


Nitric Oxide Induces a Janus Kinase-I-Dependent Inflammatory Response in Primary Murine Astrocytes

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Abstract

Nitric oxide (NO) is a versatile free radical that has been implicated in many biological processes (i.e., vasodilation, neurotransmission, and smooth muscle relaxation). High levels of NO, such as those produced by inducible NO synthase, are associated with innate immunity as well as tissue damage and disease pathology. Previous studies have characterized many stimuli that lead to NO production following central nervous system (CNS) infection, ischemia, and during neurodegeneration, but less is known about the effects of NO on the CNS resident astrocytes. Previously, excessive NO has been shown to impair protein folding leading to endoplasmic reticulum (ER) stress and initiation of the unfolded protein response. Previous studies have shown that ER stress drives activation of protein kinase R-like ER kinase (PERK) and Janus kinase-I (JAKI) leading to inflammatory gene expression. We hypothesized that NO drives inflammatory processes within astrocytes through a similar process. To test this, we examined the effects of exogenous NO on primary cultures of murine astrocytes. Our data suggest that NO promotes a pro-inflammatory response that includes interleukin-6 and several chemokines. Our data show that NO induces phosphorylation of eukaryotic initiation factor 2 alpha; however, this and the inflammatory gene expression are independent of PERK. Knockdown of JAKI using small interfering RNA reduced the expression of inflammatory mediators. Overall, we have identified that NO stimulates the integrated stress response and a JAKI-dependent inflammatory program in astrocytes.

Summary statement: Murine astrocytes in culture respond to NO with increased expression of stress and inflammatory genes. The inflammatory stress response is independent of the ER stress-activated kinase PERK and is, in part, mediated by JAKI.

Keywords

JAK/STAT, glia, unfolded protein response, integrated stress response, ISRIB, IL-6, chemokines

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Background

Inflammation in the central nervous system (CNS) is associated with most neurodegenerative diseases. CNS resident cells, including microglia and astrocytes, as well as infiltrating peripheral immune cells produce cytokines, chemokines, and reactive species. These molecules have physiological roles in the brain; however, when chronically or excessively produced they can also cause tissue damage or disruption of neuronal function. Interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ are among the major cytokines increased in the CNS during disease or following traumatic injury. These cytokines drive gene expression changes that dramatically alter the cellular and extracellular environment (Chitnis & Weiner, 2017; Ransohoff, 2016; Ransohoff & Brown, 2012).

Along with cytokine production, there is often increased production of nitric oxide (NO). Under physiological conditions, NO is produced at low levels and has various roles ranging from vascular relaxation to neurotransmission. Typically, low levels of NO exert influence by signaling through soluble guanylate cyclase leading to the production of cyclic

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guanosine monophosphate and activation of protein kinase G. Under inflammatory conditions, the inducible NO synthase (iNOS) is increased in cells such as microglia, macrophages, and neutrophils. Using arginine as a substrate, iNOS catalyzes the production of NO and citrulline (Calabrese et al., 2007; Picón-Pagès et al., 2019). iNOS produces high levels of NO and, when produced by many cells in close vicinity, potentially reaches high concentrations in the local environment. Through the regulation of various enzymes, NO impacts many cellular signaling pathways and posttranslational modifications (PTMs) (Hess & Stamler, 2012). iNOS is robustly expressed in multiple sclerosis lesions, predominantly in astrocytes (Liu et al., 2001). Excessive NO is also associated with neurodegenerative diseases including Alzheimer's and Parkinson's diseases (Calabrese et al., 2007). NO, or its reaction products such as peroxynitrite, contributes to neuronal injury and death through several mechanisms. NO can stimulate N-methyl-D-aspartate receptor activation leading to neuronal excitotoxicity (Dawson et al., 1991; Zhang et al., 2006). NO can drive the PTMs S-nitrosylation and tyrosine nitration, which can interfere with protein folding and enzyme function (Nakamura et al., 2013; Radi 2013). Additionally, NO can disrupt metabolic enzymes involved in oxidative phosphorylation (Stewart et al., 2000; Martínez-Ruiz et al., 2011). NO can also cause DNA damage resulting in aberrant activation of poly(ADP-ribose) polymerase which depletes nicotinamide adenine dinucleotide leading to an energetic failure (Ha & Snyder, 1999; Zhang et al., 2006).

The damaging effects of NO can stimulate cell stress responses. Included among these are the unfolded protein response (UPR) and the related integrated stress response (ISR) (Ill-Raga et al., 2015; Nakato et al., 2015; Pakos-Zebrucka et al., 2016; Uehara et al., 2006). The UPR signals through inositol-requiring enzyme 1, protein kinase R-like ER kinase (PERK), and activating transcription factor (ATF) 6 to concomitantly attenuate protein translation and increase expression of molecular chaperones to reduce the burden of misfolded proteins (Hetz & Saxena, 2017). Attenuation of protein translation occurs through PERK-dependent phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) in response to endoplasmic reticulum (ER) stress. The ISR is stimulated by various stressors and, depending on the stress, activates other eIF2 α kinases (protein kinase R, heme-regulated inhibitor [HRI], or general control nonrepressible 2) to reduced protein translation as part of an adaptive response (Pakos-Zebrucka et al., 2016).

Previous work has shown that astrocytes, the most abundant cell in the CNS, do not undergo apoptosis or cell death in response to ER stress (Meares et al., 2014). Similarly, astrocytes are less vulnerable than neurons to NO-induced cell death (Almeida et al., 2001, 2004). Moreover, astrocytes respond to ER stress with an inflammatory response that includes the production of IL-6 and chemokines such as chemokine ligand 2 (CCL2), chemokine ligand 20 (CCL20), and CXC chemokine ligand 10 (CXCL10), among others. This inflammatory response is mediated by PERK and involves the tyrosine kinase Janus kinase-1 (JAK1) and

P-eIF2 α -mediated attenuation of protein translation (Guthrie et al., 2016; Meares et al., 2014; Sims & Meares, 2019). Previous work has suggested that NO S-nitrosylates and activates PERK leading to eIF2 α phosphorylation (Nakato et al., 2015). Based on these findings, we hypothesized that NO would stimulate an inflammatory reaction in astrocytes dependent on PERK and JAK1. Our data show that NO does drive cytokine and chemokine production in astrocytes in a JAK1-dependent fashion. However, the inflammatory gene expression is independent of PERK, as is eIF2 α phosphorylation. Collectively, our data further show JAK1 is a key protein driving stress-induced inflammatory responses in astrocytes.

Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM; MT10013CV), HEPES (MT25060CI), nonessential amino acids (MT25025CI), L-glutamine (MT2500CI), and penicillin–streptomycin (MT30002CI) were from Cellgro. Gentamicin (G1522-10ML) was purchased from Lonza. Fetal bovine serum (FBS; S11150) was from Atlanta Biologicals. Diethylamine NONOate (DEA/NO; 82100) and propylamine NONOate (PAPA/NO; 82140) were from Cayman Chemical. ISR inhibitor (ISRIB) was from Sigma (SML0843). AZD1480 was from Tocris (5617). All antibodies were from Cell Signaling except GAPDH which was from Millipore.

Astrocyte and Microglia Isolation and Culture

C57BL/6J (Jackson Laboratories, RRID:IMSR_JAX:000664) PERK floxed (RRID:IMSR_JAX:023066 Jackson Laboratories) and CAGG-CreERTM mice (RRID:IMSR_JAX:004682 Jackson Laboratories) were bred and housed in the vivarium under the care of the Office of Lab Animal Resources. Mice were housed at 21°C on a 12 h light-dark cycle with free access to food and water. Our breeding strategy used PERK fl/fl crossed with PERK fl/fl x CAGG-CreERTM. The Cre allele is always maintained as hemizygous, therefore the offspring produced are homozygous for the floxed PERK allele and ~50% of the offspring are Cre positive. One-day-old pups were euthanized by decapitation. All procedures were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the institutional animal care and use committee. Primary murine astrocyte cultures were prepared as previously described (Meares et al., 2014). Briefly, cerebra were removed from one-day-old pups and placed in cold media. Meninges were removed and tissue triturated to generate a cell suspension. The cerebra from all of the wild-type mice in a litter are pooled together, therefore these cultures contain cells from both male and female mice. To isolate PERK fl/fl astrocytes without or with Cre, cells from each mouse in a litter were cultured individually with genotyping

post-hoc to identify Cre + cultures. The sex of the animal from which the cells were derived was not determined. The cell suspension was passed through a 100 μ m strainer and washed 2X with media, centrifuging at 300g between washes. Astrocytes were then cultured in DMEM with 10% FBS, 16 mM HEPES, 1x nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamicin. Astrocytes were separated from microglia by shaking at 200 rpm for 2 h and microglia plated in 12-well plates. Subsequently, astrocytes were trypsinized and plated into 6-well plates. Astrocytes are enriched to ~90% GLAST1+ cells as determined by flow cytometry (Miltenyi Biotec# 130-118-483, RRID:AB_2733722). Experimental treatments were initiated 48–72 h after plating.

Genotyping and Cre Activation

Genomic DNA was obtained from tail biopsies and analyzed by standard polymerase chain reaction (PCR). The genotyping primers used for CAGG-CreERTM are (forward primer GCT AAC CAT GTT CAT GCC TTC) and (reverse primer AGG CAA ATT TTG GTG TAC GG). Genotyping primers used for PERK floxed are (forward primer TTG CAC TCT GGC TTT CAC TC) and (reverse primer AGG AGG AAG GTG GAA TTT GG). Primers were synthesized by Integrated DNA Technologies. Cre-ERTM is a tamoxifen inducible cre. To activate cre in cultured astrocytes, 4-OH tamoxifen (1 μ M) was added for 48 h after the astrocytes reach confluency (~12–14 days in culture).

Small Interfering RNA (siRNA) Knockdown

Primary astrocytes were transfected with the indicated small interfering (si) RNA (50 pmols per 35 mm well) (siRNA Silencer Select, ThermoFisher) using Lipofectamine RNAiMAX (ThermoFisher) according to the manufacturer's protocol. Cells were used for experiments 48–72 h after transfection. The siRNAs used in this study include control (nontargeting) siRNA, JAK1 siRNA #1 (sequence: GCUCCG AACCGAAUCAUCA) (s68538), JAK1 siRNA #2 (sequence: CACUGAUUGUCCACAAUAUTT) (s68537), JAK2 siRNA (sequence GGACUAUAUGUGCUACGAUTT).

Preparation of NO Donors

Compounds were stored as a powder at -80°C in a desiccator. NO donors were dissolved in tissue culture media at a stock concentration of 20 mM immediately before adding to the astrocytes at the indicated final concentration. To prepare the decomposed donors (d-), compounds were dissolved in tissue culture media and incubated overnight at 37°C prior to adding to the astrocytes.

Reverse Transcription-Quantitative PCR (qPCR)

RNA was isolated using 1 ml of TRIzol according to the manufacturer's protocol. RNA was quantified using a NanoDrop system (Fisher). One microgram of RNA was used for cDNA synthesis. RNA was mixed with 0.5 μ g of oligo dT primer and incubated at 70°C for 5 min followed immediately by 5 min on ice. A mix containing reaction buffer, Moloney murine leukemia virus reverse transcriptase, deoxynucleotide triphosphates, and RNasin was added and incubated at 42°C for 60 min. The reaction was terminated by incubation at 95°C for 5 min. The cDNA was then analyzed by qPCR performed using probe-based gene expression assays using Quant studio 3 qPCR machine. Reactions were carried out in 20 μ l and analyzed using the threshold cycle method.

Immunoblotting

Cells were washed twice with phosphate buffered saline and lysed with immunoprecipitation (IP) lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% NP-40, and 1X phosphatase/protease inhibitor cocktail [Pierce]). Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Equal amounts of protein from each sample were solubilized in Laemmli sample buffer and heated for 5 min at 95°C . Proteins were separated by 8% or 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the membranes were blocked in 5% milk in wash buffer (20 mM Tris base, 137 mM NaCl and 0.05% Tris buffered saline Tween-20 [TBST]) followed by an overnight incubation at 4°C with primary Ab diluted in 5% bovine serum albumin or milk, according to the manufacturer's recommendation. Primary antibodies were diluted as follows; JAK1 (1:1000, 3344, RRID:AB_2265054), P-STAT3 (1:2000, 9145, RRID:AB_2491009), STAT3 (1:3000, 12640, RRID:AB_2629499), P-p38 (1:2000, 4511, RRID:AB_2139682), p38 (1:6000, 8690, RRID:AB_10999090), P-JNK (1:2000, 4668, RRID:AB_823588), JNK (1:2000, 9252, RRID:AB_2250373), P-eIF2 α (1:2000, 3398, RRID:AB_2096481), eIF2 α (1:6000, 5324, RRID:AB_10692650), PERK (1:2000, 3192, RRID:AB_2095847), and GAPDH (1:25000, MAB374, RRID:AB_2107445). Membranes were washed for 1 h with frequent changes to TBST. Horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse (1:2000 dilution) secondary Ab was incubated for 1 h at room temperature. Membranes were washed for 1 h with frequent changes to TBST, followed by detection with enhanced chemiluminescence and imaging using a ChemiDoc touch (Biorad).

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 was measured using 100 μ l of cell culture medium according to the manufacturers' protocol. Mouse IL-6 ELISA was from R&D systems (DY406-05).

Statistical Analysis

Statistical differences between experimental groups were determined using GraphPad Prism. Student's *t*-test was used to determine statistically significant differences between the means of two groups. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of three or more independent biological groups. Post-hoc Dunnett test was used for multiple comparisons. Two-way ANOVA with Bonferroni's multiple comparisons test was used to determine statistically significant differences between groups with two independent variables. Data are

means of 3 or more independent experiments \pm standard deviation. $p \leq .05$ was considered statistically significant.

Results

To examine the impact of NO on astrocytes, we treated cultured primary murine astrocytes with increasing concentrations of the NO donor DEA/NO. We anticipated a response similar to ER stress in astrocytes, which is dominated by the expression of IL-6 and several chemokines (Meares et al., 2014). As shown in Figure 1, DEA/NO stimulated increased expression of IL-6 in a concentration-dependent fashion. To ensure the response

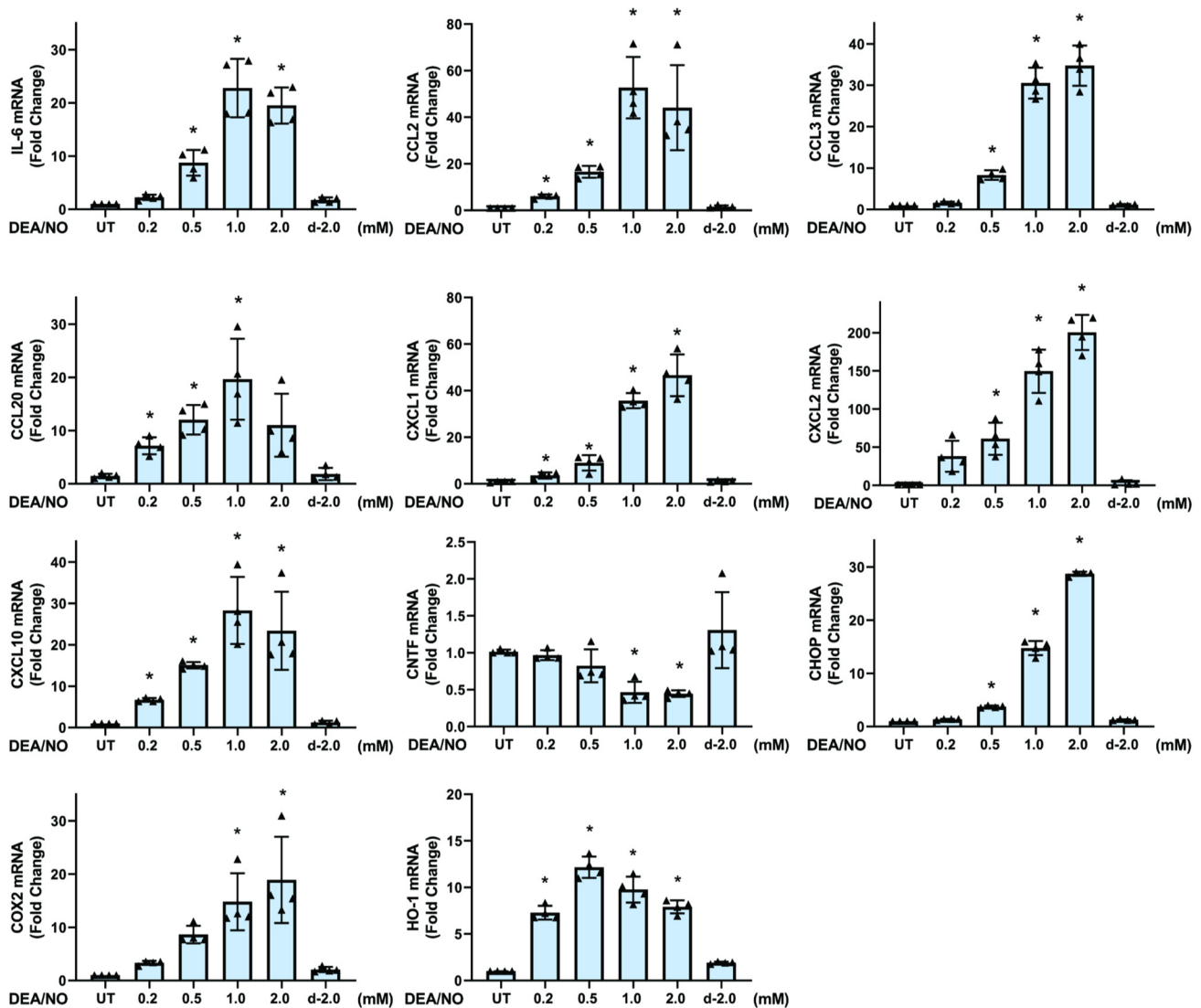


Figure 1. NO drives expression of inflammatory and stress-responsive genes in astrocytes. Primary murine astrocytes were treated with the NO donor DEA/NO at the indicated concentrations for 4 h. Gene expression was then measured by qPCR. The decomposed (d-) donor was also used as a control. UT indicates the untreated control. Data are the means \pm standard deviation, $N=4$ independent experiments. * $p \leq .05$ by one-way ANOVA with Dunnett's multiple comparisons test.

Abbreviations: NO = nitric oxide; DEA/NO = diethylamine NONOate; qPCR = quantitative polymerase chain reaction; ANOVA = analysis of variance.

was due to liberated NO, we also treated astrocytes with the decomposed (d-) donor which had no effect on the genes measured. Treatment of astrocyte with DEA/NO also led to an increase in several chemokines including CCL2, CCL3, CCL20, CXCL1, CXCL2, and CXCL10 (Figure 1). In contrast to IL-6, exposure to NO led to diminished expression of ciliary neurotrophic factor (CNTF), which is similar to that observed in astrocytes under ER stress (Sanchez et al., 2019). Consistent with a stress response, there was increased expression of cyclooxygenase 2, heme oxygenase, and CCAAT/enhancer binding protein homologous protein (CHOP). Collectively, these data indicate that NO drives an inflammatory stress response in astrocytes and may also reduce trophic support.

To further confirm the inflammatory response, astrocytes were treated with an alternative NO donor, PAPA/NO. As shown in Figure 2A, astrocytes exposed to 1 mM PAPA/NO had increased expression of IL-6, CXCL1, and CXCL10. These genes were not altered by the decomposed donor (d-). A similar response was also observed in primary microglia. As shown in Figure 2B, microglia exposed to DEA/NO had increased expression of IL-6, IL-1 β , and CHOP. These data suggest that the inflammatory stress response provoked by high levels of NO also occurs in microglia.

ER stress drives a PERK-dependent inflammatory response in astrocytes (Guthrie et al., 2016; Meares et al., 2014; Sanchez et al., 2019; Sims & Meares, 2019). Having observed that NO induces an inflammatory response concomitant with

increased expression of the ER stress-induced gene CHOP, we hypothesized that NO-induced inflammatory gene expression would be PERK dependent. To test this, we isolated astrocytes from PERK floxed (fl/fl) mice without or with tamoxifen-inducible CAGG-CreERTM. Exposure to 4-OH tamoxifen effectively deletes PERK (Figure 3A). ER stress-induced PERK activation leads to phosphorylation of eIF2 α (Harding et al., 1999). As shown in Figure 3A, DEA/NO rapidly induces phosphorylation of eIF2 α to a similar level as the ER stress inducer, thapsigargin. Interestingly, NO-induced phosphorylation of eIF2 α was unaffected by PERK deletion. Although thapsigargin-induced eIF2 α phosphorylation was reduced, as expected (Figure 3A, thapsigargin treated samples, lanes 6 and 12 indicated by \wedge). NO-induced phosphorylation of the stress-inducible c-jun N-terminal kinase (JNK) was also unaffected by PERK deletion. Additionally, PERK knockout did not affect DEA/NO-induced IL-6, CCL2, CCL20, or CXCL1 but did significantly reduce these genes under ER stress conditions (Figure 3B). CXCL10 was modestly but significantly reduced by PERK deletion. CHOP and ATF4 were increased in response to DEA/NO and thaps. PERK deletion had no effect on DEA/NO-induced ATF4 or CHOP but did abrogate the expression of these genes in response to ER stress (Figure 3B). Together, these data indicate that PERK is not activated or driving inflammatory gene expression in response to NO in primary astrocytes. ER stress-induced inflammatory gene expression requires

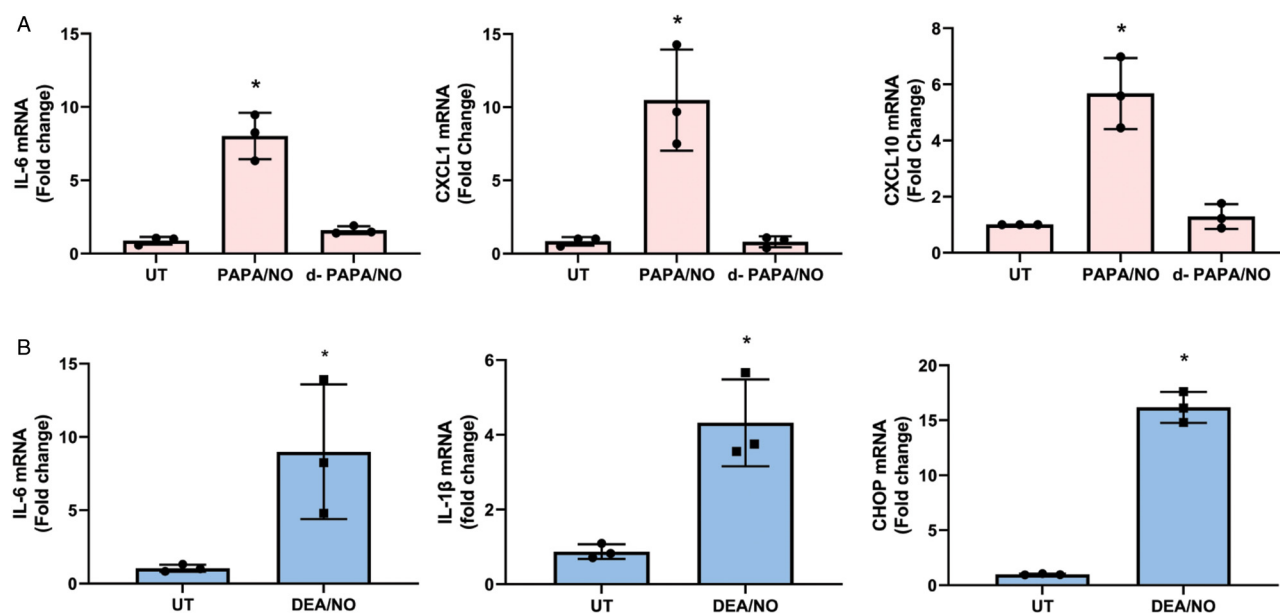


Figure 2. Astrocytes and microglia respond to NO with increased inflammatory gene expression. (A) Primary murine astrocytes were treated with the NO donor PAPA/NO (1 mM) or the decomposed donor (d-) for 4 h. Gene expression was then quantified by qPCR. Data are the means \pm standard deviation, $N = 3$ independent experiments. * $p \leq .05$ by one-way ANOVA with Dunnett's multiple comparisons test. (B) Primary microglia were treated with DEA/NO (1 mM) for 4 h followed by gene expression analysis. Data are the means \pm standard deviation, $N = 3$ independent experiments. * $p \leq .05$ by Student's t -test. Abbreviations: NO = nitric oxide; PAPA/NO = propylamine NONOate; DEA/NO = diethylamine NONOate; qPCR = quantitative polymerase chain reaction; ANOVA = analysis of variance.

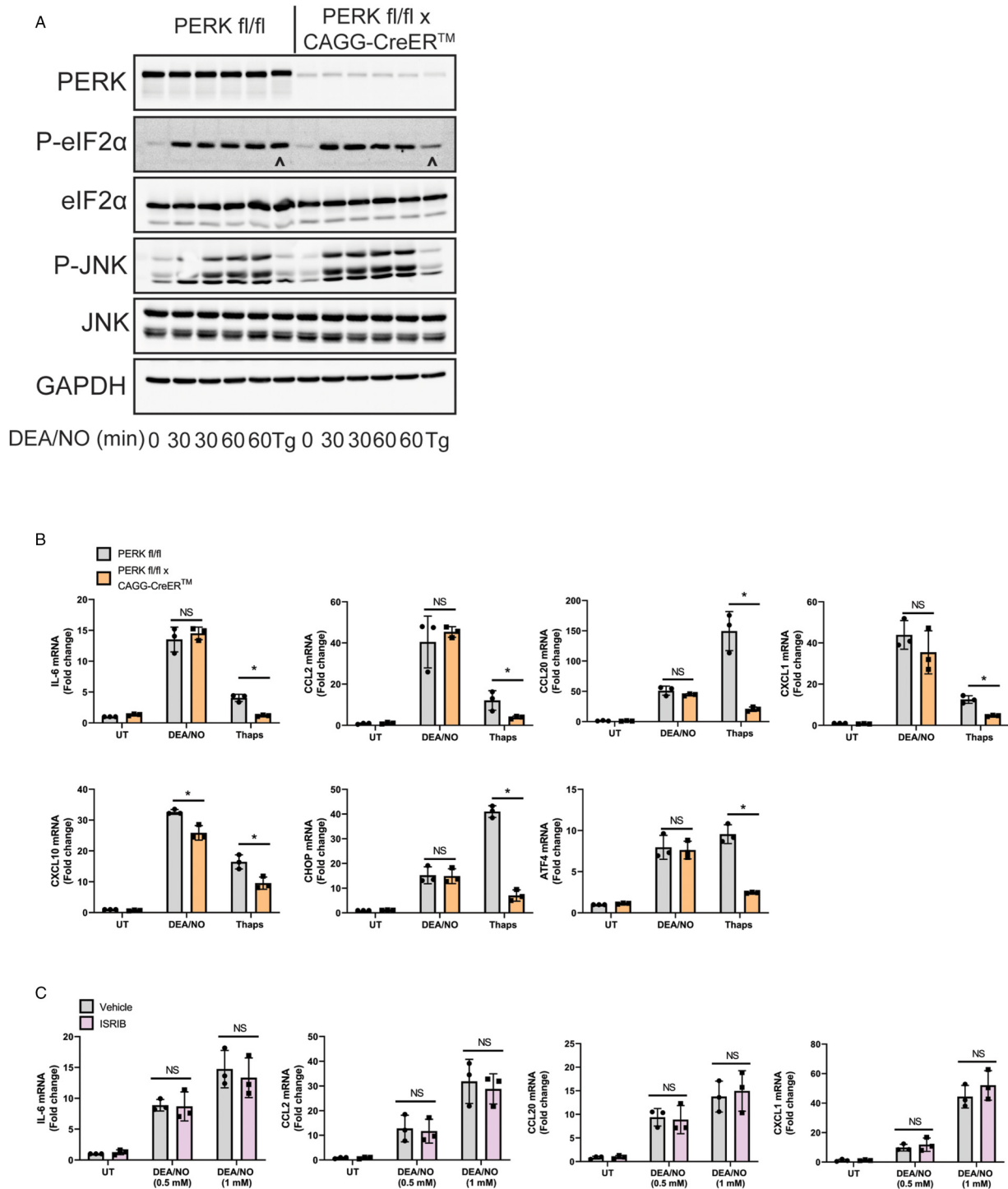


Figure 3. The astrocyte response to NO is independent of PERK and translational repression. (A) Astrocytes from PERK floxed (fl/fl) mice without or with the tamoxifen-inducible CAGG-CreERTM were isolated and cultured. The cells were treated with 4-OH tamoxifen (1 μ M) for 48 h to delete PERK. A total of 48 h after removing tamoxifen from the media, the astrocytes were treated with DEA/NO (1 mM) for 30 or 60 min or thapsigargin (Tg, 1 μ M) for 60 min. Cell lysates were then immunoblotted. (B) PERK was deleted from astrocytes as in Figure 3A, then treated with DEA/NO (1 mM) or thapsigargin (thaps, 1 μ M) for 4 h. Gene expression was measured by qPCR. (C) Astrocytes were treated with ISRIB (0.5 μ M) for 30 min followed by the addition of DEA/NO at the indicated concentrations for 4 h. Gene expression was measured by qPCR. Data are the means \pm standard deviation, $N=3$ independent experiments. * $p \leq .05$ by two-way ANOVA with Bonferroni's multiple comparisons test.

Abbreviations: NO = nitric oxide; PERK = protein kinase R-like ER kinase; ISRIB = integrated stress response inhibitor; DEA/NO = diethylamine NONOate; qPCR = quantitative polymerase chain reaction; ANOVA = analysis of variance.

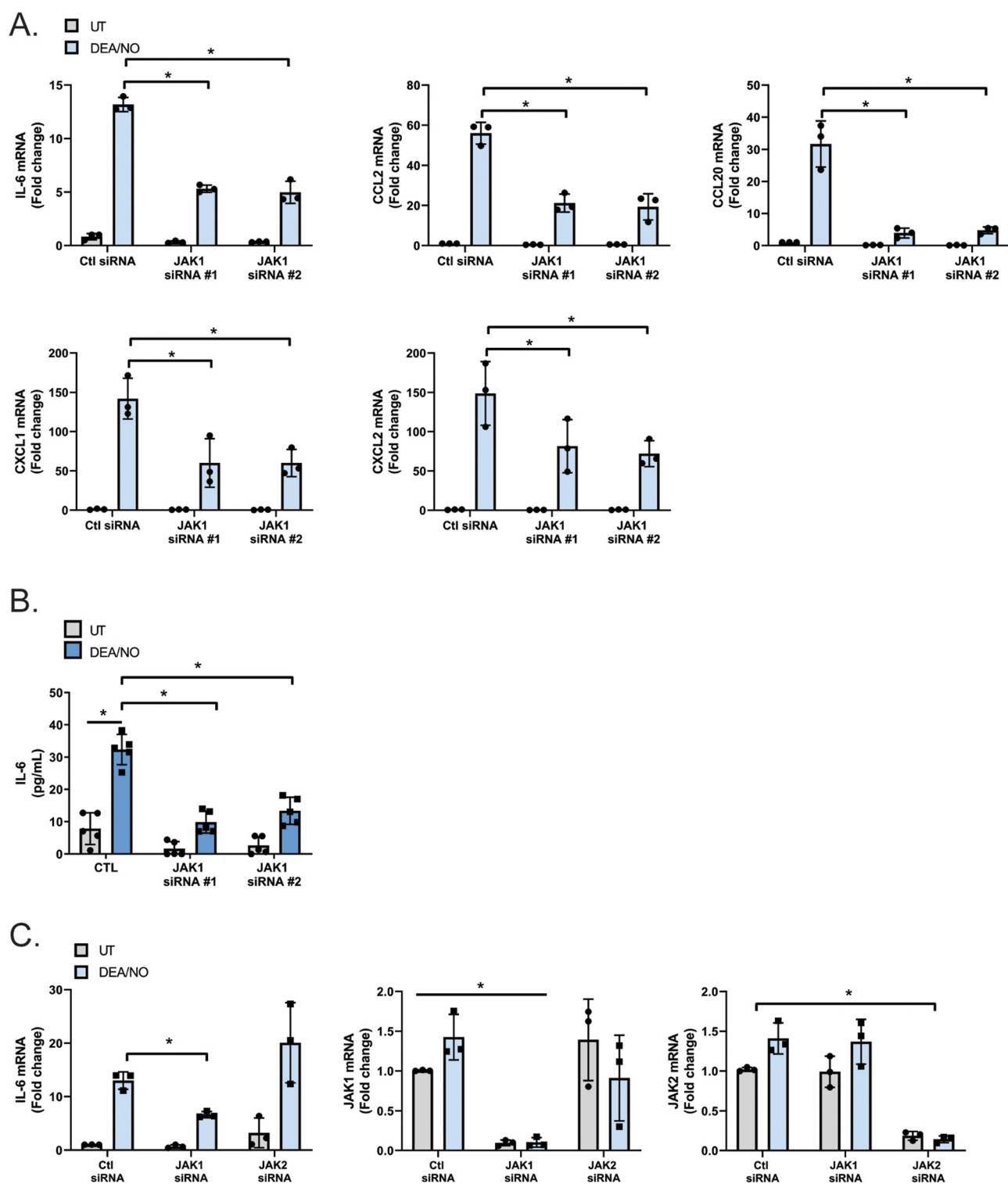


Figure 4. JAK1 is required for the NO-induced inflammatory response. (A) Astrocytes were transfected with control (Ctl) siRNA or two different siRNA targeting JAK1. The cells were then treated DEA/NO (1 mM) for 4 h followed by analysis of gene expression. (B) JAK1 was knocked down as in Figure 4A. Cells were treated with DEA/NO (1 mM) for 24 h and the cell culture media was collected to measure IL-6 by ELISA. (C) Astrocytes were transfected with Ctl siRNA, JAK1 (#2), or JAK2 siRNA. Cells were treated with DEA/NO (1 mM) for 4 h followed by qPCR. Data are the means \pm standard deviation, $N = 3-4$ independent experiments. * $p \leq .05$ by two-way ANOVA with Bonferroni's multiple comparisons test.

Abbreviations: JAK1 = Janus kinase-1; JAK2 = Janus kinase-2; NO = nitric oxide; siRNA = small interfering RNA; ELISA = enzyme-linked immunosorbent assay; DEA/NO = diethylamine NONOate; qPCR = quantitative polymerase chain reaction; ANOVA = analysis of variance.

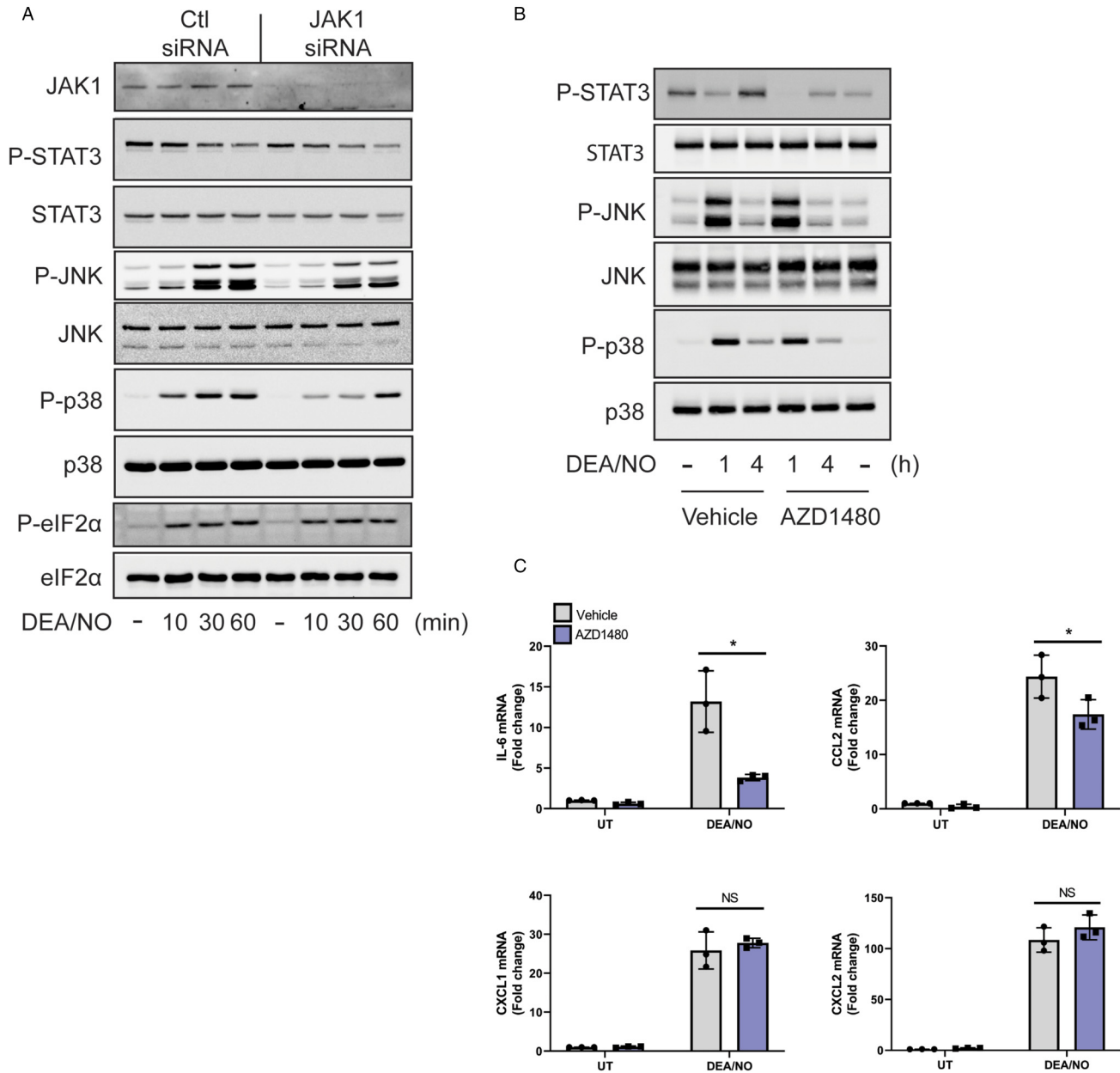


Figure 5. NO promotes delayed activation of JAK1/STAT3 signaling that is required for IL-6 expression. (A) Astrocytes were transfected with control (Ctl) siRNA or siRNA targeting JAK1 (#2). Cells were treated with DEA/NO (1 mM) for the indicated times followed by immunoblotting. (B) Astrocytes were treated with the JAK1/2 inhibitor AZD1480 (1 μ M) for 30 min followed by treatment with DEA/NO for 1 or 4 h followed by immunoblotting. (C) Astrocytes were treated as in Figure 5B (DEA/NO 4 h), and gene expression was measured by qPCR.

Abbreviations: JAK1 = Janus kinase-1; STAT3 = signal transducers and activators of transcription 3; IL-6 = interleukin-6; JAK2 = Janus kinase-2; NO = nitric oxide; siRNA = small interfering RNA; DEA/NO = diethylamine NONOate; qPCR = quantitative polymerase chain reaction.

translational suppression mediated by phosphorylation of eIF2 α (Guthrie et al., 2016). Therefore, we tested if reversing the effects of eIF2 α phosphorylation could similarly attenuate NO-induced inflammatory gene expression. Astrocytes were pretreated with the eIF2B agonist, ISRIB, to preserve protein translation (Sidrauski et al., 2013). As shown in Figure 3C,

ISRIB did not reduce DEA/NO-induced inflammatory gene expression. These data indicate that P-eIF2 α mediated translational repression is not critical to NO-induced inflammatory gene expression in astrocytes.

We have recently established JAK1 as a key molecule linking astrocyte ER stress responses to inflammatory gene

expression. Here, we used siRNA to test the involvement of JAK1 in response to NO. As shown in Figure 4A, two different siRNAs were used to knockdown JAK1, which significantly reduced DEA/NO-induced IL-6, CCL2, CCL20, CXCL1, and CXCL2. We measured IL-6 by ELISA to confirm these effects at the protein level (Figure 4B). Additionally, we found that the effect on IL-6 was JAK1 specific as knockdown of JAK2 had no effect on IL-6 expression (Figure 4C).

Interestingly, DEA/NO stimulation did not increase but rather reduced the phosphorylation of the JAK1 substrate signal transducers and activators of transcription 3 (STAT3) at early time points, suggesting JAK1 activity is not increased. These data are consistent with previous observations that NO inhibits JAK activity (Duhé et al., 1998). However, JAK1 knockdown did substantially reduce the DEA/NO-induced phosphorylation of JNK and p38 at 30 and 60 min. The DEA/NO-induced phosphorylation of eIF2 α was unaffected by JAK1 knockdown, indicating similar exposure to the stressor (Figure 5A). By 4 h following NO exposure, P-STAT3 was modestly increased suggesting delayed activation of JAK1. Consistent with this, the JAK1/2 inhibitor, AZD1480, blocked STAT3 phosphorylation. In contrast to the effects of JAK1 knockdown, AZD1480 had no effect on DEA/NO-induced phosphorylation of JNK or p38 (Figure 5B). AZD1480 blocked DEA/NO-induced IL-6 expression and modestly attenuated CCL2 induction. While JAK1 knockdown reduced DEA/NO-induced CXCL1 and CXCL2 expression by ~50% (Figure 4A), JAK inhibition had no effect on the induction of these chemokines (Figure 5C). Collectively, these data indicate the JAK1 is important for the proinflammatory response to NO in astrocytes.

To examine how this astrocyte-driven inflammatory response could impact other CNS cells, we exposed the BV2 murine microglial cell line to astrocyte conditioned medium (ACM). JAK1 was knocked down in astrocytes using siRNA followed by stimulation without or with DEA/NO for 24 h.

Importantly, by 24 h DEA/NO is decomposed and no longer stimulates inflammatory gene expression (Figure 1). The media, along with any astrocyte-produced molecules, was then transferred to BV2 cells for 6 h. As shown in Figure 6, ACM from DEA/NO treated astrocytes promoted IL-6 expression in the BV2 cells. IL-6 expression was reduced when JAK1 was knocked down in astrocytes. iNOS was also elevated by DEA/NO ACM and reduced by JAK1 knockdown in astrocytes. These data indicate that NO stimulates JAK1-dependent expression of inflammatory molecules that promote IL-6 and iNOS expression in microglia.

Discussion

The immune system, through the actions of iNOS, produces high concentrations of NO as an innate defense against pathogens. With this, comes the potential risk of tissue damage to the host. The production of NO has been observed in the CNS under a myriad of conditions (Calabrese et al., 2007). However, the glial-specific responses to NO have not been fully examined. As summarized in Figure 7, we have identified that NO drives an inflammatory response in astrocytes and microglia. Here, we have found that JAK1 mediates an inflammatory gene expression program in primary cultures of murine astrocytes in response to pathophysiological levels of NO. We observed robust activation of the ISR that included increased P-eIF2 α and expression of ATF4 and CHOP. The phosphorylation of eIF2 α and induction of inflammatory genes were independent of the ER stress-inducible kinase, PERK. This disproved our hypothesis that NO would activate PERK to drive inflammatory gene expression. We speculate that NO-induced eIF2 α phosphorylation is from direct activation of HRI, as previously shown (Tong et al., 2011), however additional studies are needed to directly test this in astrocytes and its potential role in inflammation. ER stress can stimulate PERK and JAK1-dependent expression of inflammatory

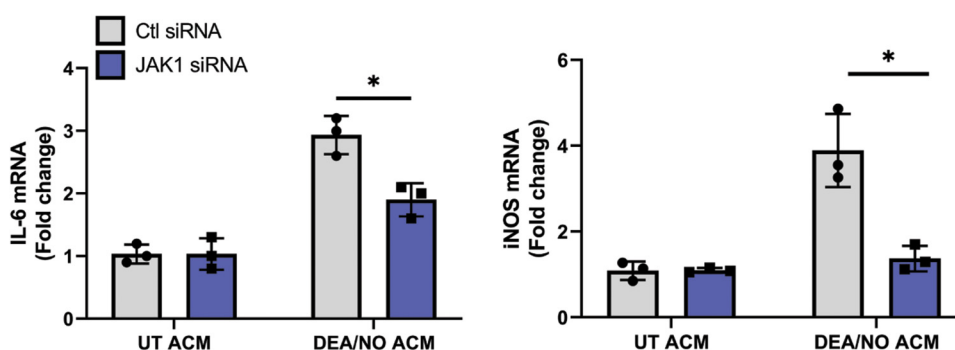


Figure 6. Astrocytes exposed to NO activate microglia. Astrocytes were transfected with control (Ctl) siRNA or siRNA targeting JAK1 (#2). Cells were treated without or with DEA/NO (1 mM) 24 h. The ACM was then collected and transfer to BV2 cells. The BV2 cells were incubated with ACM for 6 h followed by gene expression analysis by qPCR. Data are the means \pm standard deviation, N = 3 independent experiments. * $p \leq .05$ by two-way ANOVA with Bonferroni's multiple comparisons test.

Abbreviations: JAK1 = Janus kinase-1; NO = nitric oxide; siRNA = small interfering RNA; DEA/NO = diethylamine NONOate; ACM = astrocyte conditioned media; qPCR = quantitative polymerase chain reaction; ANOVA = analysis of variance.

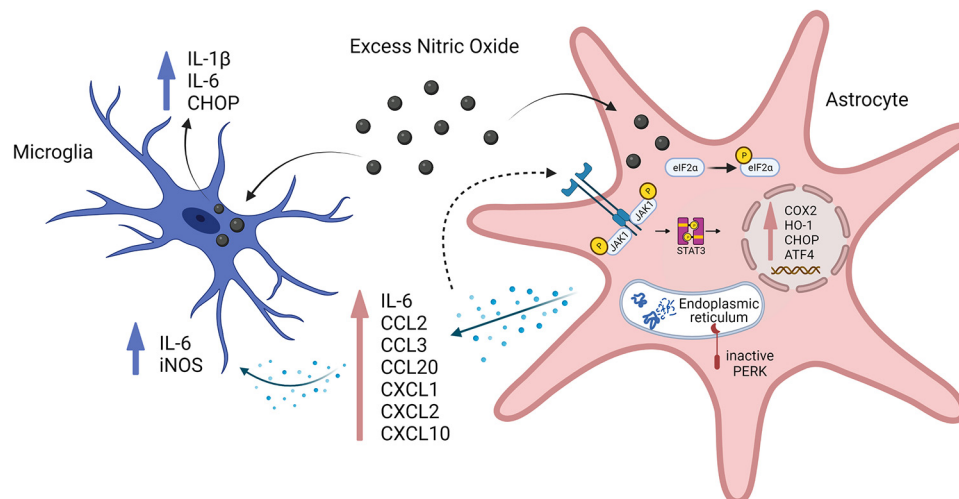


Figure 7. Summary of NO-mediated effects on astrocytes and microglia. The data suggest that NO stimulates an inflammatory reaction in astrocytes that is, in part, dependent on JAK1 and independent of PERK. Similarly, NO promotes the expression of stress-related and inflammatory genes in microglia. The astrocyte-derived inflammatory molecules also increase IL-6 and iNOS expression in microglia and may drive autocrine activation (dotted arrow) of canonical JAK/STAT signaling in astrocytes. Created with BioRender.com. Abbreviations: JAK1 = Janus kinase-1; NO = nitric oxide; PERK = protein kinase R-like ER kinase; IL-6 = interleukin-6; iNOS = inducible nitric oxide synthase; STAT=STAT3 = signal transducers and activators of transcription.

and stress-inducible genes (Meares et al., 2014; Sims & Meares, 2019). The present work reveals that JAK1 can also drive inflammatory responses to cell stressors independently of PERK.

Previously, ER stress has been shown to stimulate a reactive state in astrocytes that relies on PERK and includes induction of inflammatory transcripts and failure to support neuronal synaptogenesis (Smith et al., 2020). We observed that NO similarly induces a reactive state in astrocytes that includes the induction of IL-6 and many chemokines. While we did not examine the impact of NO-stressed astrocytes on neurons, we did find a reduction in CNTF expression suggesting reduced trophic support. Collectively, our data suggest that astrocytes may mount an inflammatory response to stressful stimuli that promote the ISR. The data shown in Figure 3, indicate the inflammatory response to NO is not dependent on translational repression, but does not rule out the involvement of the upstream eIF2 α kinases. ER stress-induced inflammatory gene expression is suppressed by ISRIB (Guthrie et al., 2016), whereas NO-induced IL-6, CCL2, and CCL20 in the present study were not. Additionally, CXCL1 was PERK-dependent and JAK1-independent in response to ER stress (Sims & Meares, 2019). In this study, we found that PERK is not active and that CXCL1 is JAK1-dependent in response to NO. This suggests different mechanisms by which ER stress and NO induce these inflammatory genes and establish an inflammatory astrocyte state.

Our results showing that NO does not activate PERK in astrocytes along with previous work in which NO activates PERK in neuroblastoma cells indicate cell type-specific responses (Nakato et al., 2015). Indeed, the same NO donor

used in the current study, DEA/NO, stimulates the activation associated phosphorylation of PERK in a pancreatic β -cell line (Meares et al., 2011). This shows that the NO-induced signaling identified in one cell type is not necessarily broadly applicable, and there is a need to evaluate responses based on cell type. However, studies of purified primary cells are challenging. In this study, we used enriched (~90%) astrocytes and therefore cannot unequivocally rule out the possibility that contaminating microglia and oligodendrocyte precursor cells are also contributing to the observed responses.

Recent work has shown that cancer cells, in response to nutrient deprivation, activate the UPR and ISR to drive inflammatory cytokines and chemokine production. Further, the study provides evidence that the cancer cells are mounting a response that is similar to wound-healing, including the production of IL-6, CXCL1, and CXCL2 (Püschel et al., 2020). Similar to nutrient-starved cancer cells, we also observed activation of the ISR and increased production of IL-6, CXCL1, and CXCL2. It is likely that the astrocyte response is intended to promote tissue repair and restoration of homeostasis. The NO donors used in our study have a relatively short half-life; ~2 min for DEA/NO and 15 min for PAPA/NO (Keefer et al., 1996). We anticipate that the responses occur after much of the NO has dissipated, and represents a reparative and adaptive process to the NO-induced stress. NO is beneficial in wound repair, in part through modulation of inflammation (Luo & Chen, 2005). There may be similar effects in the CNS mediated by astrocytes in a JAK1-dependent fashion, although additional studies are needed to directly test this.

Following neural injury or during disease multiple chemokines are increased in the CNS and may contribute to

pathology (Iadecola & Anrather, 2011; Karpus, 2020). For example, CCL2 is essential for leukocyte infiltration and disease progression in the MS mouse model of experimental autoimmune encephalomyelitis (Fife et al., 2000); in which astrocyte-specific production contributes to the chronic phase of disease (Kim et al., 2014). Additionally, CXCL1 and CXCL2 have been shown to drive immune cell infiltration and worsen disease during EAE (Rumble et al., 2015; Stoolman et al., 2018). Our observation that several chemokines were expressed in response to NO may point to a multicellular feedforward mechanism. CCL2, CXCL1, CXCL2, and CXCL10 are chemoattractants for monocytes and neutrophils (Griffith et al., 2014). As such, local production of NO in the CNS could promote the recruitment of monocytes and neutrophils, which also produce NO that could further expression of inflammatory cytokines and chemokines in astrocytes.

The delayed phosphorylation of STAT3 suggests a potential autocrine mechanism in which a JAK/STAT-activating cytokine is produced, potentially IL-6 itself, and contributes to IL-6 expression. In contrast to this canonical JAK/STAT activation, the chemokines CXCL1 and CXCL2 were JAK1-dependent but completely insensitive to the JAK1/2 inhibitor AZD1480. This is similar to the observation that ER stress induces the ATF4 target genes growth arrest and DNA-damage-inducible protein 45 (GADD45) and tribbles homolog 3 (TRIB3) through a JAK1-dependent mechanism that is insensitive to JAK1/2 inhibition (Sims & Meares, 2019). Further, we observed that the activating phosphorylation of the mitogen-activated protein kinases (MAPKs) p38 and JNK was JAK1-dependent and inhibitor insensitive. JAK signaling in the context of cytokine and growth factor signaling is well known to activate and integrate with the MAPK pathways (Bousoik & Montazeri Aliabadi, 2018; Winston & Hunter, 1996). Our data indicate that JAK1 also plays a role in MAPK activation in response to NO. The differential regulation of MAPKs by JAK1 knockdown or inhibition may also contribute to the expression of inflammatory genes. Mechanistically, it is possible that knockdown of JAK1, which occurs over 48–72 h, also down-regulates the JAK1-dependent basal expression of proteins that are important for MAPK signaling. This may not occur over the short duration (e.g. < 5 h) of JAK inhibition with AZD1480. However, the underlying mechanism(s) remains to be determined. Overall, our data provide further evidence that JAK1 is a critical protein in the inflammatory astrocyte response to cell stress.

Vertebrate animal use

The study was approved by the animal care and use committee at West Virginia University.

Author Contributions

J.D.N. conducted most of the experiments and edited the manuscript. R.N.C. conducted experiments and edited the manuscript. J.W.F.

conducted experiments and edited the manuscript G.P.M. directed the study and wrote the manuscript.



Declaration of Conflicting Interests

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