by DUSP2 and PP1

To investigate the possible mechanism by which salubrinal modulates inflammation, DUSP2, PP1, eIF2α, and phosphorylated-eIF2α levels were analyzed by immunoblotting (Figure 6). DUSP2 and PP1 are both important for cellular homeostasis and LPS significantly increased the levels of both proteins (1.22 ± 0.007, 0.994 ± 0.062; $p < .001$, respectively; Figure 6(A)). Salubrinal significantly decreased both DUSP2 and PP1 expression (0.926 ± 0.06, 0.71 ± 0.109; F(3,16)=93.9, F(3,16)=73.4; $p < .001$ and $p = 06$, respectively; Figure 6(A,B)). Furthermore, our results show that LPS significantly decreased eIF2α phosphorylation (0.266 ± 0.0383; $p < .001$; Figure 6(D)), whereas as a known phosphatase inhibitor, salubrinal significantly prevented that decrease (0.401 ± 0.02; F(3,16)=116; $p < .001$; Figure 6(D)).





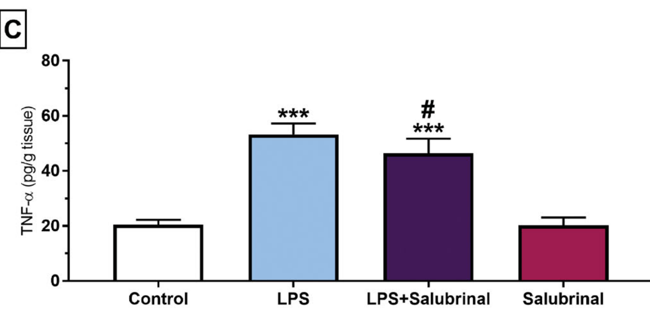


Figure 4. Results of the total (A) IL-1β, (B) IL-6, and (C) TNF-α levels in all experimental groups. LPS significantly increased IL-1β, IL-6, and TNF-α levels. Salubrinal attenuated the LPS-induced increase in the pro-inflammatory cytokines. All data are expressed as mean ± SD. $^{***}p < .001$ versus control group; $^{###}p < .001$, $^{##}p < .01$, and $^{#}p < .05$ versus LPS group.

Discussion

ERS in the central nervous system can serve as a prosurvival response to engage adaptive stress signaling events and maintain protein homeostasis. However, ERS can also result in unmanageable alteration and neuronal death [11]. It is becoming clear that ERS is intertwined with neuroinflammation and can cause dopaminergic neuronal loss in PD pathology [27]. Here we explored if reducing ERS and thereby

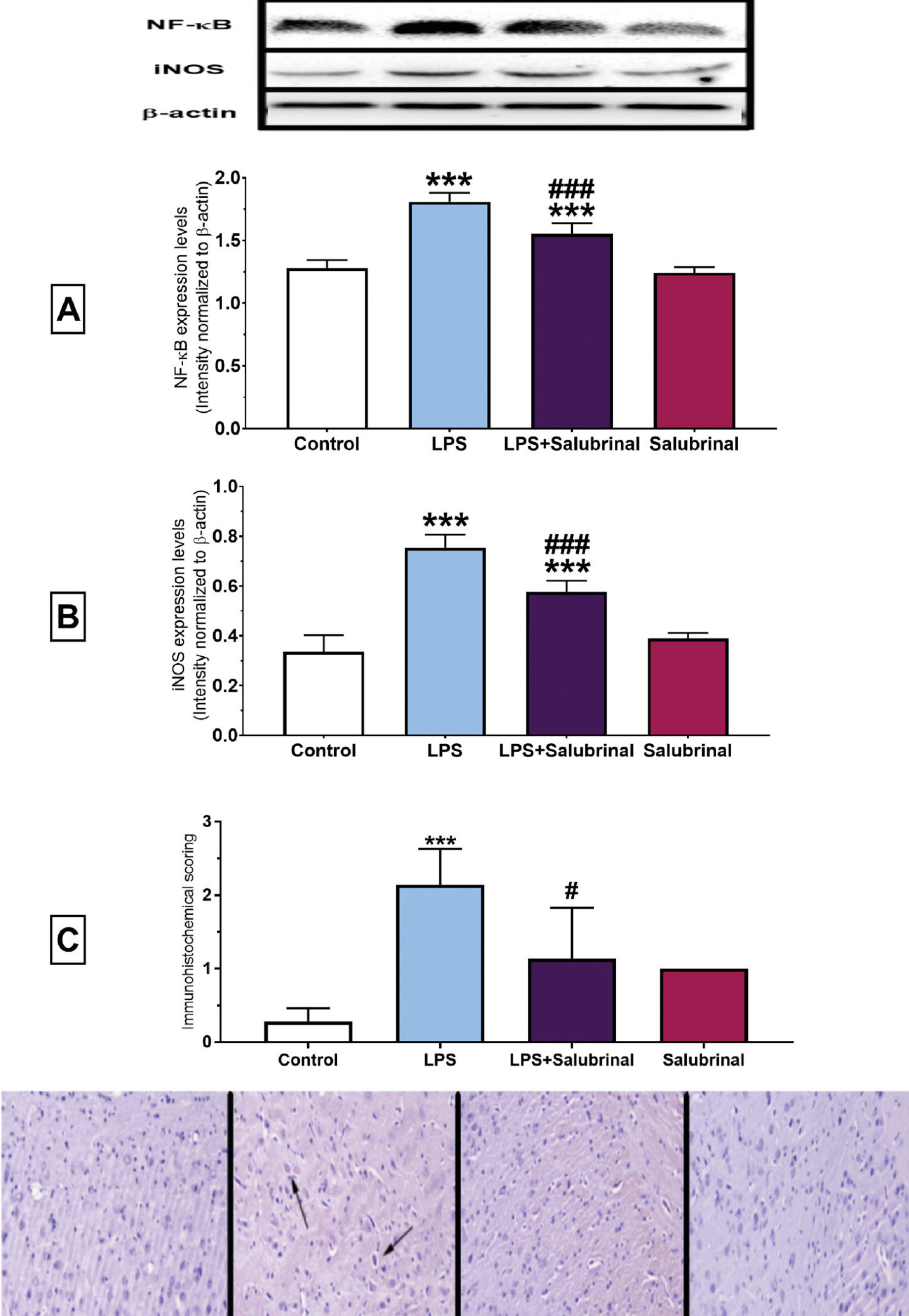


Figure 5. Immunoblotting of (A) iNOS, (B) Nf-κB and immunohistochemical analysis of (C) COX-2 expression. LPS significantly increased the levels of iNOS, Nf-κB, and COX-2. Salubrinal suppressed the increase in all these inflammatory markers. All data are expressed as mean ± SD The blot shown is representative for three independent experiments immunopositive neurons are indicated by black arrows, Streptavidin biotin peroxidase method, scale bars = 50μm. Panels were ordered as control, LPS, LPS + salubrinal, and salubrinal. $^{***}p < .001$ versus control group; $^{###}p < .001$ and $^{#}p < .05$ versus LPS group.

neuroinflammation is beneficial for PD progression in a hemi-Parkinsonian animal model.

Our behavioral studies revealed that salubrinal alleviates LPS-induced dopaminergic desensitization, motor impairment, bradykinesia, and forelimb akinesia, which are all symptoms of PD. This shows that consistent with previous studies salubrinal improved motor coordination [15,18]. The reduced decrease in the dopamine levels and TH expression observed in the LPS + salubrinal group compared with the LPS group is in concordant with the behavioral test.

Our data also reveal that intranigral administration of LPS induces neuronal cell death, whereas salubrinal attenuated this caspase-dependent neuronal loss in the SN. Intra-nigral LPS-induced neuroinflammation is associated with reactive microgliosis and IL-1β production [28]. In addition, long-term presence of reactive microgliosis and IL-1β can result in neuronal cell death [29]. Recent studies demonstrated that ERS induces the production of IL-6 and TNF-α to strengthen the inflammatory response and to stimulate other molecular pathways that are related to the ER [30]. In the context of

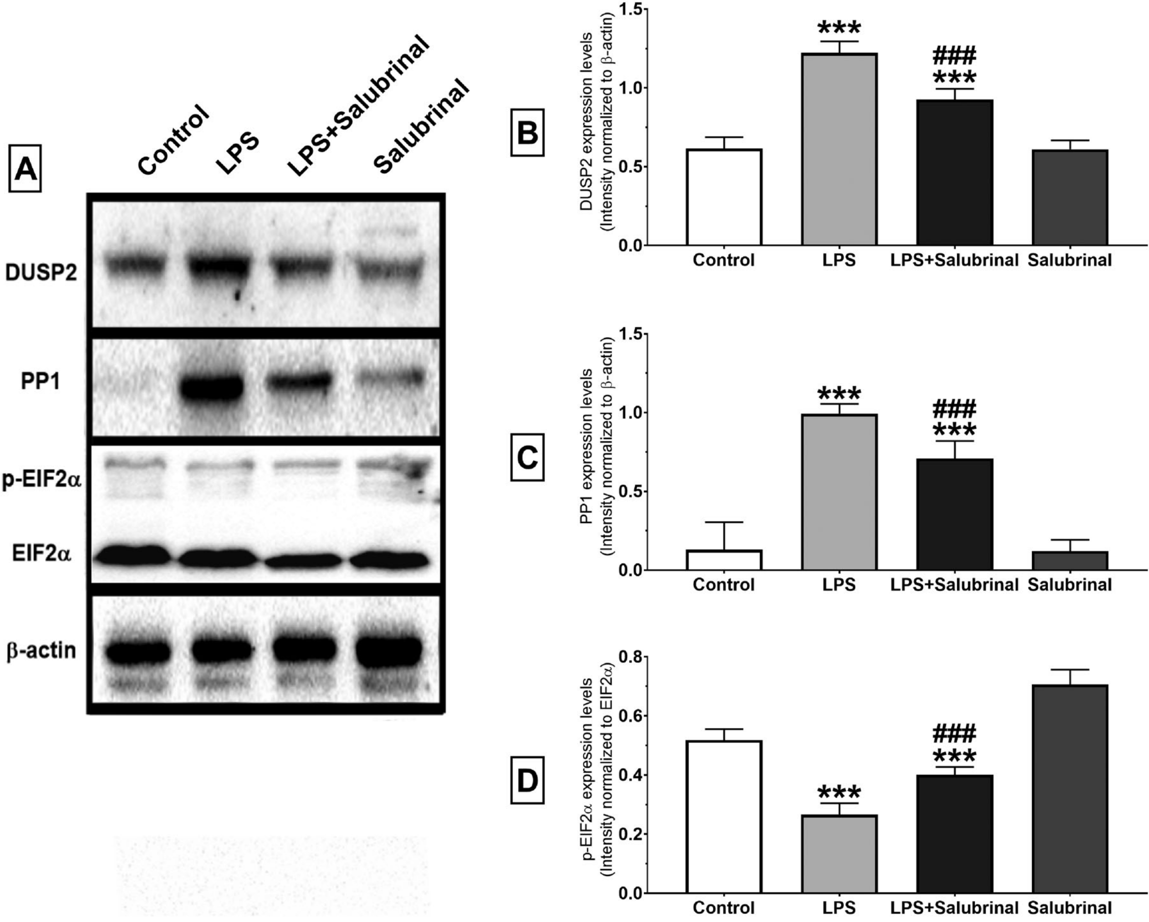


Figure 6. Expression levels of DUSP2, PP1, eIF2α, and p-eIF2α were evaluated. LPS significantly increased DUSP2 and PP1 expression. But caused a decrease in the p-eIF2α/eIF2α ratio. Salubrinal significantly lowered this LPS-induced DUSP2 and PP1 increase. Additionally, salubrinal significantly increased the p-eIF2α/eIF2α ratio. All data are expressed as mean ± SD. The blot shown is representative for three independent experiments $^{***}p < .001$ versus control group; $^{\#\#\#}p < .001$ and $^{\#\#}p < .01$ versus LPS group.

neuroinflammation, loss of TH positive dopaminergic neurons, that are the result of the long-term activation of these cytokines, has been associated with disease progression [31]. Our results show that salubrinal treatment alleviated the LPS-induced increase in cytokine levels, which might explain the observed reduced loss of dopamine levels in the SN.

Several studies have shown that increased levels of iNOS, Nf-κB, and COX-2 are contributing to neuroinflammation and ERS [32,33]. Therefore, reducing this inflammatory response could be a way to decrease the severity of neuronal injury [34,35]. During neuroinflammation, Nf-κB directly regulates IL-1β, IL-6, and TNF-α. Additionally, Nf-κB has been shown to regulate cellular iNOS activity [36,37]. Our results show consistence with previous studies [38] that salubrinal treatment decreased the increased levels of IL-1β, IL-6, and TNF-α, which might be related to the inhibitory action of salubrinal on Nf-κB expression [39].

Our results also revealed that the increased COX-2 immunoreactivity after LPS injection was attenuated by salubrinal treatment, which contrasts with previously published results. Choi et al. [40] have shown that salubrinal increases COX-2 and iNOS expression in an intestinal inflammatory model. Although LPS is a strong inducer of COX-2 expression, ERS is a negative modulator of COX-2 expression [41]. LPS is a very potent inflammatory stimulus that both directly and indirectly increases COX-2 levels, which suppressed with salubrinal treatment in our study. Additionally, Soto et al. [42] showed that cotreatment of rofecoxib, a COX-2 selective nonsteroidal anti-inflammatory drug, improved the use and potency of salubrinal. However, they also concluded that these effects differ depending on the salubrinal treatment longevity. Therefore, as seen in our study, repetitive salubrinal treatment might have different effects on COX-2 activity, which needs further investigations.

Previous studies have shown that salubrinal inhibits the PP1 phosphatase which results in increased eIF2α phosphorylation and chondroprotective effects [43]. The antiapoptotic action of salubrinal was shown to be mediated *via* the eIF2α-ATF4-CHOP signaling pathway [15]. Furthermore, recent studies revealed that salubrinal lowers the expression of PP1 and DUSP2 [43]. DUSP2 is a MAPK phosphatase that is predominantly expressed in immune cells, where it regulates cytokine release during inflammation [44]. PP1 regulates eIF2α phosphorylation thereby inducing apoptosis *via* the eIF2α-ATF4-CHOP pathway. Consistent with these studies our data show that salubrinal suppressed the LPS-stimulated PP1and DUSP2 expression. Our data thus suggests that salubrinal regulates

neuroinflammation and neuronal cell death *via* the PP1 and DUSP2 pathway.

Conclusion

Our results show that salubrinal treatment attenuated neuroinflammation in LPS-induced PD rats. Salubrinal treatment decreased the motor impairment and dopamine-related behavioral deficits provoked by LPS injection. Salubrinal increased the inflammatory cytokine/protein expression levels, thereby protecting the neurons for caspase-dependent neuronal cell loss. Our data also suggests that salubrinal regulates neuroinflammation and neuronal cell death *via* the PP1 and DUSP2 pathway. Together this suggest that inhibition of ERS in ongoing neuroinflammation in PD might be beneficial to shorten and reduce the disease progression.

Author contributions

Conceptualization: FNC, CG, SSB, and AK; Data curation: FNC, CG, and MSK; Formal analysis: FNC, CG, ET, and OO; Investigation: FNC and CG; Methodology: FNC, CG, SSB and AK; Resources: FNC, CGand AK; Animal Study: FNC, CGand SŞ; Supervision: FNC, SSB and AK; Validation: FNCand CG; Visualization: FNC, MSK and CG; Roles/Writing-original draft: FNC, MSK, CG and AK; Writing-review and editing: FNC, MSK, CG, SŞ, SSB, OO, ET, and AK.

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