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J Immunol 2011; 187:4788-4799; Prepublished online 30 September 2011; doi: 10.4049/jimmunol.1004111

http://www.jimmunol.org/content/187/9/4788

Supplementary Material http://www.jimmunol.org/content/suppl/2011/09/30/jimmunol.1004111.1.DC1

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Neuroinflammation and Endoplasmic Reticulum Stress Are Coregulated by Crocin To Prevent Demyelination and Neurodegeneration

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Endoplasmic reticulum (ER) stress is a homeostatic mechanism, which is used by cells to adapt to intercellular and intracellular changes. Moreover, ER stress is closely linked to inflammatory pathways. We hypothesized that ER stress is an integral component of neuroinflammation and contributes to the development of neurological diseases. In autopsied brain specimens from multiple sclerosis (MS) and non-MS patients, XBP-1 spliced variant (XBP-1/s) was increased in MS brains (p < 0.05) and was correlated with the expression of the human endogenous retrovirus-W envelope transcript, which encodes the glycoprotein, Syncytin-1 (p < 0.05). In primary human fetal astrocytes transfected with a Syncytin-1–expressing plasmid, which was suppressed by crocin treatment (p < 0.05). Crocin also protected oligodendrocytes exposed to cytotoxic supernatants derived from Syncytin-1–expressing astrocytes (p < 0.05) and NO-mediated oligodendrocytotoxicity (p < 0.05). During experimental autoimmune encephalomyelitis (EAE), the transcript levels of the ER stress genes XBP-1/s, BiP, PERK, and CHOP were increased in diseased spinal cords compared with healthy littermates (p < 0.05), although CHOP expression was not involved in the EAE disease phenotype. Daily treatment with crocin starting on day 7 post-EAE induction suppressed ER stress and inflammatory gene expression in spinal cords (p < 0.05), which was accompanied by preserved myelination and axonal density, together with reduced T cell infiltration and macrophage activation. EAE-associated neurobehavioral deficits were also ameliorated by crocin treatment (p < 0.05). These findings underscored the convergent roles of pathogenic ER stress and immune pathways in neuroinflammatory disease and point to potential therapeutic applications for crocin. The Journal of Immunology, 2011, 187: 4788–4799.

The unfolded protein response is an evolutionarily conserved cellular mechanism by which misfolded proteins are modulated and eventually discarded from cells (1, 2). On accumulation of misfolded proteins within a cell, multistep pathways are activated such that unwanted proteins are eliminated by several pathways, termed endoplasmic reticulum (ER) stress, which is accompanied by translational repression (3, 4). Aggre-

gation of misfolded or unfolded proteins has been implicated in several neurological diseases including Alzheimer’s and Parkinson’s diseases, often resulting in cellular death or activation of innate immune pathways (2). There is now substantial evidence to indicate that ER stress is linked to inflammation through several intersecting cellular pathways, particularly reactive oxygen species (ROS) and proinflammatory cytokines (5, 6). Protein folding is an energy-demanding process that occurs in oxidizing conditions (5). Although most neurodegenerative diseases involve inflammation, ER stress is also a critical mechanism that can regulate inflammation (7).

Several studies of animal retroviruses have reported the induction of inflammation and ER stress-related genes in the brain (8–12). Similarly, glycoproteins encoded by other viruses, such as hepatitis C virus, are also capable of inducing pathogenic ER stress in other tissues (13–15). There has been an increasing appreciation of the different ER stress markers, reflecting parallel pathways that contribute to the eventual cellular outcomes (apoptosis and inflammation). For example, the spliced XBP-1 transcript derived from inositol-requiring enzyme-1 induction represents a pivotal marker and effector of ER stress with the resulting activation of NF-κB and ensuing production of inflammatory molecules (16, 17). Other indicators of ER stress include the early sensor of ER stress, BiP, and CHOP/GADD153, which mediates apoptosis (18). Although these latter ER stress genes are recognized in CNS diseases, the spliced XBP-1 variant (XBP-1/s) has not been well studied in the CNS.

Multiple sclerosis (MS) is the prototypic inflammatory neurodegenerative disease of the CNS, defined by inflammatory demyel-
lation and ensuing neurodegeneration (19, 20). Several exogenous and endogenous infectious agents have been implicated in MS pathogenesis including human herpes viruses, human coronaviruses, and human endogenous retroviruses (HERVs) (21). Among the HERVs, the MS retrovirus, HERV-H, and the HERV-W env-encoded glycoprotein, Syncytin-1, have been reported to participate in MS neuropathogenesis (22–26). Syncytin-1, encoded by the HERV-W env gene, ERVWE1, on chromosome 7q21.2 is expressed in the placenta, and its role in health is likely to mediate syncytial fusion of the villous trophoblast (27). Previous studies also indicate that Syncytin-1 also contributes to neuroinflammation and oligodendrocyte death (23, 28). Syncytin-1 was found to be more highly expressed in the glial cells of MS lesions, specifically in astrocytes and microglia (28). Moreover, Syncytin-1 has been shown to induce ER stress in astrocytes (23), in keeping with the observations from other groups suggesting ER stress is involved in MS-related disease mechanisms (29–31).

Given the above circumstances, the present working hypothesis was that inflammation and ER stress are closely coupled and contribute to inflammatory demyelination and neurodegeneration in MS, which might be modulated by the free radical scavenger crocin. Overexpression of Syncytin-1 in astrocytes confirmed a role for Syncytin-1 in glia activation and cytotoxicity, which was inhibited by crocin. These findings were complemented by evidence of ER stress and inflammation in the MS model, experimental autoimmune encephalomyelitis (EAE), which was also abrogated by crocin treatment.

### Materials and Methods

#### Human brain tissue samples

Frontal brain white matter was collected at autopsy with consent from age- and sex-matched MS patients (total: n = 8; median age: 63 y; chronic progressive MS: n = 3; secondary progressive MS: n = 5) and non-MS (control) patients (total: n = 7; median age: 58 y; sepsis: n = 2; myocardial infarction: n = 2; HIV/AIDS: n = 1; cancer/leukemia: n = 2) for RT-PCR analyses, as previously reported (32–34). Normal-appearing white matter (NAWM) was used from all clinical samples. These studies were approved by the University of Alberta Ethics Committee.

#### Animals

C57BL/6 female wild type and male CHOP−/− mice were purchased from The Jackson Laboratory and maintained in the Health Sciences Laboratory Animal Services facility of the University of Alberta under conventional housing conditions. CHOP+/+ (wild type), CHOP+/−, and CHOP−/− female littermates were generated for EAE studies. All experiments were approved by the University of Alberta Animal Care Committee.

#### Cell proliferation assay

Splenocytes were isolated from C57BL/6 mice and the RBCs were lysed with Ack lysing buffer (Lonza Walkersville). Cells were stained with Cell Trace CFSE cell proliferation kit (C34554; Invitrogen), according to manufacturer’s protocol. The splenocytes were seeded at 2 × 10⁵ cells/well in a 96-well round-bottom plate that was precoated with anti-CD3ε.

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**FIGURE 1.** ERVWE1 and XBP-1/s are induced during MS. A, Heat map of immune gene transcript levels in MS and non-MS patients. B, XBP-1/s and ERVWE1 transcript levels in the brains of MS and non-MS patients presented as mean ± SEM (*p < 0.05). C, Spearman correlation of the transcript levels of XBP-1/s and ERVWE1 Syncytin-1 immunoreactivity in frontal cortex of (D) NAWM and (E) MS lesion. Ei, Syncytin-1+ astrocyte (GFAP, blue; Syncytin-1, brown). Arrow points to Syncytin-1+ glia. *p < 0.05, Mann–Whitney U test. Original magnification ×400 (D, E), ×600 (Ei).
Ab (16-0031-86; eBioscience) at 10 μg/ml with or without 100 μM crocin (17304; Sigma) (34). For myelin oligodendrocyte glycoprotein (MOG)-specific proliferation assay, splenocytes were isolated from EAE-induced mice at peak of disease and were cultured in the presence of 20 μg/ml MOG35–55 peptide (prepared by the Peptide Synthesis Facility, University of Calgary) with or without 100 μM crocin. The final well volume (250 μl) consisted of enriched DMEM (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, and 1% nonessential amino acids).

Flow cytometry

The surface phenotype was determined by surface staining with PerCP anti-mouse CD3ε mAb (100326; BioLegend) at the manufacturer’s recommended concentrations. After 3-d stimulation, the splenocytes were harvested, stained, and washed twice with PBS plus 2% FBS. The immunolabeled cells were analyzed on BD FACSCanto flow cytometer using FACSDiva software for acquisition. Anti-CD3ε Ab was used with compbeads (552845; BD Biosciences) for single-color compensation controls. The total percentage (%) of the CD3ε+ T lymphocyte proliferation population was analyzed using FlowJo (Tree Star software) (34).

Cell cultures

Mature oligodendrocytes were isolated from adult Sprague–Dawley rat brains and plated in polyornithine-coated chamber slides (Nunc). Cells were maintained in MEM containing 10% FBS, 0.1% dextrose, 2 mM L-glutamine, and 1 mM sodium pyruvate, 1% penicillin, 1% streptomycin, 8.7 ng/ml gentamicin, and 909 pg/ml Fungizone for 5 d, allowing oligodendrocytes to differentiate as evidenced by process extension (28). Human neural cell cultures were prepared from 15–19 wk fetal brains obtained with consent (approved by the University of Alberta Ethics Committee), as previously described (35). In brief, fetal brain tissues were dissected, meninges were removed, and a single-cell suspension was prepared by titration through serological pipettes, followed by digestion for 30 min with 0.25% trypsin (Life Technologies, Burlington, ON, Canada) and 0.2 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany), and passage through a 70-μm cell strainer (BD Biosciences, Mississauga, ON).

FIGURE 2. Syncytin-1 induced XBP-1 splicing in human astrocytes and NOS2 production. A, Western blot detection of Syncytin-1 and β-actin immune reactivity in human astrocytes transfected with vector containing ERVWE1 or control vector. B, ERVWE1 and (C) BiP, XBP-1/s, IFN-α, and Mx1 gene transcript levels, 12 and 24 h posttransfection with the ERVWE1-containing plasmid. D, Dual luciferase assay of human astrocytes transfected with ERVWE1 or control vector treated with either PBS or crocin for 16 h, starting 8 h posttransfection. ERVWE1 or control vector was cotransfected with Renilla luciferase and Firefly luciferase promoters. Renilla luciferase is constitutively expressed and was used as an internal control. Firefly luciferase is expressed only when XBP-1 is spliced during ER stress. Firefly luciferase was normalized to Renilla luciferase. Data are presented as mean ± SEM. E, BiP, (F) IFN-α, (G) NOS2, and (H) HIF-1α transcript levels in astrocytes transfected with ERVWE1-containing vector or control vector and treated with varying concentrations of crocin for 24 h. Data are presented as mean ± SEM (*p < 0.05, **p < 0.01).
Ca\(^{2+}\) imaging

Human primary astrocytes were plated in 35-mm Nunclon tissue culture dishes (VWR). On the following day, the cells were transfected with either ERVWE1 or control vector as described earlier in this article. Crocin (200 μM) was added to the cell cultures 6 h after transfection. After 48–72 h following transfection, cells were treated with 5 μM Fluo-4 acetoxyethyl ester (ABD Bioquest, Sunnyvale, CA) for 45 min. Changes in Fluo-4 acetoxyethyl ester fluorescence intensity evoked by glutamate (1 mM, 2 min) were measured using an inverted microscope (IX81 microscope; Olympus, Markham, ON, Canada) and illuminated using a xenon lamp (Lambda DG-4; Sutter Instruments, Novato, CA) with appropriate filters (Ex: 482/35, Em: 536/40). During recording, the cells were perfused with a solution containing 127 mM NaCl, 2.5 mM KCl, 1.2 mM NaH\(_2\)PO\(_4\), 1.3 mM MgSO\(_4\), 26 mM NaHCO\(_3\), 25 mM d-glucose, and 2.5 mM CaCl\(_2\). Images were recorded using a CCD camera (Rolexa-XR F-M-12-C; QImaging, Surrey, BC, Canada) at 3 s/frame. Selected regions of interest were drawn around distinct cells, and traces of time course of changes in Fluorescent (Fluo-8 acetoxymethyl ester fluorescence intensity evoked by glutamate (1 mM, 2 min)) were measured using an inverted microscope (IX81 microscope; Olympus, Markham, ON, Canada). Cells were washed twice with fresh medium and plated in T-75 flasks coated with poly-L-ornithine (Sigma-Aldrich, Oakville, ON, Canada) at 6–8 × 10\(^4\) cells/flask, and medium for growing human fetal astrocytes (HFAs) and microglia cells was subsequently added (named HFN medium), which was MEM supplemented with 10% FBS (Life Technologies), 2 mM l-glutamine (Life Technologies), 1 mM pyruvate (Life Technologies), 1 × nonessential amino acids (Life Technologies), 0.1% dextrose (Sigma-Aldrich), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 0.5 μg/ml amphotericin B (Life Technologies), and 20 μg/ml gentamicin (Life Technologies).


crocin attenuates glutamate-induced calcium signal in Syncytin-1–transduced astrocytes. A, Representative traces showing changes in [Ca\(^{2+}\)]\(_i\) in response to glutamate (1 mM) exposure in cells transfected with control (dotted line) and ERVWE1 [Syncytin-1 encoding] (solid line) vectors. B, Representative traces showing change in [Ca\(^{2+}\)]\(_i\) after glutamate (1 mM) application in cells that were treated with crocin (200 μM) after transfection with control and ERVWE1 vectors. C, Graphic display of relative fluorescence change in ERVWE1-transfected compared with control vectors with or without crocin treatment. Bars represent mean ± SEM (*p < 0.05, Student t-test). a.u., arbitrary units.

**FIGURE 3.**

Calcium imaging

Induction and assessment of EAE

Animals were injected s.c. with 50 μg MOG (MOG\(_{35–55}\) peptide; prepared by the Peptide Synthesis Facility, University of Calgary) emulsified in 100 μl CFA (Sigma-Aldrich) supplemented with 5 mg/ml heat-killed Mycobacterium H37 RA (Difco Laboratories) at 12 wk of age. Animals received i.p. injections of pertussis toxin (0.3 μg; List Biological Laboratories) at the same time as MOG immunization and repeated 48 h later. Control animals were only injected with CFA and pertussis toxin. Animals were assessed daily for EAE severity for 25 d using a 0–14 rating scale (34). EAE mice received crocin (100 mg/kg/d; Sigma-Aldrich) daily and i.p. starting at day 7 post-EAE induction, whereas control EAE animals received daily i.p. PBS (32–34).

**FIGURE 4.**

C roc in prevents oligodendrocyte cytotoxicity induced by supernatants from astrocytes overexpressing Syncytin-1. A, Oligodendrocytes were cultured for 6 d, fixed with 4% formaldehyde, stained with CNPase, and viewed under fluorescent microscopy. Original magnification ×400. Ai, CNPase immunostained oligodendrocytes viewed by fluorescent microscopy at 488-nm wavelength. Aii, CNPase immunostained oligodendrocytes viewed by fluorescent microscopy at 570-nm wavelength. B, Oligodendrocytes were differentiated for 6 d and exposed to supernatants from astrocytes or microglia transfected with the vector expressing Syncytin-1 with or without crocin, fixed with 4% formaldehyde, stained with DAPI, and measured semiquantitatively using a fluorescent plate reader. C, In-cell Western analysis of rat oligodendrocytes treated with astrocyte supernatants from either a plasmid containing ERVWE1 gene encoding Syncytin-1 or control plasmid and treated with either crocin or PBS. Human astrocytes were transfected with either the Syncytin-1–expressing plasmid or control plasmid. Medium was changed 8 h posttransfection, and supernatants were collected 24 h after changing the medium. Astrocyte supernatants were applied to oligodendrocytes cultured in a 96-well plate with increasing concentrations of crocin for 24 h. Cells were fixed and stained for CNPase. Data are presented as mean ± SEM; experiments were performed in triplicate and repeated thrice; data are presented as a percentage of DAPI or immunoreactivity (IR) fluorescence in samples transfected with the control vector for both panels. **p < 0.01, ***p < 0.001, Dunnett’s test.
Western blotting

Cells were lysed with Laemmlli buffer and 0.1% 2-ME. Proteins from whole-cell lysates were separated using PAGE. Protein fractions were transferred to a nitrocellulose membrane overnight using electrophoresis. Membrane was blocked with 10% milk and incubated with a polyclonal rabbit anti–Syncytin-1 overnight (1/100 dilution; Custom Polyclonal Open Biosystems raised against the peptide, TGMSDGGGGVQDQAREKHV) (36, 37). The Syncytin-1–immunolabeled membrane was then probed with anti-rabbit secondary Ab (1:10000) for 2 h. Membrane was developed with HRP and exposed to x-ray on film (Canon).

Histological analysis

Formalin-fixed spinal cords of EAE or control animals were embedded in paraffin before sectioning. Four-micrometer sections from lumbar spinal cords were stained with Bielschowsky’s silver impregnation method (38). White matter from the dorsal columns of four different animals per group was scanned and photographed using a microscope (Axioskop2; Carl Zeiss MicroImaging). In addition, cultured rat oligodendrocytes were stained with DAPI and quantified (39).

Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections of mouse lumbar spinal cord were deparaffinized and rehydrated using decreasing concentrations of ethanol. Ag retrieval was performed by boiling the slides in 0.01 M trisodium citrate buffer, pH 6, for 10 min followed by cooling at room temperature for 2 h. Sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidases for 30 min, followed by preincubation with 10% normal goat serum, 0.2% Triton X-100 for 2 h at room temperature to block nonspecific binding. Rabbit polyclonal anti–Iba-1 (1/500 dilution; Wako), rat polyclonal anti-CD3 (1/100 dilution; Serotec), rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP; 1/500 dilution; DAKO, Carpinteria, CA), mouse monoclonal anti-myelin basic protein (anti-MBP; 1/200 dilution; Sternberger Monoclonals), rabbit polyclonal anti-BiP (1/100 dilution; Santa Cruz), and mouse monoclonal anti–2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase) (1/200 dilution; Millipore), followed by appropriate secondary Abs were used to detect macrophage/microglial, T lymphocyte, astrocyte, myelin, BiP, and oligodendrocyte immunoreactivity, respectively (28, 33, 34, 40).

Immunostaining of MS brains

Formalin-fixed, paraffin-embedded sections from MS patients were deparaffinized and rehydrated using decreasing concentrations of ethanol. Ag retrieval was performed by boiling the slides in 0.01 M trisodium citrate buffer, pH 6, for 10 min followed by incubation with 0.3% hydrogen peroxide to block endogenous peroxidases. Sections were then preincubated with 10% normal goat serum, 0.2% Triton X-100 overnight at 4°C to block nonspecific binding. Sections were immunostained with polyclonal rabbit anti–Syncytin-1 overnight (1/100 dilution; Custom Polyclonal Open Biosystems raised against the peptide, TGMSDGGGGVQDQAREKHV). Subsequently, sections were stained with biotin-conjugated anti-rabbit secondary IgG Ab (1/500 dilution; Vector Biotechnologies), developed

**FIGURE 5.** Crocin does not affect T cell proliferation but abrogates SNP-induced oligodendrocytotoxicity. A. Splenocytes were isolated from EAE and control mice, and were stimulated with anti-CD3ε with or without crocin (100 nM). T cell proliferation was measured by CFSE. Dark gray area: unstimulated control; light gray: without crocin; white: with 100 μM crocin. B, Percent proliferation of CD3+ lymphocytes stimulated with anti-CD3ε with or without crocin (100 nM). C, MOG-specific percent proliferation of CD3+ lymphocytes. D, Fluorescence microscopy of rat oligodendrocytes stained with CNPase. Oligodendrocytes were isolated from Sprague–Dawley rats and allowed to differentiate for 6 d on polyornithine-coated 96-well plates. Oligodendrocytes were treated with sodium-nitroprusside (SNP), an NO donor, for 24 h to induce cytotoxicity and varying concentrations of crocin. Original magnification ×400. E, In-cell Western analysis probed for CNPase immunoreactivity of rat oligodendrocytes treated with SNP and increasing concentrations of crocin. Data are presented as mean ± SEM (*p < 0.05).
using 3,3’-diaminobenzidine (Vector Biotechnologies) and dehydrated in increasing concentrations of ethanol and, finally, in xylene. Sections were mounted with acryl and viewed under light microscope (Zeiss Axioskop 2).

Infrared imaging

Cultured oligodendrocytes derived from adult rat brains were immunolabeled with mouse monoclonal anti-CNPase (1:200; Millipore) Ab, which recognized mature oligodendrocytes, followed by a Cy3-conjugated goat anti-mouse Ab. In addition, oligodendrocyte cultures were colabeled with DAPI. Slides were imaged by infrared imaging (LICOR Biosciences) (28).

Real-time RT-PCR

Mouse or human tissues or cultured cells were homogenized in TRIzol (Invitrogen), and total RNA was purified from the aqueous phase using RNeasy mini columns (Qiagen, Germantown, MD), as per manufacturers’ protocol. After treatment of RNA with DNase I (Promega), cDNA was prepared by anchored oligo(dT) (dT18)VN-primed reverse transcription of equal quantities of RNA using either Superscript II or Superscript III reverse transcriptase (Invitrogen), according to the manufacturer’s recommended protocols. Semi-quantitative real-time PCR was performed using Bio-Rad iQ SYBR green supermix (Bio-Rad) on either an iQ or an iCycler (Bio-Rad), according to the manufacturer’s recommended protocols. All PCR primers used in this article are provided (Supplemental Table I). All data were normalized to GAPDH mRNA levels and expressed as relative fold change compared with controls together with ±SEM (23, 34).

Transfection and dual luciferase assay

HFAs were pretreated with 100, 200, or 400 μM crocin (Sigma) for 5 h before cotransfection with either pCMV-env vector containing the HERV-W envelope gene, ERVWE1, or pCMV-empty (ERVWE1 digested out with EcoRI, referred to as control) and a vector containing an internal control promoter constitutively driving the expression of Renilla luciferase and a promoter encoding firefly luciferase, which is activated only when XBP-1 is spliced. Lipofectamine LTX (Invitrogen) and PLUS reagent (Invitrogen) were used as transfection reagents. Luciferase activity only when XBP-1 is spliced. Lipofectamine LTX (Invitrogen) and PLUS reagent (Invitrogen) were used as transfection reagents. Luciferase activity was assayed with the dual-luciferase kit (Promega) using a luminometer.

Statistical analyses

Statistical tests were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA) for both parametric and non-parametric comparisons. The p values of <0.05 were considered significant.

Results

XBP-1 spliced variant and ERVWE1 are induced in MS white matter

To assess the extent of inflammation within MS white matter specimens, we investigated inflammatory gene expression revealing an upregulation of inflammatory cytokines, compared with non-MS control specimens (Fig. 1A). In particular, MS patients’ brains showed greater Mx1, IFN-α, HLA-DR2, IL12p35, IL23p19, and CD3e expression (Fig. 1A). Because these MS samples demonstrated neuroinflammation, ER stress gene abundance was investigated, disclosing significant XBP-1/s induction in MS tissues together with significant upregulation of the HERV-W env gene, ERVWE1, transcripts (Fig. 1B). XBP-1/s and ERVWE1 transcript abundance in brain were closely correlated within the present cohort of brain samples (Fig. 1C). Moreover, Syncytin-1, the protein product of ERVWE1, was not detectable in NAWM (Fig. 1D) but highly expressed in demyelinating lesions in MS patients (Fig. 1E); Syncytin-1 immunoreactivity was particularly evident in cells resembling activated glia (Fig. 1E, arrows) and showed colocalization with GFAP immunoreactivity (Fig. 1E, inset, arrow). These findings highlighted the inflammatory features of MS white matter, but also extended earlier findings of ER stress in MS brains in conjunction with Syncytin-1 expression (23).

Syncytin-1 overexpression induces the XBP-1 spliced transcript

XBP-1/s is a hallmark of ER stress in different cell types; thus, the impact of HERV-W env (ERVWE1) expression on XBP-1/s levels was further investigated. Transfection of HFAs with an ERVWE1-encoding vector disclosed Syncytin-1 immunoreactivity at the predicted molecular mass (72 kDa; Fig. 2A). Astrocytes transfected with the ERVWE1 containing vector showed increased ERVWE1 transcripts at 12 and 24 h posttransfection (Fig. 2B). In addition, ER stress gene transcripts (BIP and XBP-1/s) and antiviral response genes transcripts (IFN-α and Mx1) were induced at 12 and 24 h, respectively, posttransfection with ERVWE1 vector compared with the control vector (Fig. 2C). Whereas ERVWE1-transfected HFAs exhibited increased XBP-1/s transcript levels, crocin treatment disclosed a concentration-dependent suppression of XBP-1/s levels (Fig. 2D). Thapsigargin, a prototypic ER stress-inducing compound, also induced XBP-1/s levels (Fig. 2D). Transfected astrocytes also exhibited increased BIP (Fig. 2E), IFN-α (Fig. 2F), and NOS2 (Fig. 2G), which were significantly suppressed by crocin treatment. Conversely, ERVWE1 transfection did not induce hypoxia-inducible factor-1α expression in HFAs (Fig. 2F). These results underscored the capacity of Syncytin-1 to induce inflammatory cytokines and antiviral response genes in HFAs, further highlighting the potential role of Syncytin-1 in the pathogenesis of MS.

FIGURE 6. ER stress genes are induced during EAE. A, Lumbar spinal cords were isolated from EAE and control mice at peak disease severity. RNA was isolated from the lumbar spinal cord and a cDNA library was synthesized. RT-PCR using primers designed to amplify XBP-1 spliced and unspliced transcripts was performed. Amplicons were cut with PstI endonuclease. The XBP-1 unspliced transcript variant lacks a PstI restriction site and will not be cleaved during PstI treatment. B, XBP-1 transcript expression on 9 bp bands. Ctrl EAE

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induce specific inflammation and ER stress-related genes, which were suppressed by crocin.

**Syncytin-1 induces cytosolic calcium in astrocytes**

Astrocytes display excitability based on intracellular calcium elevation, which may be present spontaneously or can be evoked and modulated by neurotransmitters (43). Intracellular calcium \([\text{Ca}^{2+}]\) levels were investigated in presence or absence of ERVWE1 overexpression. Astrocytes transfected with ERVWE1 or the control vector formed giant multinucleated cells (Supplemental Video 1). Both control- and ERVWE1-transfected cells showed increase in fluorescence in response to glutamate exposure (Fig. 3A). However, ERVWE1-transfected cells exhibited an increase in peak amplitude and accelerated onset of fluorescence intensity compared with the control cells \((p < 0.05)\). When the transfected cells (both control and ERVWE1) were treated with crocin (200 \(\mu\text{M}\)), the change in the peak amplitude difference in ERVWE1 and control-transfected cells were attenuated and the onset of fluorescence change was delayed (Fig. 3B). Graphic representation of these observations disclosed that peak fluorescence induced by glutamate exposure in ERVWE1-transduced cells was diminished by crocin treatment. These results highlighted that crocin treatment could reduce ERVWE1-induced calcium fluxes.

**Syncytin-1 transduced astrocytes mediate oligodendrocytotoxicity**

Astrocytes are the most abundant cell type in the brain and are responsible for a diversity of homeostatic functions (44). Among these roles, oligodendrocyte maintenance and support are highly important. Hence HFAs were transfected with matched control or ERVWE1-containing vectors; harvested supernatants were applied to differentiated CNPase-immunopositive oligodendrocytes (Fig. 4A). DAPI staining of oligodendrocyte cultures disclosed a cytotoxic effect caused by Syncytin-1 overexpression in astrocytes with concurrent cytoprotective actions of crocin (Fig. 4B). Because microglia are also recognized to secrete proinflammatory cytokines such as TNF-\(\alpha\), which cause neuroinflammation and degeneration, the effects of ERVWE1 transfection of microglia were also investigated in terms of oligodendrocyte viability and DAPI staining. Oligodendrocytes exposed to supernatants from microglia transfected with the control plasmid or Syncytin-1–expressing plasmid demonstrated similar levels of DAPI staining without evidence of cytotoxicity (Fig. 4B). However, crocin exerted a protective effect on oligodendrocytes after exposure to...
supernatants from astrocytes overexpressing Syncytin-1 (Fig. 4B). The cytotoxic effect of supernatants from astrocytes overexpressing Syncytin-1 on oligodendrocytes was confirmed by a decrease in CNPase immunoreactivity in oligodendrocytes cultured with supernatants from ERVWE1-transfected astrocytes, compared with control vector-transfected astrocytes (Fig. 4C). When crocin was added to supernatants from astrocytes transfected with ERVWE1, the resulting CNPase immunoreactivity was preserved in a concentration-dependent manner. These data suggested that astrocytes overexpressing Syncytin-1 reduced oligodendrocyte viability, which could be ameliorated by crocin treatment.

**T cell proliferation and NO-mediated cytotoxicity during crocin treatment**

Although crocin exerted apparent protective effects on oligodendrocytes, it was unclear whether it was influencing cellular division or preventing cell death. Murine splenocytes were stimulated with an anti-CD3ε Ab, and ensuing proliferation was measured by CSFE in the presence or absence of crocin (100 μM; Fig. 5A). Crocin did not affect resting T cell viability, and similarly, it did not influence the capacity of activated cells to proliferate, based on CFSE incorporation (Fig. 5B). Similarly, crocin did not prevent MOG-specific T cell proliferation (Fig. 5C). Because crocin suppressed NOS2 levels in human astrocytes and prevented oligodendrocytotoxicity, its effects on sodium nitroprusside (SNP)-mediated oligodendrocyte cytotoxicity were investigated. SNP is a potent NO donor, and reactive nitrogen species (45) are thought to be produced in excess in the brains of MS patients and spinal cords of EAE animals as a result of inflammation (45). Moreover, NO is assumed to play a role in oligodendrocyte death and demyelination during MS (46). Oligodendrocytes were cultured with different concentrations of SNP for 24 h with or without crocin treatment (Fig. 5D). After 24-h incubation, oligodendrocytes were immunolabeled with the Ab to CNPase (Fig. 5D, 5E). Oligodendrocytes were less viable with increasing concentrations of SNP, as measured by CNPase immunostaining. However, when crocin was added to oligodendrocyte cultures, CNPase expression was maintained, suggesting crocin was protective against SNP-mediated cytotoxicity (Fig. 5D, 5E). Crocin had no protective effect on hydrogen peroxide-mediated oligodendrocytotoxicity (data not shown). As an ROS scavenger, it was plausible that crocin was quenching ROS populations, NO specifically, which was released by SNP.

**ER stress induction in EAE spinal cords**

EAE is characterized by T cell activation and entry into the CNS with neuroinflammation, recapitulating many of the events assumed to occur in MS (47). XBP-1/s and other ER stress-related gene transcripts were measured in the spinal cords of mice with and without MOG35-55-induced EAE at day 28 post-EAE induction (Fig. 6). A PstI restriction site is excised when XBP-1/u is converted to XBP-1/s (48), and thus after amplification and digestion of the resulting amplicon with PstI, it would be expected for XBP-1/s to appear as a larger amplicon compared with the two smaller XBP-1 unspliced transcript amplicons. After RT-PCR with XBP-1 primers and digestion with PstI, the XBP-1/s transcript was increased in the spinal cords of EAE mice compared with age-matched healthy mice. Image analysis was used to quantify the DNA bands, revealing the mean XBP-1/s:XBP-1/u ratio (Fig. 6A) was 7-fold higher in the spinal cords of EAE mice compared with those in healthy animals (Fig. 6B). Other ER stress gene transcript
levels were also examined in the spinal cord of EAE mice (Fig. 6C). BiP, an early ER stress marker, and CHOP, a late ER stress marker, were significantly upregulated in the spinal cords of EAE mice (Fig. 6C). Similarly, GRPs8 and OASIS transcript levels were also increased in the spinal cords of EAE animals compared with healthy animals. Together with increased XBP-1 splicing in the spinal cords of EAE mice, these data suggested that ER stress-related gene expression was increased in the CNS during EAE.

**EAE pathogenesis is CHOP independent**

ER stress has several downstream effects, of which inflammation and apoptosis are two principal outcomes. ER stress can activate CHOP, a protein responsible for ER stress-mediated apoptosis (49). To determine whether CHOP played a role in EAE pathogenesis, we induced EAE in CHOP−/−, CHOP+/−, and CHOP+/+ animals. All three CHOP genotypes exhibited similar neurobehavioral disease profiles (Fig. 7A), maximum disease severity scores (Fig. 7B), and cumulative disease scores (Fig. 7C). There was an increase in CD3ε (Fig. 7D) and F4/80 (Fig. 7E) transcripts in CHOP−/− and CHOP+/− EAE animals compared with CHOP+/+ EAE mice. Increased XBP-1s transcript levels (Fig. 7G) in CHOP−/− and CHOP+/− compared with CHOP+/+ EAE mice was not accompanied by increased BiP transcript levels (Fig. 7F). These observations implied that CHOP is not essential for the EAE disease phenotype, but other ER stress-mediated processes might participate in EAE pathogenesis.

**Neuroinflammation and ER stress in EAE spinal cords**

Given that crocin suppressed Syncytin-1–induced XBP-1 splicing in astrocytes, ER stress and immune gene expression was assessed at the time of peak disease severity (day 17 postinduction) in spinal cords from control, EAE, and crocin-treated EAE animals. XBP-1s transcript levels were diminished in crocin-treated EAE animals compared with PBS-treated animals (Fig. 8A). Similarly, crocin also suppressed BiP expression in spinal cords of EAE animals, but CHOP gene transcript level reduction in crocin-treated EAE mice was not significant (Fig. 8B, 8C). CD3ε transcript levels were also reduced in EAE animals receiving crocin, indicative of crocin’s anti-inflammatory properties (Fig. 8D). In addition, F4/80 (Fig. 8E) and TNFα (Fig. 8F) gene transcript levels exhibited diminished expression during EAE with crocin treatment. Thus, gene expression indicative of inflammatory and ER stress pathways was suppressed in EAE animals treated with crocin, recapitulating the present in vitro observations (Fig. 2).

**Neuropathological features of EAE**

Because crocin modulated gene expression during EAE, spinal cords from control, EAE, and crocin-treated EAE animals were examined revealing minimal CD3+ immunopositive T cells in control animals (Fig. 9A), but EAE animals exhibited numerous T cells at day 17 post-EAE induction (Fig. 9B), which was reduced by crocin treatment (Fig. 9C). Likewise, limited Iba-1–immunoreactive microglia were observed in control animals (Fig. 9D), but microglia were more abundant in EAE animals (Fig. 9E), although crocin appeared to suppress microglial activation (Fig. 9F). MBP immunoreactivity was consistently observed in white matter tracts of control animals (Fig. 9G), in contrast with EAE animals, which exhibited patchy loss of MBP reactivity (Fig. 9H), but was preserved in EAE animals treated with crocin (Fig. 9I). BiP immunoreactivity was minimal in control animals (Fig. 9J) but evident in glial cells within EAE spinal cord sections (Fig. 9K), although its immunoreactivity was highly restricted in crocin-treated EAE animals. In fact, BiP immunoreactivity was apparent only in cells colabeled with anti-GFAP Abs (Fig. 9K, inset i) or anti–Iba-1 Abs (Fig. 9K, inset ii), but not in CNPase immunoreactive cells (data not shown). Axonal density was assessed in Bielschowsky-stained spinal cord sections disclosing numerous axons in white matter tracts of controls (Fig. 9M) with fewer axons evident in EAE animals (Fig. 9N) and relative preservation of axonal numbers in EAE animals receiving crocin (Fig. 9O). Quantitative analyses of Bielschowsky silver staining...
confirmed crocin’s protective effects in terms of axonal injury in the context of EAE (Fig. 9P). Thus, ER stress gene expression was concordant with immune gene expression in EAE, but crocin appeared to suppress both inflammatory and ER genes in glial cells during EAE.

**Crocin improves neurobehavioral outcomes in EAE**

EAE mice treated with PBS exhibited the expected MOG-induced disease course with a disease onset at day 7 and peak neurobehavioral impairment at days 15–18 (Fig. 10A). EAE mice showed fully paralyzed tails and hind limbs at the peak of disease, whereas those treated daily with crocin from the time of disease onset showed only limp tails and partially weak hind limbs at the peak of disease (Fig. 10A) based on neurobehavioral scores. Similarly, EAE mice treated with crocin were a median 7 points lower on the 14-point disease severity scale at the point of maximum disease compared with EAE mice treated with PBS (Fig. 10B). Similarly the median cumulative disease score of EAE mice treated with crocin was 5-fold lower than EAE mice treated with PBS (Fig. 10C). These data indicated that crocin diminished neurological impairment during EAE accompanied by suppression of neuroinflammation and ER stress.

**Discussion**

These observations provide evidence for a close association between ER stress and inflammation within the CNS in both clinical and experimental settings. XBP-1/s was induced in MS brains in conjunction with the HERV glycoprotein, Syncytin-1, which induced XBP-1 splicing in human astrocytes. Moreover, Syncytin-1 mediated XBP-1 splicing in association with enhanced transcription of inflammatory genes in human astrocytes. The induction of ER stress genes, particularly XBP-1/s, preceded induction of the antiviral-associated genes (IFN-α and Mx1; Fig. 2C), suggesting that in this model, ER stress induction contributed to induction of inflammation. The pathobiological effects of astrocytic Syncytin-1 overexpression on oligodendrocyte viability were ameliorated by concurrent treatment with crocin. Crocin has been shown to exert neuroprotective effects in several model systems, possibly through suppression of free radical production (50–52). More recently, crocin has been reported to suppress microglial activation (53) and ER stress (54). The MS animal model, EAE, also showed evidence of ER stress gene activation including XBP-1/s, BiP, PERK, and CHOP, together with inflammatory gene expression in diseased spinal cords, which was suppressed by crocin treatment and accompanied by substantial improvements in disease outcomes. Thus, these observations reflect the pathogenic consequences of ER stress and immune gene coactivation in the CNS with ensuing inflammatory demyelination and neurodegeneration, which were regulated by crocin treatment.

Oligodendrocytes are the principal target cell in EAE (and MS), frequently dying during neuroinflammation and demyelination (55). Microglia and astrocytes maintain homeostasis within the CNS during health; however, they also mediate very specific disease mechanisms as illustrated in Alexander’s disease in which GFAP polymorphisms result in demyelination, neurodegeneration, and delayed development (56, 57) or HIV-associated encephalopathy during which microglia/macrophages and astrocytes are infected by HIV-1, resulting in neuroinflammation with axonal loss (58, 59). Hence it is plausible that a retroviral env-encoded glycoprotein such as Syncytin-1 might induce ER stress and inflammation in glia, which results in impaired myelin homeostasis and limited remyelination postinjury. These speculations can be reconciled with the present observations of human astrocytes transfected with the Syncytin-1 expression vector, resulting in the
induction of cytokines and free radicals (Fig. 2), which impair oligodendrocyte viability (Fig. 4). Indeed, oligodendrocytes cultured with supernatants from astrocytes transfected with a Syncytin-1–expressing vector were less viable, measured by CNPase immunoactivity. Astrocytes can prevent oligodendrocyte precursor cells’ migration and maturation (60, 61), and astrogliosis is characteristic of chronic demyelination (62). Hence in astrocytes, Syncytin-1 might contribute to intracellular cascades that potentially alter the type of astrogliosis or limit astrocyte secretion of trophic factors. Nonetheless, the precise mechanism by which crocin affects intracellular pathways is unclear; crocin inhibits translocation of G protein-coupled receptor kinase 2 from the cytosol to membrane, whereas also diminishing ERK1/2 phosphorylation (52). Another study demonstrated crocin protects against hydrogen peroxide-induced endothelial injury by diminishing apoptosis (51). Crocin’s neuroprotective effects on TNF-α (50), hydrogen peroxide- (51), Syncytin-1–, and NO-mediated cytotoxicity in vitro underscore its potential therapeutic effects in neurodegenerative and neuroinflammatory diseases.

The current studies with CHOP wild type and null animals demonstrated that CHOP expression is not a requisite protein for the development of EAE pathogenesis (Fig. 7). CHOP has a variety of functions, but its chief contribution is to mediate apoptosis through ER stress (18). Because the EAE disease profile, together with maximum and cumulative scores, was similar among CHOP+/−, CHOP−/−, and CHOP+/+ animals, CHOP-mediated apoptosis was not an essential component for EAE. During EAE, neuroinflammation may drive ER stress, which was evident in astrocytes, as suggested by increased BIP immunostaining in EAE. Both apoptosis and astrogliosis are common outcomes to most neuroinflammatory diseases (63). Previous in vitro studies indicate that oligodendrocytes exposed to inflammatory proteins such as IFN-γ exhibit features of ER stress (64), representing yet another link between ER stress and neuroinflammation. All three ER stress cascade initiators, ATF6/OASIS, IRE1, and PERK, are revealed variable immune and ER stress transcript profiles with CHOP+/− and CHOP−/− genotypes during EAE. This suggests that inflammatory mechanisms are the chief determinants, overriding other (cell death) pathways to cause EAE.

Crocin is the active ingredient in saffron, a natural spice commonly used in Northern Indian and Persian cooking (65), and has been shown to prevent TNF-α–induced cell death of neurally differentiated PC-12 cells (50). Crocin also diminishes free radical-induced stress, evidenced by the suppression of NO and malondialdehyde, in the brain after cerebral ischemia (52). Indeed, crocin diminished Syncytin-1–induced XBP-1 splicing in astrocytes in a concentration-dependent manner (Fig. 2). A direct transcriptional role for crocin is unclear, but it is conceivable that its ER stress-modulating effects might stem from its suppression of inflammation and free radical generation. Although crocin had no apparent suppressive effects on T cell proliferation, it exerted a robust neuroprotective effect by reducing ER stress and inflammation in the CNS during EAE, underscoring its direct effects on CNS, perhaps because of its highly lipophilic properties (66). When crocin was administered to EAE mice 7 d post-EAE induction, the resulting neuropathology was reduced, as evident by the preserved axonal counts in treated EAE animals. It is unlikely that crocin impaired leukocyte infiltration initially because it was administered after leukocytes began their entry into the CNS during EAE (day 7 post-EAE induction), and moreover, T cell proliferation was unaffected by crocin treatment. Crocin might attenuate activation of T cells and microglial within the CNS during EAE. In addition, with crocin’s documented anti-inflammatory effects (50, 51), it might also modulate blood–brain barrier permeability, possibly through suppression of inflammation-induced cell adhesion molecules on brain endothelia such as ICAM2, VCAM, or one of the integrins (44, 52). Crocin also ameliorated the EAE disease profile with a concurrent decrease in maximum and cumulative scores. The reduced neurobehavioral deficits in crocin-treated EAE animals were congruent with the present findings of diminished EAE neuropathology. Despite the remaining questions regarding crocin’s mechanism of action in neuroprotection during EAE, these studies highlight a potential therapeutic role for crocin in neuroinflammation, which was previously unrecognized.

In summary, these studies support the hypothesis that neuroinflammation and ER stress contribute to the pathogenesis of inflammatory demyelination and neurodegeneration. Syncytin-1 exerted a robust effect on XBP-1 splicing in human astrocytes, which was partially abrogated by crocin. In addition, crocin was neuroprotective in vitro against Syncytin-1– and NO-induced astrocyte and oligodendrocyte cytotoxicity, respectively. Furthermore, crocin treatment diminished neuropathology in EAE with significantly less neurological impairment. These results underscore the roles of Syncytin-1 and ER stress in MS and point to potential therapeutic utility for crocin in neuroinflammation and neurodegeneration.

Acknowledgments
We thank Krista Nelles for assistance with publication preparation. We also thank F. Mallet for providing the HERV-W env encoding plasmid.

Disclosures
The authors have no financial conflicts of interest.

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