TESI DOCTORAL

LA VIA JAK/STAT COM A MEDIADORA DE RESPOSTES A L'ESTRÈS OXIDATIU, LA INFLAMACIÓ I LA IMMUNITAT INNATA EN ASTRÒCITS

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RESULTATS

RESUM DELS RESULTATS

Els resultats d'aquesta tesi es presenten organitzats en articles:

1. Article nº1

AG490 prevents cell death after exposure of rat astrocytes to hydrogen peroxide or proinflamatory cytokines: involvement of the Jak2/STAT pathway

Roser Gorina, Valérie Petegnief, Ángel Chamorro i Anna M. Planas
Journal of Neurochemistry, 2005; **92**: 505-518

En aquest treball es demostra que la via Jak2/Stat1 s'activa en resposta a peròxid d'hidrogen i citoquines proinflamatòries en astròcits, i que està directament relacionada amb la mort cel·lular. L' exposició transitòria a peròxid d'hidrogen i l'exposició perllongada a IFN-γ i a IL-6 redueix la viabilitat cel·lular en cultiu primari d'astròcits a 24h, en canvi el tractament amb la citoquina antiinflamatòria IL-10 no produeix mort. En aquest treball s'ha caracteritzat el tipus de mort cel·lular produïda per aquests estímuls utilitzant diferents mètodes de mesura de viabilitat. El pretractament amb l'inhibidor de Jak2 AG490, impedeix la mort cel·lular suggerint que la via Jak2/Stat participa en la mort.

El peròxid d'hidrogen, IL-6 i IFN-γ indueixen la fosforilació de Stat1 en tirosina 701 depenent de Jak2, mentre que la fosforilació de Stat3 en tirosina 705 té lloc després del tractament amb peròxid d'hidrogen, IL-6 i IL-10. La mort cel·lular produïda pels tractaments amb peròxid d'hidrogen, IFN-γ i IL-6 correlaciona positivament amb el grau de fosforilació de Stat1 en tirosina 701.

2. Article nº2

Exposure of glia to prooxidant agents revealed selective Stat1 activation by H_2O_2 and Jak2-independent antioxidant features of the Jak2 inhibitor AG490

Roser Gorina, Coral Sanfeliu, Aida Galitó, Àngel Messeguer i Anna M. Planas
Glia, 2007; **55**: 1313-1324

En aquest treball, els cultius d'astròcits han estat exposats a diferents agents prooxidants per estudiar l'activació de la via JAK/STAT en resposta a l'estrès oxidatiu. S'ha mesurat l'augment dels nivells de espècies reactives d'oxigen (ROS) després dels tractaments amb peròxid d'hidrogen, sulfat de ferro, nitoprussiat (donador d'òxid nítric) i

paraquat (donador d'anió superòxid). Aquests agents prooxidants també indueixen l'oxidació de proteïnes, excepte el donador de NO que produeix nitrosilació proteica. El peròxid d'hidrogen, i en menor grau el $FeSO_4$, indueixen la fosforilació de Stat1, en canvi el donador de NO i el donador d'anió superòxid no ho fan.

S'ha avaluat els efecte dels antioxidants (trolox, propilgallat i N-acetilcisteïna) i dels inhibidors de Jak2 (AG490 i Jak2-Inhibitor-II) sobre la producció de ROS i la fosforilació de Stat1 en aquests models d'estrès oxidatiu.

Els resultats mostren que el Trolox i el propilgallat redueixen de manera important els nivells de ROS, en canvi, no inhibeixen la fosforilació de Stat1 induïda per peròxid d'hidrogen. D'altra banda, la N-acetilcisteïna no té efecte sobre la formació de ROS però sí inhibeix l'activació de Stat, demostrant que la generació de ROS i la fosforilació de Stat1 són efectes independents.

Els inhibidors de Jak2 impedeixen la fosforilació de Stat1 després del tractament amb H_2O_2, suggerint que la quinasa Jak2 participa en la fosforilació de Stat1 per peròxids. En aquest treball es demostra que l'inhibidor de Jak2, AG490 presenta efectes antioxidant ja que és capaç d'inhibir la formació de ROS i la peroxidació lipídica. Aquests efectes son independents de l'inhibició de Jak2, doncs, en astròcits on s'ha silenciat l'expressió de Jak2 amb siRNA, l'efecte antioxidant es manté i també s'ha demostrat que l'altre inhibidor utilitzat, el Jak2-inhibitor-II no redueix la formació de ROS.

Finalment, aquest estudi mostra l'especificitat de la senyalització induïda per peròxid d'hidrogen i suggereix que aquesta molècula també podria jugar un paper en la senyalització fisiològica.

3. Article nº3

Atrocytes are very sensitive to develop innate immune responses to short interfering RNA

Roser Gorina, Tomàs Santalucia, Valérie Petegnief, Aroa Ejarque-Ortiz, Josep Saura i Anna M. Planas

Segona revisió a *Glia*

En aquest treball s'ha estudiat l'inducció de la resposta immune innata activada pels siRNA en astròcits. Els *small interfering RNAs* (siRNAs) s'utilitzen àmpliament en la recerca bàsica per inhibir l'expressió de proteïnes específicament. En aquest treball es demostra que diferents siRNAs indueixen efectes inespecífics no desitjats independents del seu efecte silenciador. En resposta al tractament amb diferents siRNA, s'indueixen

nivells variables de l'expressió de Stat1 i aquest augment de l'expressió és dependent de la dosi de siRNA. A part de l'increment de Stat1, altres efectes no específics, com la síntesi d'IL-6 i IL-10, s'observen en cultius glial mixtes i en astròcits purs després del tractament amb el siRNA. En canvi, en microglia i en la línia cel·lular no glial NIH3T3, tot i que el silenciament gènic i la transfecció amb el siRNA són eficients no es detecta activació del sistema immune innat.

En aquest treball es demostra que la modificació química dels siRNA afegint un grup 2'-O-metil en alguns nucleòtids inhibeix l'activació innata sense impedir el silenciament específic.

Finalment, es demostra que l'activació de la resposta immune pels siRNA està mitjançada per la senyalització dels TLRs, i es proposa que TLR3 podria ser el responsable, ja que els nivells d'expressió de TLR3 són més elevats en cultius purs d'astròcits que en cultius de microglia o en les cèl·lules 3T3. A més a més, es compara els efectes de l'agonista de TLR3, poly(I:C), amb els efectes inespecífics dels siRNAs, i s'observa que tots dos indueixen IP-10, Stat1, VCAM-1 i COX-2 i augmenten l'expressió de TLR3. Tots aquests resultats en conjunt demostren que els astròcits activen la resposta immune innata per acció d'alguns siRNA i que Stat1 participa en aquesta resposta.

4. Article nº 4

Signalling pathways mediating inflammmatory responses in brain ischaemia

Anna M. Planas, Roser Gorina i Ángel Chamorro
Biochemical Society Transactions, 2006; **34**, part 6: 1267-1270

En aquest treball es posa de manifest el paper de les citoquines en la resposta inflamatòria a la isquèmia cerebral, i l'importancia de la via JAK/STAT com efectora de la resposta cel·lular a citoquines. L'expressió de citoquines augmenta en el plasma dels pacients d'ictus i els nivells de determinades citoquines poden tenir valor pronòstic.

En el cervell isquèmic es produeixen citoquines pro i antiinflamatòries. Les citoquines antiinflamatòries, com IL-10, promouen la supervivència cel·lular, mentre les citoquines proinflamatòries, com el TNF-α, poden induir mort cel·lular. Però això no sempre és així, l'acció beneficiosa o perjudicial de determinades citoquines depèn de diferents variables com la concentració, el moment en el temps i la complexa xarxa de senyals intracel·lulars que s'activen i interaccionen.

Cal que existeixi l'equilibri just entre citoquines pro i antiinflamatòries, doncs, un augment de senyals antiinflamatòries pot comportar una disminució de la resposta immunitaria. La visió més recent és que la isquèmia cerebral pot comportar un estat d'immunosupressió que vindria donat per un excés de citoquines antiinflamatòries.

En aquest treball es resumeixen els diferents estudis sobre la via de senyalització JAK/STAT portats a terme en cultius d'astròcits per posar de manifest l'importància d'aquesta via en la mort i la supervivència cel·lular. En el cervell, l'activació de Stat1 es relaciona amb mort cel·lular, mentre que Stat3 podria jugar un paper com a promotor de la supervivència.

Article nº1

AG490 prevents cell death after exposure of rat astrocytes to hydrogen peroxide or proinflamatory cytokines: involvement of the Jak2/STAT pathway

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AG490 prevents cell death after exposure of rat astrocytes to hydrogen peroxide or proinflammatory cytokines: involvement of the Jak2/STAT pathway

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Abstract

Janus kinases/STAT pathway mediates cellular responses to certain oxidative stress stimuli and cytokines. Here we examine the activation of Stat1 and Stat3 in rat astrocyte cultures and its involvement in cell death. H_2O_2, interferon (INF)-γ and interleukin (IL)-6 but not IL-10 caused cell death. Stat1 was phosphorylated on tyrosine (Tyr)-701 after exposure to H_2O_2, INF-γ or IL-6 but not IL-10. Tyr-705 pStat3 was observed after H_2O_2, IL-6 and IL-10. Also, H_2O_2 induced serine (Ser)-727 phosphorylation of Stat1 but not Stat3. The degree of Tyr-701 pStat1 by the different treatments positively correlated with the corresponding reduction of cell viability. AG490, a Jak2 inhibitor, prevented Tyr-701 but not Ser-727, Stat1 phosphorylation. Also, AG490 inhibited Tyr-705 Stat3 phosphorylation induced by H_2O_2 and IL-6 but did not prevent that induced by IL-10. Furthermore, AG490 conferred strong protection against cell death induced by INF-γ, IL-6 and H_2O_2. These results suggest that Jak2/Stat1 activation mediates cell death induced by proinflammatory cytokines and peroxides. However, we found evidence suggesting that AG490 reduces oxidative stress induced by H_2O_2, which further shows that H_2O_2 and/or derived reactive oxygen species directly activate Jak2/Stat1, but masks the actual involvement of this pathway in H_2O_2-induced cell death.

Keywords: antioxidant, interferon-γ, interleukin-6, interleukin-10, Stat1, Stat3.

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Inflammation is involved in the pathogenesis of brain damage after several insults, such as cerebral ischaemia/reperfusion. The formation of oxygen free radicals and the release of proinflammatory cytokines contribute to this effect (Dirnagl *et al.* 1999; Chan 2001). Clinical data support a correlation between the increase in several proinflammatory cytokines in the circulation and early neurological worsening in ischaemic stroke (Vila *et al.* 2000). Cytokines, by acting on their membrane receptors, activate various intracellular signalling pathways. Ligand binding to cytokine receptors induces activation of kinases of the Janus family (JAK) which is followed by phosphorylation of the receptor tyrosine (Tyr) residues (Ihle 1995, 2001; Stahl *et al.* 1995). These phosphorylated sites entrap cytoplasmic STAT proteins, which are then directly phosphorylated by the receptor-associated JAKs (Schindler and Darnell 1995; Leonard and O'Shea 1998; Imada and Leonard 2000). Phosphorylated proteins of the STAT family can form homo- or heterodimers, translocate to the nucleus, bind to specific consensus DNA sequences and trigger gene transcription (Darnell *et al.* 1994; Darnell 1997).

The JAK/STAT pathway transduces signals from the interleukin (IL)-6 cytokine family (Gerhartz *et al.* 1996; Hibi *et al.* 1996; Heinrich *et al.* 1998, 2003). IL-6 binds to the signal transduction receptor gp130 and signals via gp130 homodimers (Heinrich *et al.* 2003). Anchorage of Jak1 to gp130 triggers Tyr phosphorylation of the kinase (Haan *et al.* 2002) and this activation is essential for the subsequent IL-6-induced signalling process (Guschin *et al.* 1995; Rodig *et al.* 1998; Heinrich *et al.* 2003). Also, the anti-inflammatory

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Abbreviations used: DCF, 2′,7′-dichlorofluorescein; IL, interleukin; INF, interferon; JAK, Janus kinases; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density; PBS, phosphate-buffered saline; PI, propidium iodide; Ser, serine; Tyr, tyrosine.

cytokine IL-10 (Gerard *et al.* 1993; Walmsley *et al.* 1996; Vila *et al.* 2003), through binding to the extracellular domain of IL-10 receptor 1, stimulates Tyr phosphorylation of receptor-JAK-associated kinases. In turn, Jak1 phosphorylates a specific Tyr residue (Y446) on the intracellular domain of IL-10 receptor 1, which then acts as a docking site for Stat3 through its Src homology 2 domain and becomes itself Tyr phosphorylated by Jak1 (Donnelly *et al.* 1999). It is now recognized that activation of this Stat3-dependent signalling pathway mediates most of the anti-inflammatory properties of IL-10 (Williams *et al.* 2004). Stat3 is constitutively expressed in brain (Planas *et al.* 1996, 1997a) and its expression is up-regulated under certain neuropathological conditions (Planas *et al.* 1996; Justicia *et al.* 2000; Gautron *et al.* 2002). Phosphorylation of Stat3 on Tyr-705 was reported under inflammatory conditions, such as those resulting from autoimmune encephalomyelitis (Jee *et al.* 2001) and focal cerebral ischaemia (Suzuki *et al.* 2001; Wen *et al.* 2001).

The expression of Stat1 is also induced after cerebral ischaemia/reperfusion (Planas *et al.* 1997b) and evidence demonstrating a detrimental role of this factor was obtained in Stat1-deficient mice as they showed smaller infarct volumes after brain ischaemia (Takagi *et al.* 2002). Stat1 is strongly activated by interferon (INF)-γ (Darnell *et al.* 1994; Darnell 1998; Ganster *et al.* 2001; Ihle 2001). In addition to cytokines, oxidative stress is a major inducer of STAT protein activation (Tacchini *et al.* 2002). However, activation of the JAK/STAT pathway is restricted to certain oxidative stress stimuli as it is induced by peroxides but not by other types of reactive oxygen species, such as superoxide (Simon *et al.* 1998). Stat1 and Stat3 are Tyr phosphorylated and translocate to the nucleus in a Jak2-dependent manner after exposure of rat vascular smooth muscle cells to H_2O_2 (Madamanchi *et al.* 2001) and exposure of MRC5 human fibroblasts to oxidized low-density lipoprotein (Maziere *et al.* 2001).

STAT proteins are recognized as important signalling molecules in astrocytes mediating cell differentiation and responses to cytokines and other extracellular signals (De-Fraja *et al.* 1998; Lee *et al.* 1999; Justicia *et al.* 2000; Dell'Albani *et al.* 2001; Jenab and Quinones-Jenab 2002; Sriram *et al.* 2004). To better understand the role of JAK/STAT in the cellular responses to cytokines and oxidative stress in astrocytes, we examine here the action of these stimuli on STAT activation and cell viability and the effect of Jak2 inhibition on these parameters. Our results show that the Jak2/Stat1 pathway mediates astrocyte cell death after exposure to pro-inflammatory cytokines or peroxides.

Materials and methods

Astrocyte cell culture preparation

Glial cell cultures enriched in astrocytes were prepared from the cerebral cortex of 1- to 2-day-old Sprague-Dawley rats as described previously (Juurlink *et al.* 1992), with slight modifications as described by Fauconneau *et al.* (2002). All products and reagents, unless otherwise stated, were from Sigma (St Louis, MO, USA). Cells were plated in 20 flasks (25 cm^2; TPP, Techno Plastic Products, Trasadingen, Switzerland) or 96-well plates (NUNC, Roskilde, Denmark) and maintained in Dulbecco's modified Eagle's medium supplemented with 20% foetal bovine serum (Gibco-BRL, Invitrogen, Paisley, UK) and 4 mL/L of a mixture of penicillin/streptomycin (10 000 U/10 000 μg/mL; Gibco-BRL) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed twice a week with the incubation medium supplemented with 10% and then 7% foetal bovine serum. After 1 week, the flasks were shaken at 200 r.p.m. for 2 h to dislodge microglia. The medium was immediately removed and replaced with a fresh medium containing 7% foetal bovine serum. The medium was then used up to confluence with 7% foetal bovine serum. Unless otherwise stated, all products and reagents were from Sigma.

Secondary astrocyte cultures were prepared from rat primary glial cultures as previously described (Aloisi *et al.* 1997) to obtain cultures more enriched in astrocytes. Cultures at 10–12 days *in vitro* were shaken for 2 h at 200 r.p.m. to detach microglia and oligodendrocytes growing on top of the astrocytic layer. The remaining adherent cells were detached with trypsin (0.05%)/EDTA (0.2 mM) and the resulting cell suspension was left at room temperature (20°C) in uncoated dishes to allow adherence of microglia to the plastic surface. After 20 min, the supernatant fluids containing the non-adherent or loosely adherent cells were collected and centrifuged; the cells were resuspended in fresh medium and reseeded on flasks.

Experimental design and treatments

Glial cell cultures enriched in astrocytes were exposed to different concentrations of H_2O_2 for 1 h and cell death was assessed 24 h later by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, lactate dehydrogenase (LDH) assay and propidium iodide (PI) staining (see below). In further experiments we chose to use 300 μM H_2O_2, which caused more than 50% cell death. Studies of STAT phosphorylation were carried out at several times after 1 h transient exposure to 300 μM H_2O_2. Cells were treated with tyrphostin AG490 (Calbiochem, San Diego, CA, USA), an inhibitor of Jak2 (Meydan *et al.* 1996), 30 min before 300 μM H_2O_2 for 1 h and in controls. AG490 was dissolved in dimethylsulfoxide to a final concentration of 0.1%. All reactions with AG490 were kept in the dark to avoid inactivation of the drug. The effects of AG490 (0.03–30 μM) on cell viability were evaluated 24 h after H_2O_2 exposure. The effect of AG490 (30 μM) on STAT phosphorylation was examined 30 min after 1 h exposure to H_2O_2.

In other experiments cells were exposed to either 0.5–5 ng/mL recombinant INF-γ (R and D Systems, Abingdon, Oxon, UK), 1–100 ng/mL recombinant IL-6 (Biosource International Inc., Camarillo, CA, USA) or 10–150 ng/mL recombinant IL-10 (Biosource International) or the corresponding vehicles and STAT phosphorylation and cell death were examined at 30 min and 24 h, respectively. The vehicle for INF-γ was the medium and that for IL-6 and IL-10 was phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. The effect of 30 μM AG490, which was given 10 min before drug exposure, on STAT phosphorylation and cell viability was tested at 30 min and at 24 h, respectively.

Protein expression

Cells were harvested to study the phosphorylation of Stat1 and Stat3 in the different treatment groups by western blot. Cell cultures were homogenized in radio immunoprecipitation assay buffer containing sodium dodecyl sulphate (0.1%), sodium deoxycholate (0.5%), the non-ionic detergent Igepal (1%) and a protease inhibitor cocktail (Complete; Boehringer Mannheim, Mannheim, Germany) in 0.01 M PBS. The protein concentration of the samples was determined with the Bradford assay (Bio-Rad, Hercules, CA, USA). Thirty μg of protein were mixed with loading buffer containing dithiothreitol, denatured at 100°C for 5 min and loaded in 10% polyacrylamide gel for electrophoresis. Samples were run together with pre-stained molecular weight markers (Bio-Rad). After sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA) and incubated overnight at 4°C with either mouse monoclonal antibodies against p-Tyr^{705} Stat3 (diluted 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Stat1 (diluted 1 : 1000; Transduction Laboratories, BD Biosciences, Madrid, Spain) or β-tubulin (diluted 1 : 10 000; Sigma) or rabbit polyclonal antibodies against Stat3 (diluted 1 : 2000; K15; Santa Cruz Biotechnology), p-Tyr^{701} Stat1 (diluted 1 : 500; Upstate, Milton Keynes, UK), p-Ser^{727} Stat1 (diluted 1 : 1000; Cell Signalling Technology, Beverly, MA, USA) or p-Ser^{727} Stat3 (diluted 1 : 500; Upstate). On the following day membranes were incubated for 1 h with Ig peroxidase-linked secondary antibodies (1 : 2000; Amersham, Madrid, Spain). The reaction was visualized using a chemiluminescence detection system based on the luminol reaction. Each membrane was first used to study the phosphorylated proteins and then it was reprobed with the corresponding antibody against either total Stat1 or Stat3 or β-tubulin as a protein loading control. The optical density (OD) of the bands was measured by densitometric analysis (Kodak Digital Science 1D; Kodak, Rochester, USA). The ratio between the measure of band intensity of the phosphorylated protein and the corresponding value for the loading control was calculated to correct for differences in protein gel loading. Values were expressed as the percentage of control.

Trypan blue dye exclusion assay

We estimated the total number of cells and the percentage of dead cells 24 h after the lesion by trypan blue dye exclusion assay, as reported by Fauconneau *et al.* (2002). Stained (taken as dead) and viable non-stained cells were counted in a haemocytometer. Mean values for each treatment group were obtained from four to six samples obtained in two to four independent experiments.

Caspase 3 activity

Activation of caspase 3 was determined using the CaspaTag™ kit (Intergen Co., Purchase, NY, USA), as previously reported by Fauconneau *et al.* (2002), at 24 h after 1 h exposure to 300 μM H_2O_2. Dissociated astrocytes were incubated with a carboxy-fluorescein analogue of benzyloxycarbonyl-aspartyl-glutamylvalyl-laspartic acid fluoromethyl ketone. This is a potent inhibitor of caspase 3 and caspase 3-like caspases. Carboxy-fluorescein-benzyl-oxycarbonyl-aspartyl-glutamylvalylaspartic acid fluoromethyl ketone enters the cell and irreversibly binds to active caspase 3. After incubation, astrocytes were fixed and analysed in a flow cytometer (Epics XL flow cytometer; Coulter Corporation, Hialeah, FL, USA).

Release of lactate dehydrogenase

Cell death was assessed 24 h after the lesions by measuring the activity of LDH released in the medium, according to a modification of the method of Wroblewski and LaDue (1955). Briefly, the decrease in 0.75 mM NADH absorbance at 340 nm was followed in phosphate buffer (50 mM, pH 7.4) in the presence of 4.2 mM pyruvic acid as the substrate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cell viability was assessed with the MTT assay for measuring cytotoxicity (Hansen *et al.* 1989). Viable cells with active mitochondria reduce the yellow tetrazolium salt MTT giving dark blue water-insoluble formazan crystals. To perform the assay, MTT was dissolved at a concentration of 5 mg/mL in PBS, sterilized by filtration and stored at 4°C for up to 1 month, protected from light, and tightly capped. The extraction solution to lyse cells and dissolve the crystals was prepared with 20% sodium dodecyl sulphate dissolved in *N,N*-dimethyl formamide/water (1 : 1), pH 4.7. Twenty-four hours after the lesion, control and treated cells were washed twice with PBS and the MTT solution was then added to 96-well plates (10 μL per well) and the plates were returned to an incubator. After 2 h, incubation was terminated by adding extraction solution (100 μL per well) and plates were tightly wrapped with Parafilm to avoid evaporation and incubated overnight at 37°C. OD was measured at 570 nm using the extraction solution as a blank. Results were calculated as follows: percent of MTT reduction = $[(OD - OD_0)/(OD_c - OD_0)] \times 100$, where OD_c is the mean of the control wells and OD_0 the mean of the control wells without added MTT for each plate.

Propidium iodide staining

Propidium iodide staining was used to microscopically identify the presence of dead cells in fresh (non-fixed) cultures. PI enters cells with damaged membranes and greatly increases its fluorescence by binding to nucleic acids. PI was added to the cultures at a final concentration of 15 μg/mL and was incubated for 30 min. Cells were then washed with warm PBS and the cultures were immediately observed under an inverted fluorescence microscope. The value corresponding to total cell death (F_{max}) was obtained after exposure to 0.02% Triton-X100. Values for the different treatment groups (F) were expressed as: $(F - F_{min})/(F_{max} - F_{min}) \times 100$. F_{min} corresponds to the mean value in the control group.

Production of reactive oxygen species

Oxidation of 2′,7′-dichlorofluorescein diacetate to fluorescent 2′,7′-dichlorofluorescein (DCF) is taken as an index of overall oxidative stress in biological systems (LeBel *et al.* 1992). Astrocytes growing at confluence in 96-well cell plates were incubated for 20 min with 2′,7′-dichlorofluorescein diacetate (10 μM) in HEPES-buffered saline solution. After this time, the 2′,7′-dichlorofluorescein diacetate was washed with the buffer and changed to fresh HEPES buffer and either 30 μM AG490 or vehicle (as above) was immediately added to the wells, followed 30 min later by the addition of either 300 μM H_2O_2 or 0.5–50 ng/mL INF-γ. One hour later DCF fluorescence was measured in a fluorimeter (Cytofluor™ 2350; Millipore) with 485 and 530-nm emission filters.

Statistical analyses

Comparisons between treatments were made with one-way ANOVA followed by post-hoc analysis with Bonferroni's multiple comparisons test.

Results

Exposure to H_2O_2 causes cell death and induces Stat1 and Stat3 phosphorylation

Astrocytes were transiently exposed to different concentrations of H_2O_2 ranging from 50 to 1200 μM for 1 h and cell death was evaluated 24 h later by means of the MTT (Fig. 1a), PI (Fig. 1b) and LDH assays (Fig. 1c). In accordance with these data, in further experiments we chose to use a concentration of 300 μM H_2O_2 which, in relation to controls, caused an 85% reduction of MTT values (Fig. 1a), around a 40% increase in PI staining (Fig. 1b) and a fivefold increase in LDH release (Fig. 1c).

Phosphorylation of STAT proteins was examined at different time points after H_2O_2 exposure. Stat1 and Stat3 were rapidly and transiently phosphorylated on Tyr residues 701 and 705, respectively, after 1 h exposure to 300 μM H_2O_2, as the signal was very intense at 30 min but decreased by 90 min (Figs 1d and e). We chose to carry out further studies on the phosphorylation of STAT proteins at 30 min after 1 h exposure to H_2O_2, when Tyr residues were highly phosphorylated (Figs 1d and e). H_2O_2 also induced phosphorylation of Stat1 at residue serine (Ser)-727, which also peaked at 30 min (Fig. 1f). This was not accompanied by significant Ser-727 phosphorylation of Stat3 (Fig. 1g).

AG490 prevents tyrosine STAT phosphorylation and protects astrocytes from H_2O_2-induced cell death

We treated the cells with the Jak2 inhibitor AG490 (30 μM) 30 min before H_2O_2 exposure. This drug prevented H_2O_2-induced Tyr phosphorylation of Stat1 (Fig. 2a) and Stat3

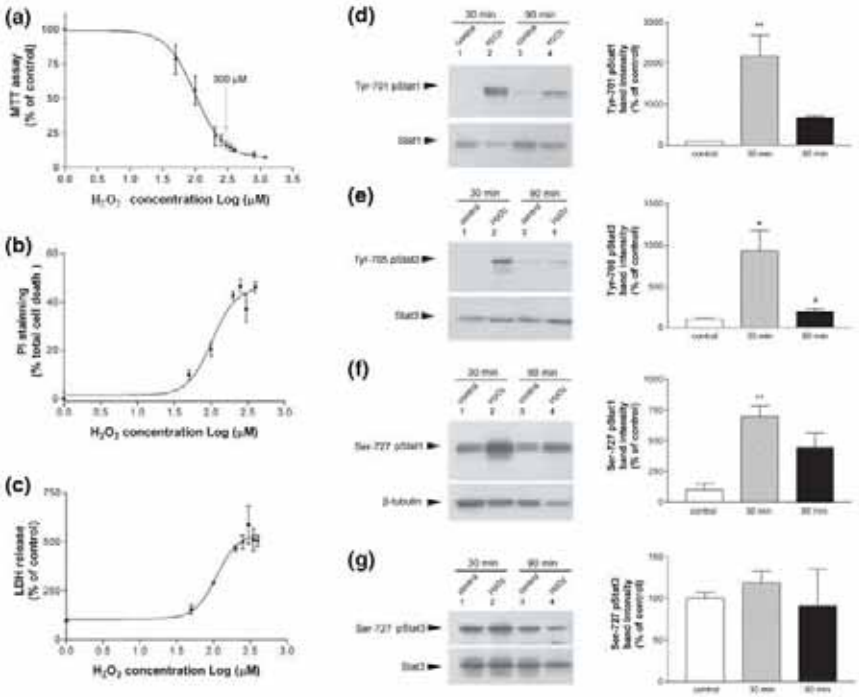

Fig. 1 H_2O_2 promotes cell death in primary astrocyte cultures and induces STAT phosphorylation. Concentration–response curves at 24 h after 1 h H_2O_2 exposure. (a) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay of cell viability. Data were fitted to a sigmoidal dose–response curve by means of non-linear regression analysis ($r^2 = 0.96$). (b) Propidium iodide (PI) staining expressed as the percentage of total cell death (as induced by exposure to Triton X-100, see Materials and methods). Data were fitted to a sigmoidal dose–response curve as above ($r^2 = 0.74$). (c) Cell death assessment by lactate dehydrogenase (LDH) release. Data were fitted to a sigmoidal dose–response curve ($r^2 = 0.77$). (d) Time course of Stat1 phosphorylation after transient exposure to 300 μM H_2O_2 for 1 h. Rapid, intense and transient tyrosine (Tyr)-701 Stat1 phosphorylation is detected at 30 min and decreases at 90 min. Image analysis of optical density in the western blot membranes shows a significant increase in band intensity at 30 min (one-way ANOVA by time $F_{2,14} = 10.04$, $p < 0.002$). Data of image analysis are expressed as percentage of control. (e) Tyr-705 Stat3 phosphorylation followed a similar temporal pattern to that of Stat3 Tyr-705 phosphorylation, with a significant increase in band intensity at 30 min ($F_{2,5} = 7.8$, $p < 0.03$). (f) Serine (Ser)-727 Stat1 phosphorylation peaks at 30 min (g), while no induction of Stat3 phosphorylation on serine residue 727 is apparent. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. control; $\#p < 0.05$ vs. 30 min.

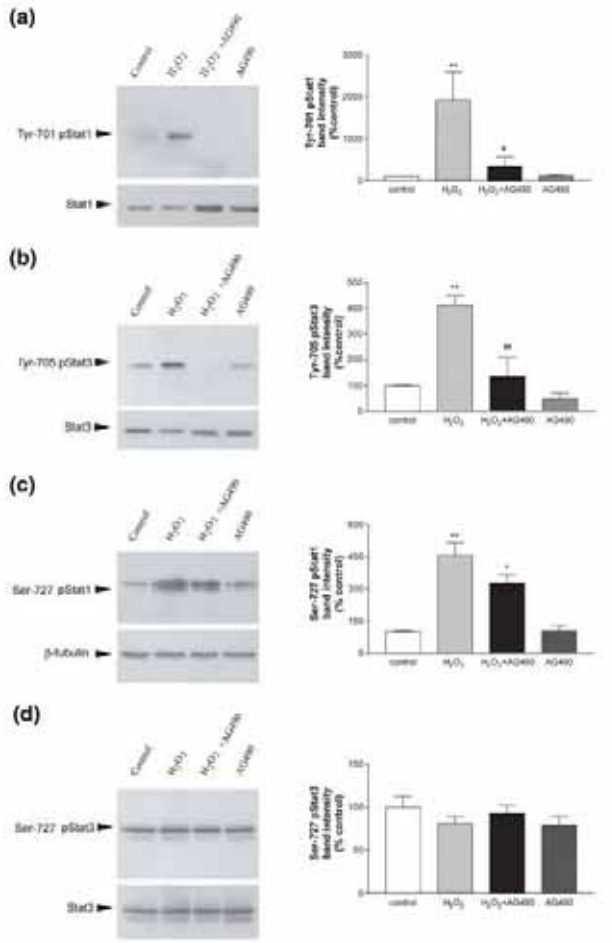

Fig. 2 AG490 reduces H_2O_2-induced Stat1 and Stat3 phosphorylation. Primary cultures of astrocytes were treated with 30 μM AG490 30 min before 1 h exposure to 300 μM H_2O_2 and 30 min later cells were collected to study STAT phosphorylation. (a) Tyrosine (Tyr)-701 Stat1 phosphorylation induced by H_2O_2 ($p < 0.001$) is prevented by AG490 ($p < 0.001$) (one-way ANOVA; $F_{3,16} = 6.06$, $p < 0.005$). (b) Tyr-705 Stat3 phosphorylation induced by H_2O_2 ($p < 0.01$) is prevented by AG490 ($p < 0.01$) (one-way ANOVA; $F_{3,8} = 15.15$, $p < 0.001$). (c) Serine (Ser)-727 Stat1 phosphorylation induced by H_2O_2 ($p < 0.01$) is not significantly reduced by AG490 and band intensity values remain higher than control in the presence of AG490 ($p < 0.05$) (one-way ANOVA; $F_{3,11} = 14.04$, $p < 0.0004$). (d) Ser-727 Stat3 phosphorylation is not significantly increased by H_2O_2 and is not affected by AG490. Results are the mean of three to five independent experiments. *Comparison vs. control; # comparison vs. H_2O_2. One symbol indicates $p < 0.05$ and two symbols indicate $p < 0.01$.

(Fig. 2b) but did not significantly reduce the phosphorylation of the Ser-727 residue of Stat1 (Fig. 2c) and did not affect Ser-727 Stat3 phosphorylation (Fig. 2d).

We studied the effect of various concentrations of AG490 (ranging from 30 nM to 30 μM) on the cell death induced by H_2O_2 in astrocytes. This agent effectively improved cell viability as revealed by the MTT assay ($p < 0.001$) (Fig. 3a),

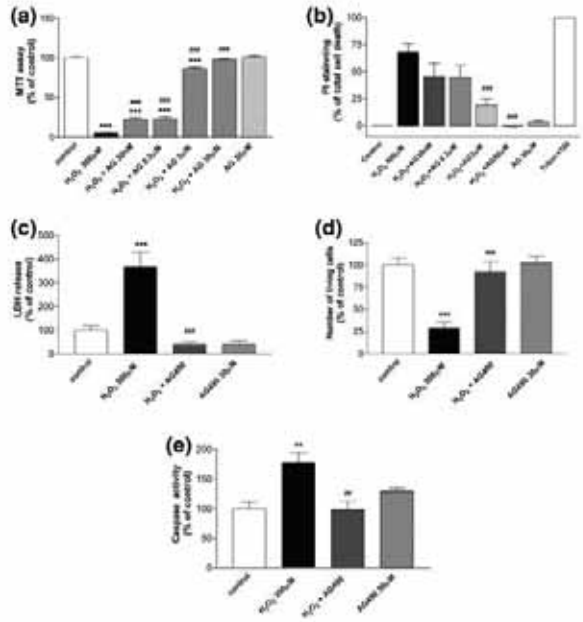

Fig. 3 The Jak2 inhibitor AG490 prevents H_2O_2-induced cell death in astrocytes. (a) AG490 (30 min before H_2O_2) prevents H_2O_2-induced cell death in a concentration-dependent manner, as assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay at 24 h. Statistical analysis shows significant differences between groups (one-way ANOVA; $F_{6,202} = 845.5$, $p < 0.0001$). (b) Cell death, as assessed at 24 h with propidium iodide (PI) staining assay, increases after H_2O_2 and is reduced by AG490. Total cell death was determined by exposing the cells to Triton-X100 (see Materials and methods). (c) 30 μM AG490 prevents H_2O_2-induced lactate dehydrogenase (LDH) release at 24 h (one-way ANOVA; $F_{3,15} = 21.72$, $p < 0.0001$). (d) 30 μM AG490 prevents H_2O_2-induced reduction of live cells as assessed by the trypan blue dye exclusion assay (one-way ANOVA; $F_{3,26} = 16.25$, $p < 0.0001$). (e) H_2O_2-induced caspase 3 activation at 24 h ($p < 0.001$) is no longer seen in cells pre-treated with 30 μM AG490 ($p < 0.01$) (one-way ANOVA; $F_{3,11} = 8.54$, $p = 0.0033$). ***$p < 0.001$ and **$p < 0.01$ vs. control; ###$p < 0.001$ and ##$p < 0.01$ vs. H_2O_2.

in which a significant protective effect against 300 μM H_2O_2-induced MTT reduction was already observed from 30 nM to 30 μM, and by the PI assay using 400 μM H_2O_2 (Fig. 3b), in which again significant protection was seen from concentrations of 3–30 μM AG490. A concentration of 30 μM AG490 gave full protection against H_2O_2 in the MTT and PI assays (Figs 3a and b). We then assessed H_2O_2-induced cell death and the protective effect of 30 μM AG490 by other procedures, including measures of LDH release (Fig. 3c) by counting the number of live cells with the trypan blue dye exclusion assay (Fig. 3d). AG490 showed a protective effect against peroxides in all of the assays (Fig. 3). We previously showed that H_2O_2 induces activation of the apoptotic enzyme caspase 3 in astrocytes (Fauconneau *et al.* 2002). Here we found that H_2O_2-induced caspase 3 activation was prevented by AG490 (Fig. 3e).

Primary cultures of astrocytes normally contain some contamination by other glial cells. We carried out secondary cultures of astrocytes (see Materials and methods), which are further enriched in astrocytes in order to verify that the responses that we found were located in astrocytes. H_2O_2 also induced Tyr-701 Stat1 (Fig. 4a) and Tyr-705 Stat3 (Fig. 4b) phosphorylations in these purified astrocyte cultures and this was prevented by pre-treatment with 30 μM AG490 (Figs 4a and b). Here again we observed a strong ($p < 0.001$) protective effect of AG490 against H_2O_2-induced astrocyte cell death ($p < 0.001$) (Fig. 4c). This experiment demonstrates that STAT phosphorylations and cell death after exposure to H_2O_2 took place in astrocytes.

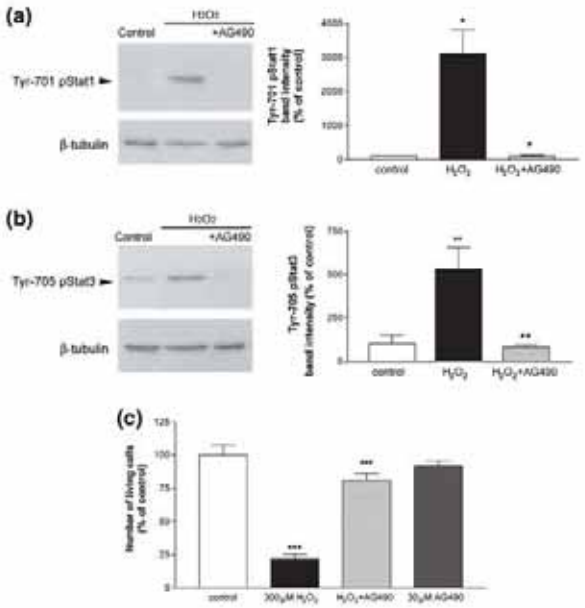

Fig. 4 Purified secondary cultures of astrocytes show a similar response to H_2O_2 exposure as primary cultures. Secondary cultures of astrocytes essentially behave like the primary cultures demonstrating that the responses examined here in the primary astroglia cultures are attributable to astrocytes. (a and b) 1 h exposure to 300 μM H_2O_2 induced tyrosine (Tyr) phosphorylation of Stat1 and Stat3 at 30 min and these effects were prevented by pre-treatment with 30 μM AG490. Statistical analyses (one-way ANOVA by treatment) of pStat1 band intensity showed ($F_{2,3} = 18.46$, $p < 0.02$) that the increase in Tyr-701 Stat1 phosphorylation (post-hoc Bonferroni multiple comparison test; $^*p < 0.05$) is significantly reduced by AG490 (Bonferroni test; $\#p < 0.05$). Likewise, a statistically significant increase ($F_{2,6} = 10.7$, $p < 0.05$) is found for Tyr-705 pStat3 induced by H_2O_2 (Bonferroni test; $^{**}p < 0.01$), which again is prevented by AG490 ($\#\#p < 0.01$). (c) Studies of cell viability with the trypan blue dye exclusion assay at 24 h after 1 h exposure to 300 μM H_2O_2 showed a significant (one-way ANOVA; $F_{3,12} = 44.15$, $p < 0.0001$) reduction in the number of live cells in relation to controls (Bonferroni test; $^{***}p < 0.001$). This effect was strongly attenuated by 30 μM AG490 (Bonferroni test; $\#\#\#p < 0.001$).

Interferon-γ induces Stat1 phosphorylation and causes cell death which is prevented by AG490

A 30-min exposure to 0.5–5 ng/mL INF-γ induced a strong Tyr-701 Stat1 phosphorylation (Fig. 5a) but caused no major Tyr-705 phosphorylation of Stat3 (Fig. 5b) and we did not detect an increase in STAT Ser phosphorylations (Fig. 8e and 8f). AG490 (30 μM; applied 10 min before 0.5 ng/mL INF-γ) prevented Tyr phosphorylation of Stat1 (Fig. 5a). We exposed astrocyte cultures to several concentrations of INF-γ (ranging from 0.5 to 5 ng/mL) and cell death was assessed at 24 h with the trypan blue dye exclusion assay. INF-γ significantly ($p < 0.05$) reduced the number of live cells in a concentration-dependent manner (Fig. 5c). AG490 (30 μM) protected ($p < 0.05$) astrocytes against cell death induced by INF-γ (0.5 ng/mL; $p < 0.05$) in relation to controls, as assessed by the trypan blue dye exclusion assay (Fig. 5d).

Interleukin-6 induces Stat1 and Stat3 phosphorylation and causes cell death which is prevented by AG490

Cells were exposed to several concentrations of IL-6 ranging from 1 to 100 ng/mL. Significant Tyr-705 Stat3 and Tyr-701 Stat1 phosphorylations were detected at a concentration of 100 ng/mL after 30 min exposure (Figs 6a and b). Tyr-705 Stat3 phosphorylation was also detected after 10 min exposure to 100 ng/mL IL-6 (not shown). No effect of IL-6 on Ser phosphorylation of Stat1 or Stat3 was detected (Fig. 8e and 8f). Tyr phosphorylation of Stat3 and Stat1 was prevented by 30 μM AG490 (applied 10 min before IL-6) (Figs 6a and b). Exposure of astrocytes to 100 ng/mL IL-6 for 24 h caused some toxicity as assessed with the trypan blue dye exclusion assay, which showed around a 30% reduction in the number of live cells ($p < 0.001$) (Fig. 6c). Again, IL-6-induced cell death was prevented by 30 μM AG490 ($p < 0.01$) (Fig. 6c).

Interleukin-10 induces Stat3 but not Stat1 phosphorylation and does not cause cell death

Interleukin-10 (10–100 ng/mL) induced Tyr-705 phosphorylation of Stat3 (Fig. 7a) at 30 min but phosphorylation of Stat1 on residue Tyr-701 was not detected at the doses tested here (up to 150 ng/mL) (Fig. 7b). Likewise, we did not detect Ser phosphorylations under the present conditions (Figs 8e and f). Pre-treatment with 30 μM AG490 10 min before IL-10 exposure did not prevent Tyr-705 Stat3 phosphorylation (Fig. 7a), thus demonstrating that Jak2 did not mediate it. In order to discover whether Stat3 phosphorylation was at all associated with cell death after exposure to IL-10, astrocyte cultures were exposed to concentrations of IL-10 ranging from 10 to 100 ng/mL for 24 h and cell death was evaluated with the trypan blue dye exclusion assay (Fig. 7c) and LDH release (Fig. 7d). No effect of IL-10 on cell viability was detected with these procedures (Figs 7c and d).

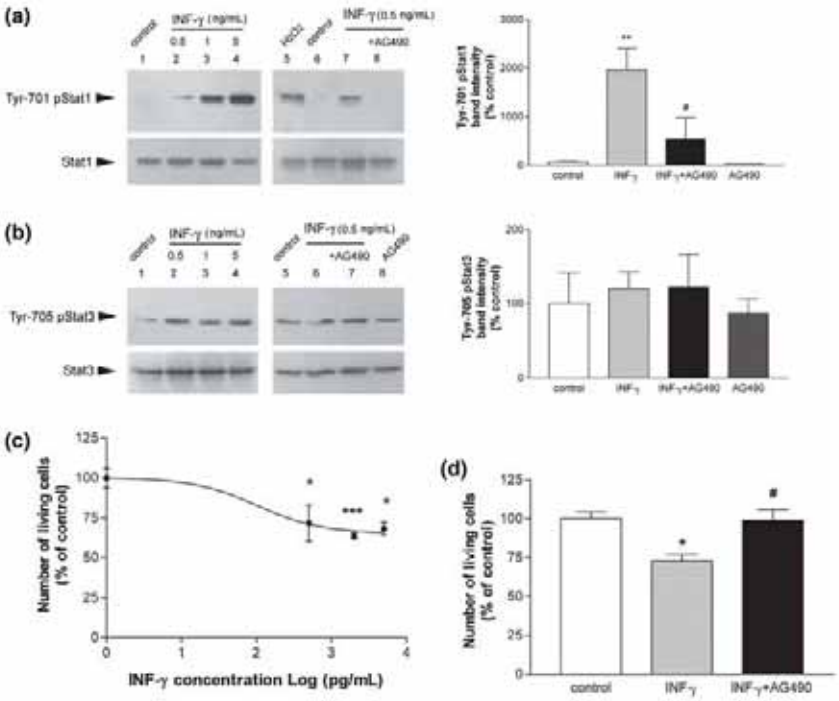

Fig. 5 Interferon (INF)-γ induces Stat1 phosphorylation and causes cell death in astrocytes, which is prevented by AG490. (a) Phosphorylation of Stat1 is induced by INF-γ in a concentration-dependent manner (lanes 2–4) in relation to controls (lanes 1 and 6). Stat1 phosphorylation (lane 7) is abolished by pre-treatment (10 min before INF-γ) with 30 μM AG490 (lane 8). Exposure to H_2O_2 is used as a positive control (lane 5). Measures of band intensity ($n = 3$–4 samples per group) show statistically significant differences between groups (ANOVA; $F_{2,12} = 7.95$, $p < 0.006$) with an increase after INF-γ (**$p < 0.01$), which is reduced by AG490 ($p < 0.05$). (b) Compared with Stat1, only minor phosphorylation of Stat3 is observed after INF-γ and data analysis shows that differences against controls did not reach statistical significance. (c) Cells were exposed to various concentrations of INF-γ and the number of live cells was assessed at 24 h by means of the trypan blue dye exclusion assay. The concentration–response curve was fitted to a one-phase exponential decay equation (Graphpad software); the goodness of the fit was assessed by $r^2 = 0.83$. INF-γ significantly (one-way ANOVA; $F_{3,12} = 5.75$; $p = 0.011$) reduces the number of live cells. Post-hoc analysis reveals a significant effect at concentrations of 0.5 ng/mL (*$p < 0.05$), 2 ng/mL (***$p < 0.001$) and 5 ng/mL (*$p < 0.05$). (d) AG490 (30 μM) prevents the reduction of the number of cells induced by 0.5 ng/mL INF-γ (one-way ANOVA; $F_{2,9} = 8.23$; $p < 0.01$). Post-hoc analysis showed a significant reduction in the number of live cells by INF-γ in relation to controls (*$p < 0.05$) and a complete prevention of this effect by AG490 (#$p < 0.05$). Tyr, Tyrosine.

Comparative levels of STAT phosphorylation induced by H_2O_2, interferon-γ, interleukin-6 and interleukin-10

The different experimental conditions used here could be ranked according to their ability to phosphorylate STAT proteins and to their toxicity. For STAT protein phosphorylation the effect of oxidative stress derived from peroxides was examined at 30 min after 1 h exposure to 300 μM H_2O_2 and the effect of cytokines was examined after 30 min exposure to either INF-γ (0.5 ng/mL), IL-6 (100 ng/mL) or IL-10 (100 ng/mL). Cell viability, as assessed with the trypan blue dye exclusion assay, was studied at 24 h after 1 h exposure to H_2O_2 or after 24 h exposure to the cytokines at the same doses as above. H_2O_2 was the largest inducer of Tyr-701 Stat1 phosphorylation followed by INF-γ and IL-6, while IL-10 did not show a noticeable effect in relation to control (Fig. 8a). The extent of Tyr-701 Stat1 phosphorylation was related to the extent of cell death by examining the correlation between the measure of Tyr-701 pStat1 (band intensity) and the reduction of cell viability induced by exposure to these agents. A negative correlation was found between the induction of Tyr-701 Stat1 phosphorylation and cell viability (Fig. 8b), thus suggesting that activation of Stat1 was associated with cell death. The largest inducer of Tyr-705 Stat3 phosphorylation was again H_2O_2 treatment followed by IL-10 and IL-6, whereas the effect of INF-γ was very small (Fig. 8c). Comparison of the level of Tyr-705 phosphorylation with the corresponding cell viability after the different treatments illustrated that the extent of Stat3 phosphorylation was not clearly associated with cell death (Fig. 8d). Indeed, 24 h treatment with 0.5 ng/mL INF-γ caused a similar reduction in cell viability as 24 h exposure to 100 ng/mL IL-6 in spite of the fact that significant induction of Tyr-705 Stat3 phosphorylation was only detected after IL-6 but not after INF-γ. Also, 100 ng/mL IL-10

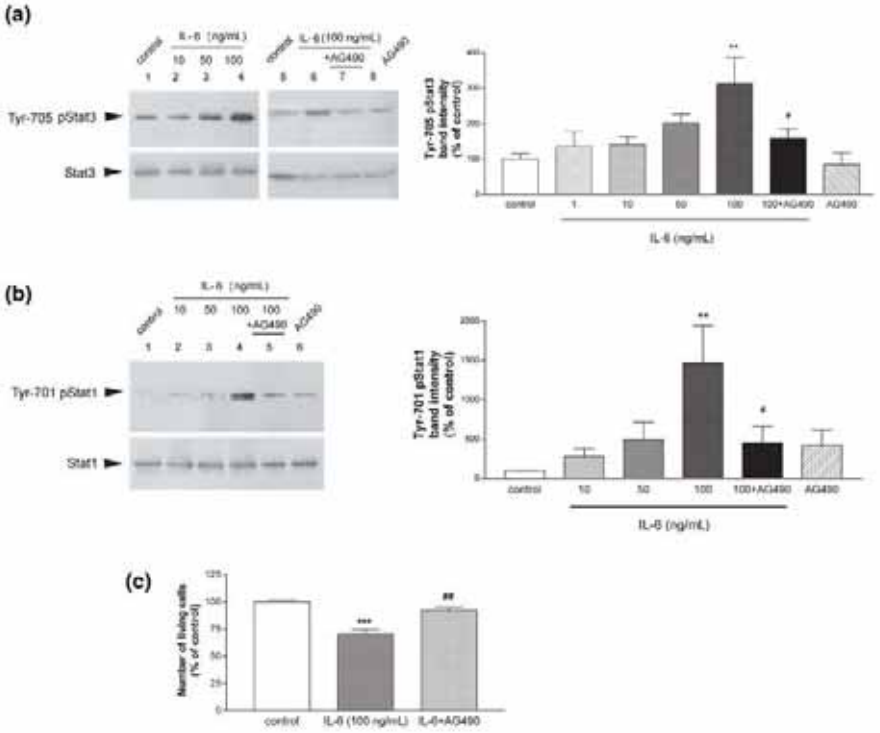

Fig. 6 IL-6 induces Stat1 and Stat3 phosphorylation and causes cell death in astrocytes, which is prevented by AG490. (a) Tyrosine phosphorylation of Stat3 was examined after 30 min exposure to various doses of IL-6 (1 to 100 ng/ml) (lanes 2–4,6), and the effect of 30 μM AG490 (given 10 min prior to IL-6) was tested (lane 7). 100 ng/mL IL-6 significantly increases (ANOVA: $F_{(6,49)} = 2.96$; $p < 0.02$) Tyr-705 Stat3 phosphorylation in relation to controls (lanes 1,5) (**$p < 0.01$). This effect is abolished by AG490 (lane 7) (#$p < 0.05$), which alone has no effect (lane 8). (b) Tyrosine phosphorylation of Stat1 was studied in controls (lane 1) and after IL-6 exposure (lanes 2–4) under the same conditions as in (a). 100 ng/mL IL-6 cause a significant increase (**$p < 0.01$) of Tyr-701 Stat1 phosphorylation, and this is prevented by AG490 (lane 5) (#$p < 0.05$) (one-way ANOVA: $F_{(5,19)} = 2.95$; $p < 0.05$). AG490 alone has no effect (lane 6). (c) Cells were exposed IL-6 (100 ng/mL) and the number of live cells was assessed at 24 h with the trypan blue dye exclusion assay. IL-6 reduces the number of live cells, an effect that is prevented by AG490 (30 μM) (one-way ANOVA: $F_{(2,9)} = 26.27$; $p = 0.002$). Post-hoc analysis (Bonferroni's test) shows a significant reduction of live cells by IL-6 in relation to controls (***$p < 0.001$), and prevention of this effect by AG490 (##$p < 0.01$).

induced a similar level of Stat3 phosphorylation as 100 ng/mL IL-6 while the reduction in cell viability was only detected after 24 h exposure to IL-6 but not to IL-10. Ser-727 Stat1 phosphorylation was only detected after H_2O_2 treatment (Fig. 8e) and this might promote Stat1 activity induced by Tyr-701 phosphorylation after this treatment. Basal levels of Ser-727 Stat3 phosphorylation remained unaffected by these treatments (Fig. 8f), suggesting that this was not involved in the activation of Stat3 induced by H_2O_2 or certain cytokines. Altogether, this experiment supports the view that phosphorylation of Stat1 on residue Tyr-701 is associated with cell death.

Production of reactive oxygen species

In order to discover whether peroxides could contribute to the reported actions of cytokines on STAT phosphorylation we examined the production of reactive oxygen species by measuring the conversion of 2′,7′-dichlorofluorescein diacetate to fluorescent DCF (see Materials and methods). Exposure of astrocytes to H_2O_2 caused a 3.5-fold increase in DCF fluorescence [mean ± SD 3158 ± 388 fluorescence units (FU)] in relation to controls (mean ± SD 990 ± 153 FU) ($n = 10$ per group; *t*-test, $p < 0.001$). In contrast, exposure to several concentrations of INF-γ did not cause changes in relation to control values. Mean ± SD DCF measured fluorescence values ($n = 6$ per group) were: control, 793 ± 139 ng/mL; 50 ng/mL INF-γ, 669 ± 106 ng/mL; 5 ng/mL INF-γ, 766 ± 294 ng/mL; 0.5 ng/mL INF-γ, 597 ± 164 ng/mL. This shows that INF-γ did not increase the cellular production of peroxides and therefore that peroxides did not contribute to the STAT phosphorylation induced by INF-γ.

We then tested whether AG-490 had any effect on H_2O_2-mediated DCF production. In spite of the fact that, to our knowledge, any antioxidant effects of AG-490 were not previously reported, we observed that this drug prevented the increase of DCF fluorescence by H_2O_2. Mean ± SD ($n = 10$ per group) DCF fluorescence values by H_2O_2 ($n = 10$) decreased around sixfold in the presence of AG-490

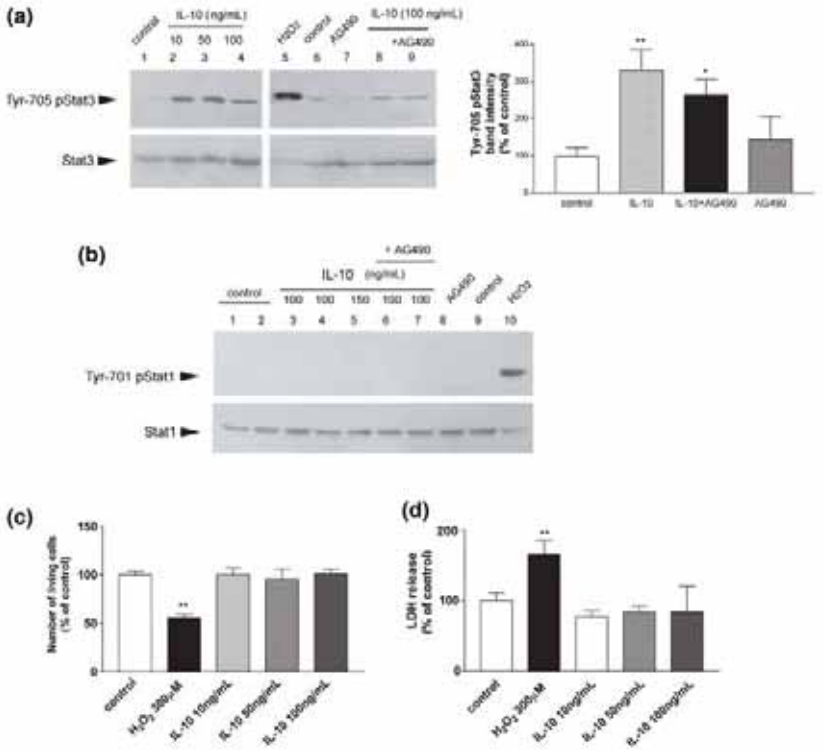

Fig. 7 Interleukin (IL)-10 induces Jak2-independent Stat3 tyrosine (Tyr) phosphorylation, which is not accompanied by Stat1 Tyr phosphorylation and does not cause cell death in astrocytes. (a) IL-10 (10–100 ng/mL) (lanes 2–4) induces Tyr-705 Stat3 phosphorylation 30 min after exposure in relation to untreated controls (lane 1). IL-10-induced Stat3 activation (lane 8) is not prevented by 30 μM AG490 (lane 9), which was applied 10 min before IL-10. Statistical analysis of band intensity data shows significant differences between groups ($F_{3,31} = 4.27$, $p < 0.0014$), with an increase in Stat3 phosphorylation by 100 ng/mL IL-10 above control values ($p < 0.01$), which remain higher than control ($p < 0.05$) in the presence of AG490. (b) IL-10 (100–150 ng/mL) does not induce significant Tyr-701 Stat1 phosphorylation (lanes 3–7) in relation to controls (lanes 1, 2 and 9). AG490 does not appear to affect this parameter, both in combination with IL-10 (lanes 6 and 7) or alone (lane 8). Tyr-701 Stat1 phosphorylation after H_2O_2 (lane 10) is shown as a positive control. (c) Cells were exposed to various concentrations of IL-10 ranging from 10 to 100 ng/mL or to 300 μM H_2O_2 as a positive control and the number of live cells was assessed at 24 h by means of the trypan blue dye exclusion assay. No differences are found after IL-10 exposure in relation to controls, while H_2O_2 significantly (Bonferroni test; $^*p < 0.05$) reduces the number of live cells (ANOVA; $F_{4,15} = 8.91$; $p < 0.001$). (d) Lactate dehydrogenase (LDH) release was measured at 24 h after exposure to various concentrations of IL-10 or 300 μM H_2O_2. IL-10 does not increase LDH release in relation to controls, while H_2O_2, which is used as a positive control, significantly ($^*p < 0.05$) increases it (ANOVA test; $F_{4,15} = 3.307$; $p < 0.05$).

(433 ± 49 ng/mL; $n = 10$; $p < 0.001$). This suggests that AG490 might exert some antioxidant effect against the oxidative stress caused by H_2O_2 in astrocytes.

Discussion

Astrocyte viability was compromised by transient exposure to H_2O_2 and by prolonged exposure to INF-γ or IL-6. We found that, under these conditions, astrocyte cell death was prevented by the Jak2 inhibitor AG490 (Meydan *et al.* 1996), thus strongly suggesting that cell death was mediated through activation of the Jak2/Stat1 pathway. Cytokines trigger activation of Stat1 and/or Stat3 mediated by various JAKs. Jak2 activates Stat1, as for instance in primary astroglial cell cultures after treatment with IFN-γ and LPS (Dell'Albani *et al.* 2001), and Stat3, as seen in myeloma cells (De Vos *et al.* 2000) and in adult ventricular myocytes (Yu *et al.* 2003). Also, Jak1 plays an important role in the activation of Stat3, as found in Src-transformed mouse fibroblasts (Zhang *et al.* 2000), and of Stat1, as shown in thyroid cells exposed to INF-γ (Chung *et al.* 2000). Cytokines can induce activation of both Stat1 and Stat3 but certain agents favour selective activation of one or another STAT protein. Here in cultured astrocytes, INF-γ caused Tyr-701 Stat1 phosphorylation while IL-6 caused Tyr-705 Stat3 and Tyr-701 Stat1 phosphorylations, in agreement with previous data (Heinrich *et al.* 1998), and IL-10 did not activate Stat1 but caused Tyr-705 Stat3 phosphorylation, concordant with Stat3 activation (Makuta *et al.* 2003). Nevertheless, the balance of STAT activation can be switched by combined stimuli, e.g. activation of Stat3 induced by IL-10 is switched to Stat1 activation by INF-γ, which subsequently induces the

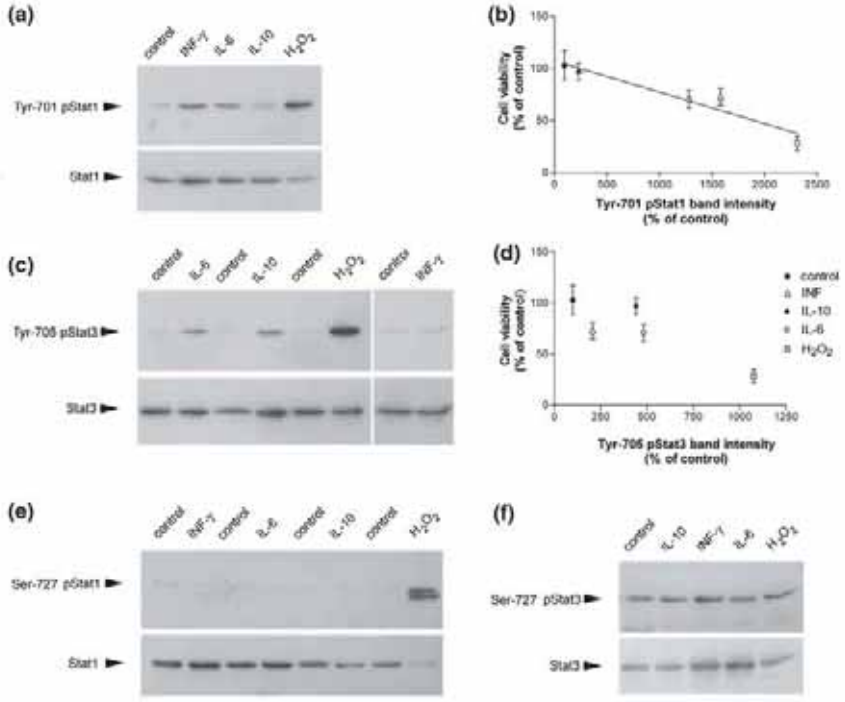

Fig. 8 Comparative levels of STAT protein phosphorylation after exposure to either oxidative stress or cytokines. Cells were exposed to either interferon (INF)-γ (0.5 ng/mL), interleukin (IL)-6 (100 ng/mL) or IL-10 (100 ng/mL) for 30 min or H_2O_2 for 1 h and STAT phosphorylation was examined 30 min later. (a) The highest level of tyrosine (Tyr)-701 Stat1 phosphorylation is found with H_2O_2 followed by similar increases induced by INF-γ and IL-6. (b) Mean ± SEM values of Tyr-701 pStat1 band intensity were plotted against mean ± SD values of cell viability at 24 h, as assessed with the trypan blue dye exclusion assay. Values are expressed as percentage of the corresponding controls. A significant correlation is found between induction of Tyr-701 Stat1 phosphorylation and reduction of cell viability (data were fitted with linear regression analysis, the goodness of the fit was assessed by $r^2 = 0.92$). (c) H_2O_2 also causes the highest induction of Tyr-705 Stat3 phosphorylation followed by IL-10 and IL-6, while the effect of INF-γ is very minor. (d) Values of Tyr-705 Stat3 phosphorylation were plotted against values of cell viability, as above. IL-10 causes no cell death but a similar induction of Tyr-705 Stat3 phosphorylation as IL-6, which induces around 30% reduction in cell viability, while INF-γ causes very minor Tyr-705 Stat3 phosphorylation but a similar reduction in cell viability as IL-6. (e) Appreciable levels of serine (Ser)-727 Stat1 phosphorylation are only observed after H_2O_2. (f) Basal levels of Ser-727 Stat3 phosphorylation are not affected by these treatments.

expression of inflammatory genes (Herrero *et al.* 2003). This suggests that a fine equilibrium between the activation of the different proteins of the STAT family underlies the specificity of the regulation of gene transcription and of their subsequent biological effects. Furthermore, a cross-talk between different cytokine pathways influencing the cellular responses to inflammatory stimuli has been reported, e.g. Stat1 interacts with the tumour necrosis factor receptor 1 (Wesemann and Benveniste 2003). Besides inflammatory stimuli, chemically generated oxidative stress induces Stat3 activation in rat liver (Tacchini *et al.* 2002) and oxidative stress generated with oxidized low-density lipoprotein activates Stat1 and Stat3 in MRC5 human fibroblasts (Maziere *et al.* 2001). Likewise, we found here phosphorylation of Stat1 on Tyr-701 and Ser-727 and of Stat3 on Tyr-705 after H_2O_2 exposure.

Stat3 was activated in astrocytes after exposure to H_2O_2, IL-6 and IL-10 whereas Stat1 was activated under those conditions that later caused cell death, i.e. H_2O_2, INF-γ and IL-6, but not by exposure to the anti-inflammatory cytokine IL-10. Indirect evidence favouring Stat1, rather than Stat3, being associated with cell death was obtained by comparing the extent of STAT phosphorylation under the different treatments with the corresponding reduction of cell viability. This showed that Tyr-701 Stat1 phosphorylation was negatively correlated with cell viability. In the conditions inducing astrocyte cell death, Stat1 and Stat3 activation was prevented by Jak2 inhibition, while Jak2 did not mediate activation of IL-10-induced Stat3. Therefore, activation of Stat1 by Jak2 was identified as a crucial upstream signal inducing astroglial cell death. Caspase 3 is activated after exposure to H_2O_2 (Fauconneau *et al.* 2002), an effect which was fully prevented here by inhibiting Jak2, thus suggesting that this pathway has pro-apoptotic properties. Accordingly, active Stat1 has been identified as a pro-apoptotic factor after various insults, such as LPS/D-galactosamine-induced liver apoptosis (Kim *et al.* 2003) and ischaemia/reperfusion in cardiac murine myocytes (Stephanou *et al.* 2002), by promoting the transcription of pro-apoptotic and pro-inflammatory genes,

as shown in lung epithelial adenocarcinoma cells (Kristof *et al.* 2003) and in foetal astrocytes (Lee *et al.* 2003). However, redox-dependent activation of Stat1 in cultured rat hepatocytes subjected to hypoxia/reoxygenation has a dual effect as it triggers apoptotic signals shortly (during the first 24 h) after the insult, whereas it mediates resistance to apoptotic cell death, possibly through induction of the heat-shock stress protein HSP70, at later time points (48–72 h) (Terui *et al.* 2004). Therefore, targeting Stat1 activation might be beneficial in the acute phase of certain oxidative stress stimuli or pro-inflammatory insults, whereas intervention at late phases might be self-defeating.

In contrast to Stat1, Stat3 has been mainly attributed antiapoptotic properties (Fukada *et al.* 1996; Hirano *et al.* 1997; Catlett-Falcone *et al.* 1999). Stat3 activation is associated with survival in a paradigm of ischaemic pre-conditioning in the myocardium (Hattori *et al.* 2001) and the JAK/STAT pathway is regarded as a protective agent in cardiovascular diseases (Bolli *et al.* 2003) and after focal cerebral ischaemia (Suzuki *et al.* 2001). Nonetheless, evidence suggesting that phosphorylation of Stat3 might be involved in neuronal death after cerebral ischaemia was reported (Wen *et al.* 2001). However, one key factor underlying this apparent controversy about the biological effect of Stat3 might be whether, and to what extent, Stat1 is concomitantly activated. Here, evidence that activation of Stat3 is associated with astrocyte survival was obtained from the observation that IL-10 activated Stat3, but not Stat1, in a Jak2-independent manner and did not cause cell death. In fact, IL-10 has been reported to promote survival in retinal ganglion cells mediated through Stat3 (Boyd *et al.* 2003). IL-6, a well-known activator of Stat3 (Schumann *et al.* 1999; Debonera *et al.* 2001; Niehof *et al.* 2001), also caused Stat3 activation in astrocytes together with Stat1 activation, both mediated through Jak2. The biological effect of IL-6 against CNS injury is controversial. While there is a certain body of evidence for a neuroprotective action of IL-6 (Bolin *et al.* 2002; Leu *et al.* 2003; Park *et al.* 2003; Pavelko *et al.* 2003; Penkowa *et al.* 2003), other data suggest that it has a pro-inflammatory action and participates in the acute-phase response triggered by stroke (Beamer *et al.* 1995; Tarkowski *et al.* 1995; Vila *et al.* 2000). In patients, high serum levels of IL-6 correlate with early neurological worsening after stroke (Vila *et al.* 2000). Here, only 24 h exposure of astrocytes to high concentrations of IL-6 (100 ng/mL) induced cell death, which again was prevented by AG490. Therefore, the toxic effect of IL-6 on astrocytes might only become apparent at high doses and would be dependent on activation of Jak2.

In addition to Tyr phosphorylation, STAT proteins can suffer Ser phosphorylation (Wen *et al.* 1995; Wen and Darnell 1997). Here, exposure of astrocytes to H_2O_2 induced Ser-727 phosphorylation of Stat1 but not of Stat3. Ser-727 STAT phosphorylation can be mediated by the MAPK Erk1/2 (Chung *et al.* 1997). Although we previously showed that H_2O_2 causes Erk1/2 phosphorylation in astrocytes (Fauconneau *et al.* 2002), it is unlikely that this signal mediated Ser-727 Stat1 phosphorylation as Stat3 is a much better Erk substrate than Stat1 (Chung *et al.* 1997). Also, AG490 was reported to down-regulate the activation of Erk2 in an IL-6-dependent human myeloma cell line (De Vos *et al.* 2000), which subsequently indicates that phosphorylation of Stat1 on Ser would be affected by AG490. Here, AG490 did not prevent H_2O_2-induced Ser-727 Stat1 phosphorylation as levels were significantly above control levels, thus suggesting that kinases other than Erk contribute to the Ser phosphorylation of Stat1 reported here. p38 is a candidate kinase involved in phosphorylating Stat1 as it has been reported to play a key role in the Ser phosphorylation of Stat1 (Goh *et al.* 1999) and is activated in primary cultures of astrocytes after exposure to H_2O_2 (Robinson *et al.* 1999). In spite of the fact that Stat1 was Ser phosphorylated after H_2O_2, here Tyr, rather than Ser, was identified as the crucial phosphorylation signal on Stat1 involved in promoting cell death, as it was prevented by the protective treatment with AG490.

Although AG490 has long been regarded as a specific Jak2 kinase inhibitor (Duhé *et al.* 2002), recent evidence indicates that AG490 exerts some inhibition on the Tyr kinase activity of the epidermal growth factor receptor (Luo and Laaja 2004) and on Jak3 activity (Wang *et al.* 1999). In addition to these described effects of AG490, the present results suggest that this drug might have some antioxidant action against peroxides. Although AG490 fully inhibited STAT Tyr phosphorylation, it did not affect Stat1 Ser phosphorylation induced by H_2O_2, thus showing that AG490 did not prevent all of the effects of H_2O_2 in astrocyte cultures. Further studies are needed to unravel the extent and nature of the effects of AG490 against oxidative stress and the actual involvement of Jak2/Stat1 in peroxide-induced cell death.

Altogether these findings suggest that abrogating Jak2/Stat1 activation might be a therapeutic approach in the acute phase of diseases involving inflammation in neural cells. However, Stat1 is an essential signalling molecule mediating the cytokine responses necessary to overcome viral and bacterial infections (Durbin *et al.* 1996; Meraz *et al.* 1996). In CH235-MG astroglioma cells, Stat1 signalling mediates IFN-γ-induced class II major histocompatibility complex (MHC) expression, which is critical in the immune responses (Lee and Benveniste 1996). Nonetheless, hypothetical treatments in human diseases targeting Stat1 activation might induce immunodepression conveying increased susceptibility to infection. A challenge for future studies will be the design of specific cell-targeted therapeutic approaches aimed at acutely inhibiting the activation of the Jak2/Stat1 pathway in particular cells.

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Article nº 2

Exposure of Glia to Pro-Oxidant Agents Revealed Selective Stat1 Activation by H_2O_2 and Jak2-Independent Antioxidant Features of the Jak2 Inhibitor AG490

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Exposure of Glia to Pro-Oxidant Agents Revealed Selective Stat1 Activation by H_2O_2 and Jak2-Independent Antioxidant Features of the Jak2 Inhibitor AG490

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reactive oxygen species; protein oxidation; peroxide; superoxide; siRNA

ABSTRACT
The JAK/STAT pathway is activated in response to cytokines and growth factors. In addition, oxidative stress can activate this pathway, but the causative pro-oxidant forms are not well identified. We exposed cultures of rat glia to H_2O_2, $FeSO_4$, nitroprussiate, or paraquat. We assessed oxidative stress by measuring reactive oxygen species (ROS) and oxidated proteins, we determined phosphorylated Stat1 (pStat1), and we evaluated the effect of antioxidants (trolox, propyl gallate, and *N*-acetylcysteine) and of Jak2 (Janus tyrosine kinases) inhibitors (AG490 and Jak2-Inhibitor-II). Pro-oxidant agents induced ROS and protein oxidation, excluding nitroprussiate that induced protein nitrosylation. H_2O_2, and to a lesser extent $FeSO_4$, increased the level of pStat1, whereas nitroprussiate and paraquat did not. Trolox and propyl gallate strongly prevented ROS formation but they did not abolish H_2O_2-induced pStat1. In contrast, NAC did not reduce the level of ROS but it prevented the increase of pStat1 induced by H_2O_2, evidencing a differential effect on ROS formation and on Stat1 phosphorylation. H_2O_2 induced pStat1 in mixed glia cultures and, to a lesser extent, in purified astroglia, but not in microglia. Jak2 inhibitors reduced H_2O_2-induced pStat1, suggesting the involvement of this kinase in the increased phosphorylation of Stat1 by peroxide. Unexpectedly, AG490, but not Jak2-Inhibitor-II, reduced ROS formation, and it abrogated lipid peroxidation in microsomal preparations. Furthermore, AG490 reduced ROS in glial cells that were transfected with siRNA to silence Jak2 expression. These findings reveal previously unrecognized Jak2-independent antioxidant properties of AG490, and show that Jak2-dependent Stat1 activation by peroxide is dissociated from ROS generation.

INTRODUCTION

STATs are latent transcription factors in the cytoplasm that become phosphorylated by Janus tyrosine kinases (JAK) after ligand binding to cytokine and growth factor receptors. In this manner, the JAK/STAT pathway mediates the transduction of signals from the cell membrane to the nucleus and it activates gene transcription (Aaronson and Horvath, 2002; Darnell, 1997; Levy and Darnell, 2002; Rawlings et al., 2004). This pathway is thought to be essential for the biological responses to cytokines (de Prati et al., 2005; Gao, 2005; Hebenstreit et al., 2005; Leonard and O'Shea, 1998; O'Shea et al., 2002; Platanias, 2005). In addition, certain types of pro-oxidant conditions have been reported to activate Stat1 and/or Stat3 (Carballo et al., 1999; Gorina et al., 2005; Madamanchi et al., 2001; Maziere et al., 1999, 2001; Simon et al., 1998; Tacchini et al., 2002). Indeed, STAT activation was found to be induced by peroxide, but not by superoxide or nitric oxide (NO), and it was associated to an increase of the tyrosine-kinase activity of Jak2 and Tyk2 (Simon et al., 1998). Furthermore, exposure to hydrogen peroxide has been reported to induce phosphorylation of Jak2 (Tawfik et al., 2005; Yu et al., 2006). However, the specific pro-oxidant agents and the molecular mechanism underlying the latter effects remain to be clarified.

Oxidative stress is believed to contribute to inflammation and neuronal damage in several neurological diseases, including stroke, Parkinson's, and Alzheimer's diseases. Therefore, understanding the molecular mechanisms activated by pro-oxidant agents in brain cells may help to unravel oxidative stress-related pathological events leading to brain damage. Oxidative stress activates the JAK/STAT pathway in cultured astrocytes (Gorina et al., 2005). JAK/STAT is recognized to play important roles in astrogliogenesis (He et al., 2005) and to mediate the cellular response to proinflammatory stimuli in cultured glia (Dell'Albani et al., 2001; Gorina et al., 2005; Kahn et al., 1997; Kim et al., 2002; Natarajan et al., 2004). Also, several lines of evidence suggests its involvement in the activation of glia after brain injury, such as in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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model of neurodegeneration (Sriram et al., 2004), and in cerebral ischemia (Justicia et al., 2000; Planas et al., 1996, 1997).

Here we investigated the capacity of several pro-oxidant agents to activate Stat1 in cultured glia, and whether the effects of these compounds could be attenuated by antioxidants and Jak2 inhibitors. We identified peroxide, among various reactive oxygen species (ROS) sources, as the main trigger of Stat1 activation, and that this effect was mediated by Jak2. While performing this study we evidenced that one of the Jak2 inhibitors tested, AG490, showed antioxidant properties that were independent of its action on Jak2. These features might be relevant to the biological effects of AG490 when used as a Jak2 inhibitor.

MATERIALS AND METHODS

Glia Cultures

Mixed glial cell cultures were prepared from cerebral cortex of 1- to 2-day-old Sprague-Dawley rats as described previously (Gorina et al., 2005), with slight modifications. Briefly, for each culture 10 pups were decapitated, their brain was aseptically removed, and placed on ice in sterile culture dishes with phosphate-buffered saline (PBS) (NaH_2PO_4 and K_2HPO_4 10 mM pH 7.4 in isotonic NaCl solution). The midbrain, meninges, and blood vessels were removed by dissection. The remaining cerebral cortices were mechanically dissociated by pipetting for 2 min with a Pasteur pipette in 6 mL of DMEM (Dulbecco's modified Eagle medium, Gibco-BRL, Invitrogen, Paisley, UK). The suspension was filtered through a 70-μm pore size nylon mesh cell strainer (Becton Dickinson, Franklin Lakes, NJ). Then cells were plated in 6 or 96-well plates (NUNC, Roskilde, Denmark) and maintained in DMEM supplemented with 20% foetal bovine serum (FBS; Gibco-BRL) and 4 mL/L of penicillin/streptomycin (10,000 U/10,000 μg/mL) (Gibco-BRL), at 37°C in an humidified atmosphere of 5% CO_2 and 95% air. Medium was changed once per week with the incubation medium supplemented with 10%, then 7% FBS. Confluence was achieved after 12–13 days *in vitro* (DIV). At this point mixed glia cultures typically consisted of 75% type-I astrocytes and 25% microglia.

To further enrich glia cultures in astrocytes, mouse primary mixed glial cultures on 10–12 DIV were treated with 10 μM of the antimitotic cytosine arabinoside (Ara-C, Sigma-Aldrich) for 4 days to eliminate dividing cells, i.e. mostly microglia and progenitors, but not quiescent cells, i.e. mostly confluent astrocytes. The adherent cells were detached with trypsin 0.05%/EDTA 0.2 mM and seeded at 6×10^4 cells/mL with culture medium (see earlier). Astrocytes were used 2 days after subculturing. To estimate the purity of the cultures we did immunostaining with glial fibrillary acidic protein (GFAP, no. Z0334, Dako) diluted 1:1,000, and with CD11b (OX42, no. MCA275G, Serotec) diluted 1:500 to detect microglia. Fluorescent corresponding secondary antibodies were used and nuclei were stained with Hoechst. By cell counting under the fluorescence microscope we estimated that the purity of enriched astrocytes was above 96%.

Microglial cells were obtained by mild tripsinization with a method yielding high purity (>98%) (Saura et al., 2003), and they were used 1 day after isolation.

Treatments

Cells were exposed to the pro-oxidant agents H_2O_2 (2 μM–1.2 mM), $FeSO_4$ (1–50 μM), the NO donor nitroprussiate (NP) (10–500 μM), or the herbicide 1,1′-dimethyl-4,4′-bipyridium (paraquat, PQ) (25 μM–2 mM). All reagents, unless otherwise stated, were from Sigma. Treatments with the antioxidants Trolox (Tx) (5 nM–50 μM), propyl gallate (PG) (5 nM–50 μM), and *N*-acetylcysteine (NAC) (10 μM–100 mM) were applied 30 min before exposure to the pro-oxidant agents. Likewise, the Jak2 inhibitor AG490 (Calbiochem) was applied (5 nM–30 μM) 30 min before exposure to pro-oxidant agents, whereas Jak2-Inhibitor-II (Calbiochem) was applied (50 μM) either 30 min or 16 h earlier. AG490 and Jak2-Inhibitor-II were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture was 0.1% for AG490 and 0.6% for Jak2-Inhibitor II. Control cultures were exposed to the corresponding amount of this vehicle.

DCFH-DA Technique

Oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent DCF is taken as an index of overall oxidative stress in biological systems (LeBel et al., 1992). Cells seeded in 96-well plates were incubated for 20 min with DCFH-DA (10 μM) in HEPES-buffered saline solution. After DCFH-DA incubation, cells were washed with the buffer and the basal DCF fluorescence was measured in a fluorimeter (CytofluorTM 2350, Millipore) with 485 nm-excitation and 530 nm-emission filters. Then, antioxidants, Jak2 inhibitors, or corresponding vehicles were added. Thirty minutes later the different pro-oxidant agents were added and were incubated for 1 h. After this time, fluorescence was measured as earlier and the basal values were subtracted. To normalize between experiments, the results of each experiment were expressed as the percentage of control or as the percentage of the pro-oxidant (for antioxidants). Then the mean of n experiments was calculated (the n value is indicated in each figure legend).

DHE Technique

The intracellular generation of superoxide anion ($O_2^{-\bullet}$) was monitored by the selective oxidation of dihydroethidium (DHE) to ethidium (Bindokas et al., 1996). Briefly, cells grown in 96-well plates were loaded with 10 μm

DHE (Molecular Probes) for 10 min at 37°C in the dark. Superoxide anion oxidizes DHE intracellularly to produce ethidium, which intercalates into DNA and emits fluorescence. After 10 min incubation with 4.8 μM DHE, basal fluorescence was measured at 485-nm excitation and 590-nm emission wavelengths in a fluorimeter (CytofluorTM 2350, Millipore). Then the cells were treated with antioxidant agents or the corresponding vehicles, and 30 min later the pro-oxidant compounds were added and incubated for 1 h. After this time, fluorescence was measured again and the basal measure was subtracted. Inter-experiment normalization was carried out by expressing the values as the percentage of control for each individual experiment. Then the mean of n experiments was calculated (the n value is indicated in each figure legend).

Measurement of TBARS

Lipid peroxidation was determined with the thiobarbituric acid reactive substances (TBARS) test in rat liver microsomes. This system relies on microsomal-mediated oxidation and on addition of Fe^{2+}-ascorbate as an external reductive system (Aruoma et al., 1989). A suspension of Sprague-Dawley rat liver microsomes (Advancell, Barcelona, Spain) in Krebs-Ringer phosphate, pH 7.2, was prepared at 1 mg of microsomal protein per mL. The microsomal protein suspension was pretreated with different concentrations of AG490 (dissolved in DMSO to a final concentration <1%) for 15 min at 37°C. Then, the suspension was incubated with 50 μM $FeSO_4$ and 500 μM ascorbic acid during 40 min at 37°C, and centrifuged at 12,000g for 10 min. Then, 750 μL of thiobarbituric acid solution (17 mM thiobarbituric acid containing 2% perchloric acid) was added to 100 μL of the supernantant of the microsomal preparation and heated during 1 h at 95°C. After this time samples were cooled on ice for 10 min, and centrifuged at 12,000g for 5 min. Supernatants were neutralized with NaOH to pH 6, and immediately analyzed by high performance liquid chromatography (HPLC). The analytical procedure used for determination of MDA·2TBA compound was based on a reported method (Bird et al., 1983). Samples were immediately injected into an HPLC system equipped with a 4.6 × 150 mm^2 C_{18} column (5 μm, XTerra, MS). The eluent solution was 52% buffer (50 mM formic acid containing triethylamine) at pH 6 and 48% methanol, with 1 mL/min isocratic flow rate. Detection was carried out at 532 nm.

Western Blotting

Cell cultures were homogenized in radioimmunoassay buffer (RIPA) containing 0.01 M PBS, sodium dodecyl sulphate, sodium deoxycholate, the nonionic detergent Igepal, and the protease inhibitor cocktail Complete (Boehringer Mannheim, Mannheim, Germany), as reported (Gorina et al., 2005). The protein concentration of samples was determined with the Bradford assay (Bio-Rad, Hercules CA, USA). Twenty-five microgram of the protein extracts were run in denaturing 8% polyacrylamide gels and were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with one of the following primary antibodies: mouse monoclonal antibodies against Stat1 (BD Transduction Laboratories, BD Biosciences, Madrid, Spain); GFAP, as a marker of astroglia, (Boehringer Mannheim) diluted 1:8,000; ED1, as a marker of microglia, (Serotec) diluted 1:5,000; and β-Tubulin (Sigma, St Louis, MO, USA) diluted 1:10,000; and rabbit polyclonal antibodies against Jak2 diluted 1:1,000; and Tyr^{701}-phospho-Stat1 diluted 1:1,000 (Cell Signaling Technology, Beverly, MA). On the following day membranes were incubated for 1 h with Ig peroxidase-linked secondary antibodies (1:2,000) (Amersham, Piscataway, NJ). The reaction was visualized with a chemiluminiscence method. Membranes were reblotted with β-Tubulin or Stat1 as loading controls. Protein nitrosylation was studied by incubating the membranes with a mouse monoclonal anti-nitrotyrosine antibody (ab7048, Abcam Limited, Cambridge, UK) diluted 1:1000. Band intensity was measured using Quantity One software (Bio-Rad Laboratories), and expressed as the ratio to the corresponding band intensity of the loading control to correct for any differences in protein content between lanes. Treated samples were always run in the same gel with corresponding control samples. To validate the semiquantitative Western blot analysis of pStat1 we examined the increase in signal intensity as a function of the amount of protein loaded in the gels (ranging between 10 and 40 μg). We found that the increase of signal intensity was linear within this range of protein (not shown).

Measurement of Oxidative Protein Modification by the Oxyblot Technique

The carbonyl groups in the protein side chains produced by oxidative agents were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhidrazine (DNPH). Twenty microgram of protein sample were derivatized with DNPH solution following the instructions of the manufactures (OxyBlot™ Protein Oxidation Detection Kit, Chemicon International, CA). DNP-derivatized proteins were separated in 8% polyacrilamide gels by electrophoresis followed by Western Blotting. Membranes were incubated with a primary antibody, specific for the DNP moiety of the proteins, and incubated with a horseradish peroxidase-linked antibody (goat anti-rabbit IgG). Antibodies were supplied in the same kit. The reaction was developed and semiquantification of band intensity was carried out as explained before in Western blotting. We observed the appearance of several bands in the blots, and one of them (see Results) was chosen for semi-quantification. For semiquantitative Oxyblot blot analysis we found that the amount of protein loaded in the gels (ranging

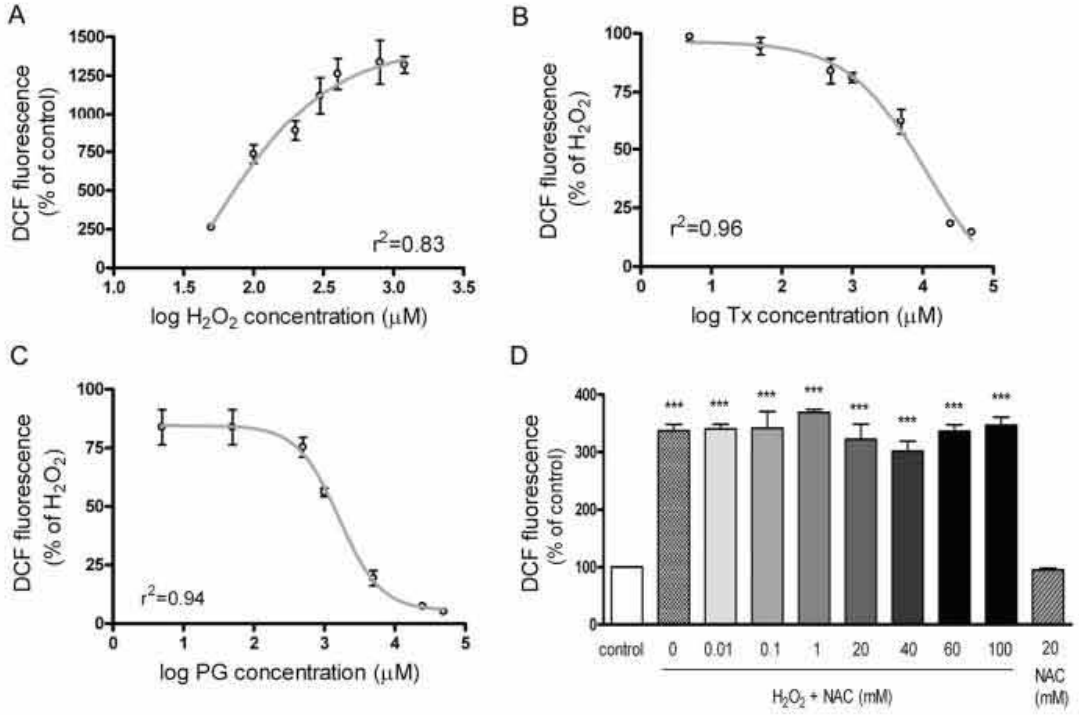

Fig. 1. Exposure of primary cultures of mixed glia cells to H_2O_2 generates ROS, which is prevented by antioxidants. Primary cultures of mixed glia were exposed to H_2O_2 and the formation of ROS was assessed with the DCFH-DA reaction. (A) Response curve to concentrations of H_2O_2 ranging from 50 μM to 1.2 mM. Values are expressed as percentage of control and are the mean of 4 independent experiments. The antioxidant trolox (Tx) (B) and propyl gallate (C) at concentrations, ranging from 5 nM to 50 μM, reduced DCF in mixed glia exposed to 300 μM H_2O_2. Values are expressed as percentage of H_2O_2 and are the mean of 4 independent experiments. The results show a concentration-dependent inhibition of H_2O_2-induced ROS. The concentrations of the drugs in the *x*-axis is expressed as the logarithm (Log) of μM. Values were fit to a sigmoidal dose-response curve using nonlinear regression analysis. The goodness of the fits was assessed by r^2. *N*-acetylcysteine (from 10 μM to 100 mM) did not reduce H_2O_2-induced DCF. Values are expressed as percentage of control and are the mean ± SEM of 3 independent experiments.

between 5 and 20 μg) gave a linear increase in band signal intensity (not shown).

Assessment of Cell Viability

Cell viability was assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay for measuring cytotoxicity, as reported (Gorina et al., 2005). MTT assay was performed at 24 h after transient (90 min) exposure to several concentrations of the pro-oxidant and antioxidant compounds.

Silencing Jak2 with siRNA on Mixed Glia or Astrocytes

Mixed glia or astrocytes were transfected with 100 nM of either siRNA against rat Jak2 (ON-TARGETplus SMARTpool L-088340-01-0010, Rat JAK2, NM_031514, from Dharmacon) or nonsilencing control siRNA (scramble from Dharmacon, ON-TARGET plus siCONTROL™, Non-Targeting siRNA, D-001810-01) using Oligofectamine (Invitrogen) at 4 μL/mL in Optimem™ Reduced-Serum Medium (Gibco-BRL). Cells were utilized 3 days after transfection. The expression of Jak2 was assessed by Western blot to verify the silencing effect of the treatment. Cells were treated with 300 μM H_2O_2 in the presence or absence of AG490. Then the DCFH-DA technique was carried out as earlier to assess oxidative stress.

Statistical Analyses

Data analysis was performed with one-way ANOVA followed by Dunnet's multiple comparison test to find out differences against the negative or positive control, or by Bonferroni's test for multiple comparisons between groups. The Student's *t*-test was applied to compare two individual groups. Comparison by two types of variables (i.e. siRNA treatment and drug treatment) was made with two-way ANOVA. Concentration-response curves were analyzed by nonlinear regression and were fit to sigmoidal dose-response curves. The goodness of the fit was assessed with r^2. Values in graphs are expressed, unless otherwise stated, as mean ± SEM.

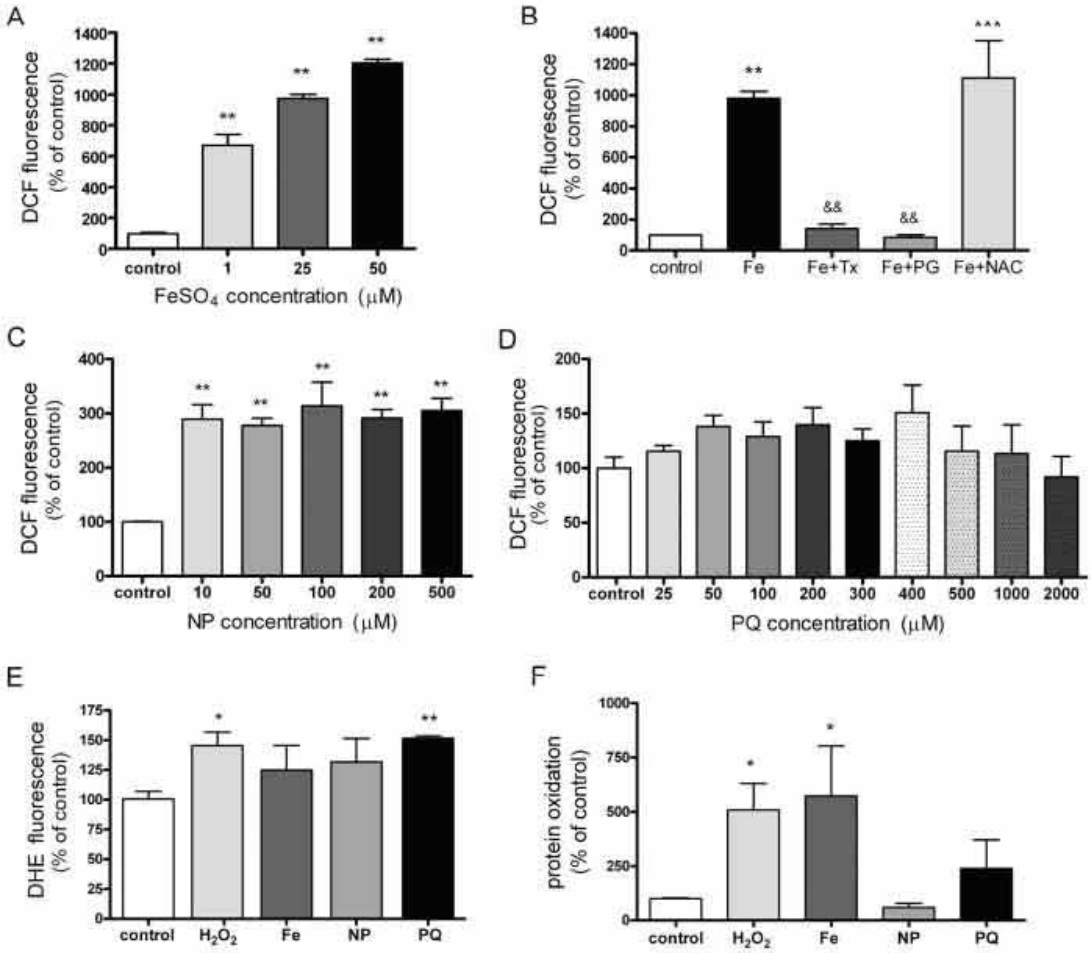

Fig. 2. Several pro-oxidant agents induce ROS and respond to antioxidants. We exposed astrocytes to $FeSO_4$, nitroprussiate (NP), and paraquat (PQ) and evaluated the formation of ROS by the DCFH-DA technique (**A**–**D**) and the DHE technique (**E**), and protein oxidation by the Oxyblot technique (**F**). (A) Rising concentrations of $FeSO_4$ from 1 to 50 μM induced increasing formation of ROS (n 4 independent experiments). (B) The formation of ROS after 25 μM $FeSO_4$ (Fe) was prevented by the antioxidants trolox (Tx, 50 μM) and propyl gallate (PG, 50 μM), but not by *N*-acetylcysteine (NAC, 100 μM) (n 3 independent experiments). (C) NP concentrations ranging from 10 to 500 μM induced similar production of ROS (n 4 independent experiments), but the level was inferior to that induced by $FeSO_4$ or H_2O_2. (D) PQ showed a tendency to increase the production of DCF, but this was not statistically significant (n 4 independent experiments). (E) The production of superoxide, as assessed with the DHE technique increased after PQ (2 mM) (n 7) and H_2O_2 (300 μM) (n 4), while $FeSO_4$ (Fe, 25 μM) (n 2) and NP (100 μM) (n 2 independent experiments) showed a nonsignificant tendency to increase. (F) Quantification of band intensity in the oxyblot technique shows significant protein oxidation after H_2O_2 (300 μM) (n 14), and $FeSO_4$ (Fe, 25 μM) (n 5), a tendency to increase after PQ (n 3), and lack of increase after NP (n 3 independent experiments) (the oxyblot band used for quantification is illustrated with an arrow in Fig. 5D). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against control; $^{\&\&}P < 0.01$ against Fe.

RESULTS

Generation of Oxidative Stress by Pro-Oxidant Compounds and Effect of Antioxidants

Primary cultures of mixed glia showed oxidative stress after exposure to increasing concentrations of H_2O_2 (Fig. 1A), as assessed with the DCFH-DA technique. The effect of H_2O_2 was inhibited by certain antioxidants, such as trolox (Tx) (Fig. 1B) and PG (Fig. 1C), in a concentration-dependent manner. However, NAC, at concentrations ranging from 10 μM to 100 mM, did not prevent the increase in DCF induced by H_2O_2 (Fig. 1D). In addition, H_2O_2 induced the formation of superoxide (Fig. 2D), as assessed with the DHE technique.

We then tested the capacity of other pro-oxidant agents to induce oxidative stress in astrocytes. $FeSO_4$ (Fig. 2A) highly increased the production of DCF in a concentration dependent manner. From these results, we used 25 μM $FeSO_4$ in further experiments. Again this effect was prevented by Tx (50 μM) and PG (50 μM), but not by NAC (100 μM) (Fig. 2B). Likewise, the NO donor NP increased the production of DCF (Fig. 2C). We decided to work with 100 μM NP in further experiments. Unlike the previous pro-oxidants, PQ, at concentrations ranging from 25 μM to 2 mM, did not produce significant amounts of DCF in astrocytes (Fig. 2D), but it generated superoxide, as assessed with DHE. The latter reaction was not significantly increased in relation to control after treatment with $FeSO_4$ or NP (Fig. 2E).

In addition, H_2O_2 and $FeSO_4$ promoted protein oxidation (PQ showed a nonsignificant tendency to increase), as assessed with the Oxyblot technique (Fig. 2F),

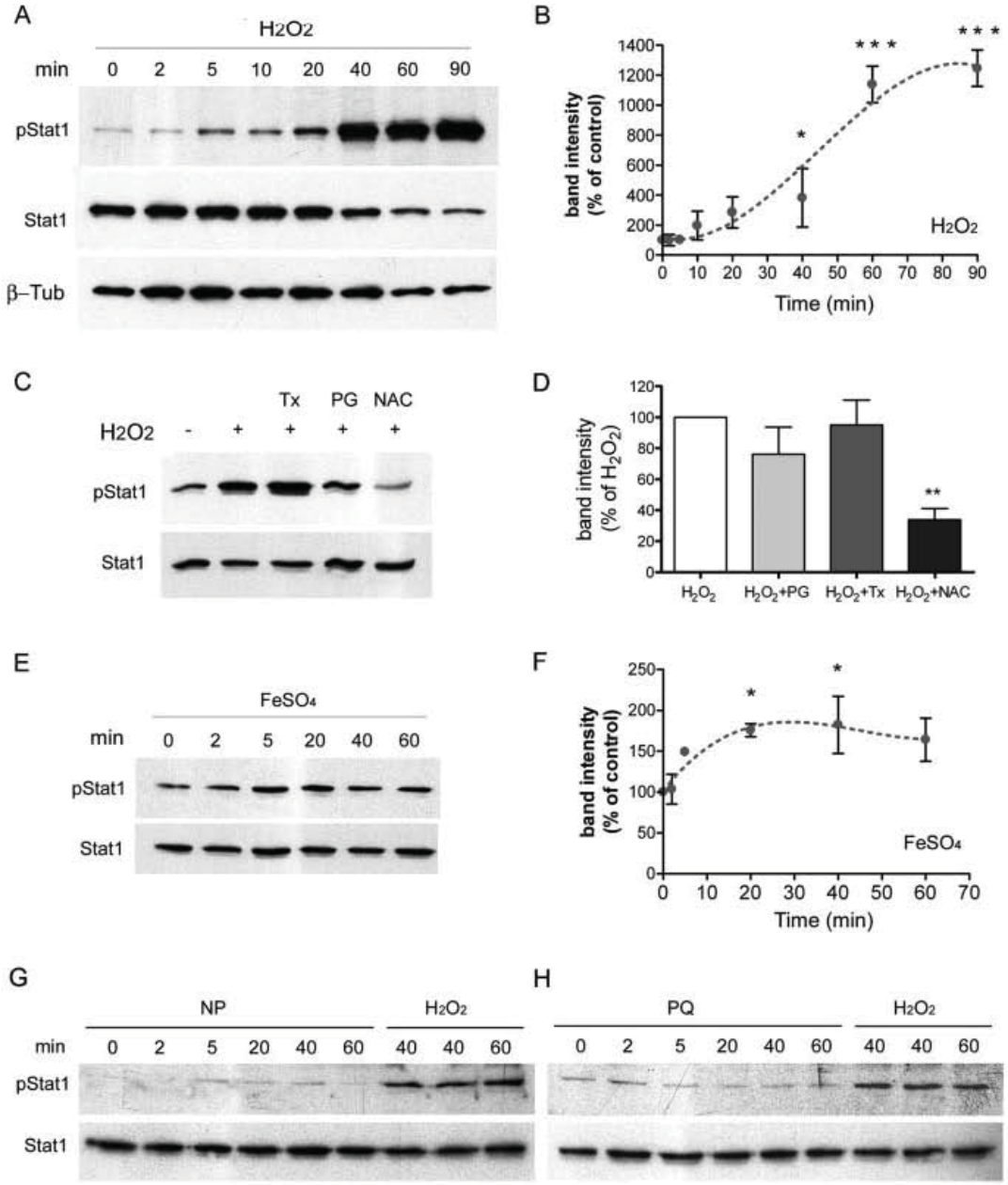

Fig. 3. Stat1 phosphorylation on tyrosine 701 does not parallel ROS formation and it is very marked after exposure to peroxide. (**A**) Time course of phosphorylated Stat1 (pStat1) by H_2O_2 (300 μM) in astrocytes. The level of pStat1 progressively increases with time. (**B**) Measure of band intensity shows significant increases in pStat1 after H_2O_2 (*P < 0.05, ***P < 0.001). (**C**) The antioxidants trolox (Tx, 50 μM) and propyl gallate (PG, 50 μM) do not prevent the increase in pStat1 induced by H_2O_2 at 90 min, while *N*-acetylcysteine (NAC, 20 mM) fully abrogates this effect. (**D**) Significant (**P < 0.01) reductions in H_2O_2-induced pStat1 by NAC become apparent after quantification of band intensity. (**E**) Exposure to $FeSO_4$ (25 μM) causes small increases in pStat1 at 20 40 min, as revealed by quantification (*P < 0.05) (**F**). Nitroprussiate (100 μM) (**G**) and paraquat (2 mM) (**H**) do not increase pStat1 from 2 to 60 min. In the later gels, lanes corresponding to H_2O_2 are shown as positive controls. Stat1 or β-Tubulin is shown for each gel as a loading control. Images show representative membranes from 3 to 4 independent experiments.

whereas NP did not cause protein oxidation (Fig. 2F) but promoted protein nitrosylation (Fig. 5E).

Exposure of glia cultures to H_2O_2 caused a concentration-dependent reduction in cell viability at 24 h, as reported (Gorina et al., 2005). PQ caused some decrease of cell viability (30% reduction at 2 mM, P < 0.01 vs. control). However, exposure to $FeSO_4$ (1–50 μM) or NP (100–500 μM) did not significantly affect cell viability. The antioxidants Tx and PG prevented H_2O_2-induced cell death in a dose-dependent manner (50 μM exerted nearly full protection), whereas no protection was provided by NAC (data not shown). We also observed that 24 h exposure to 100 mM NAC induced some cell death (20% reduction in cell viability, P < 0.01 vs. control). In

addition we found that the Jak2 inhibitor AG490 provided full protection against H_2O_2-induced cell death, as previously reported (Gorina et al., 2005).

Peroxides Increased pStat1

The level of tyrosine-phosphorylated Stat1 (pStat1) was progressively increased from 5 to 90 min of exposure to H_2O_2 (Figs. 3A,B), while total levels of Stat1 did not increase. H_2O_2-induced pStat1 was not prevented by the antioxidants trolox and PG (Figs. 3C,D), evidencing that activation of Stat1 was a particular property of peroxide that was induced even after abrogation of ROS generation. In contrast, high concentrations of NAC (20 mM) did abrogate pStat1 (Fig. 3C), in spite that they did not prevent ROS generation (Fig. 1G). $FeSO_4$ only caused a mild increase in the level of pStat1 (Figs. 3E,F), while changes in relation to control were not detected after exposure to NP (Fig. 3G) or PQ (Fig. 3H), further supporting the view that activation of Stat1 was dissociated from ROS generation.

Peroxide-Induced Increase in pStat1 Was Prevented by Jak2 Inhibitors

We then asked whether H_2O_2-induced Stat1 tyrosine phosphorylation was dependent on the upstream kinase Jak2. We used two different Jak2 inhibitors: the widely employed tyrphostin AG490 (Meydan et al., 1996), and a more recently developed compound named Jak2-Inhibitor-II (Sandberg et al., 2005) (Fig. 4A). Both compounds reduced the accumulation of pStat1 after 90 min of exposure to H_2O_2 (Figs. 4B,C), suggesting that Jak2 mediated this process.

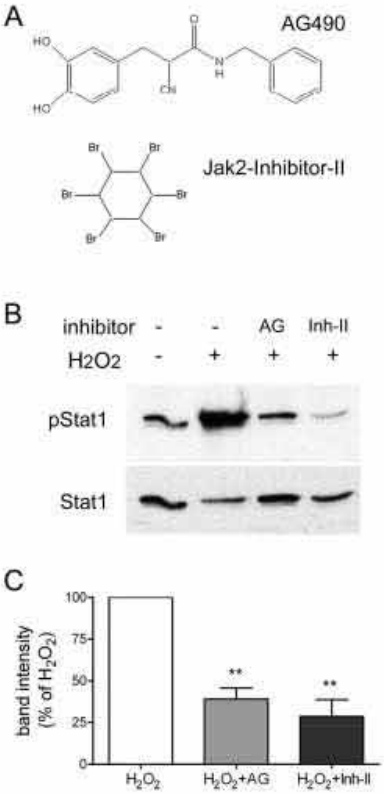

Fig. 4. Jak2 is involved in H_2O_2-induced increase in pStat1. (**A**) Chemical structure of the Jak2 inhibitors: AG490 and Jak2-Inhibitor-II. (**B**) AG490 (30 μM, added 30 min before H_2O_2) and Jak2-Inhibitor-II (Inh-II) (50 μM, added 16 h before H_2O_2) reduce the levels of pStat1 in mixed glia at 90 min of H_2O_2 (300 μM) exposure. (**C**) Quantification of band intensity shows a significant effect of these compounds (**$P < 0.01$) (n 3 independent experiments).

AG490, but Not Jak2-Inhibitor-II, Showed Antioxidant Properties

Unexpectedly, AG490 reduced H_2O_2-induced ROS (Fig. 5A) in a concentration-dependent manner. This effect was not observed with the vehicle of AG490, DMSO, which was used at 12 mM or less. However, at higher concentrations, DMSO (280 mM) has been reported to act as a hydroxyl radical scavenger (Repine et al., 1981). Here the antioxidant effect was attributable to AG490 and not to DMSO. AG490 also attenuated the formation of ROS induced by $FeSO_4$ (Fig. 5B). Moreover, it reduced ROS after NP (Fig. 5C), in spite that this compound did not induce pStat1 (Fig. 3G). Also, AG490 attenuated protein oxidation induced by H_2O_2 (Fig. 5D). However, AG490 did not prevent NP-induced protein nitrosylation (Fig. 5E) or the generation of superoxide after exposure to PQ (not shown), indicating some specificity in the effect of AG490. In contrast to AG490, the other Jak2 inhibitor that we tested (Jak2-Inhibitor-II, which was also dissolved in DMSO) did not affect ROS production after H_2O_2 (Figs. 5F,G) or $FeSO_4$ (Fig. 5H), thus demonstrating that the effect of AG490 was a specific feature of this compound that was not shared by another Jak2 inhibitor. Altogether, these findings led us to deduce that AG490 might have antioxidant features.

H_2O_2-Induced pStat1 Was Higher in Mixed Glia than in Pure Astroglia, While It Was Not Detected in Microglia

To elucidate whether the effects of H_2O_2 in the mixed glia cultures occurred in astrocytes or in microglia, we purified these cells (see Methods), which were then treated with H_2O_2. H_2O_2-induced oxidative stress, as assessed by DCFH-DA, in pure astroglia (Fig. 6A). Again, this effect was inhibited by AG490 (Fig. 6A), but not by Jak2-Inhibitor-II, both after 30 min (Fig. 6A) or 16 h incubation (not shown). Likewise, H_2O_2 increased DCF in microglia and AG490, but not Jak2-Inhibitor-II (16 h incubation), prevented it (Fig. 6B). Notably, the production of DCF after H_2O_2 was higher in astrocytes than in microglia (Fig. 6C).

pStat1 was not detected in our microglia culture after exposure to H_2O_2, while we found pStat1 in astrocytes after H_2O_2 (Fig. 6D). However, compared with mixed

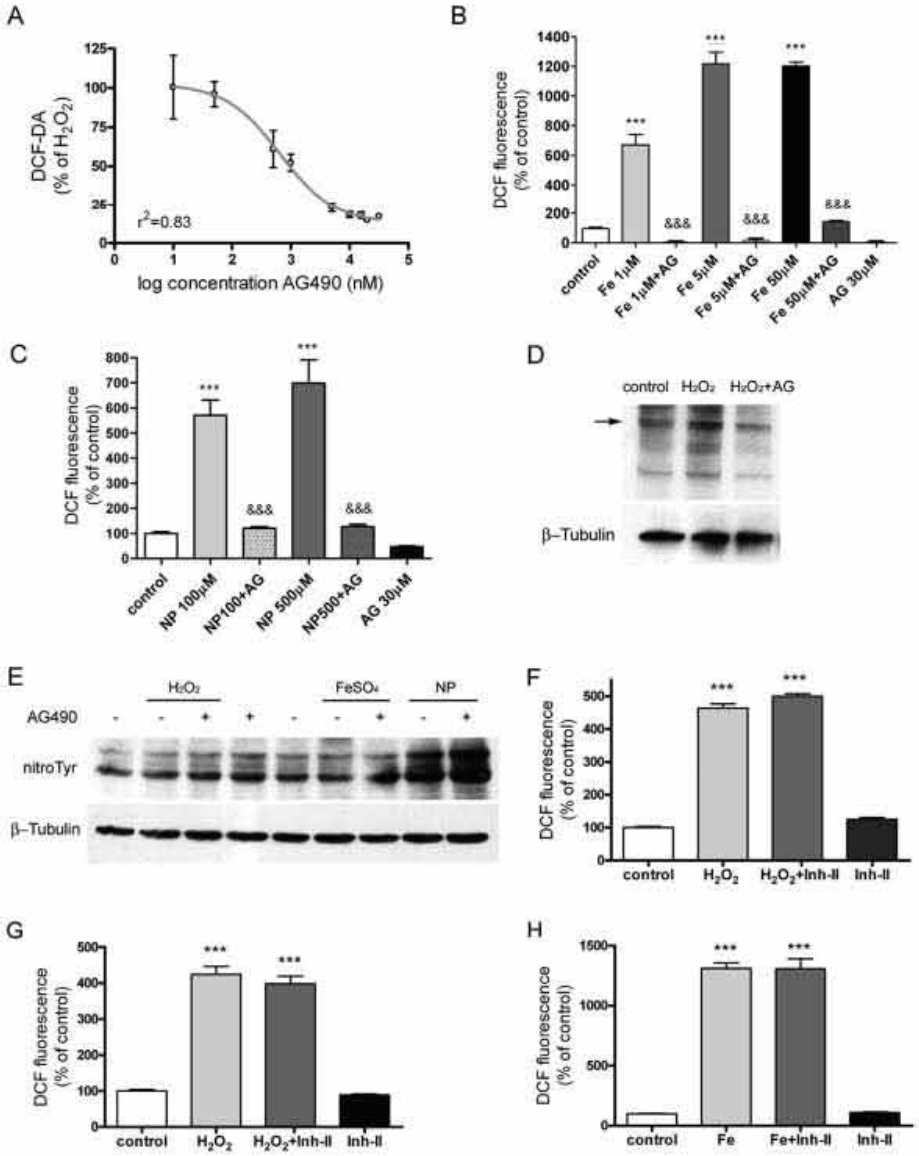

Fig. 5. AG490 reduces the oxidative stress induced by pro-oxidant agents. (**A**) AG490 reduces the production of ROS induced by H_2O_2 in a concentration-dependent manner, as assessed with the DCFH-DA technique; controls were exposed to the vehicle (DMSO); (n 9 obtained in 3 independent experiments for each cell type). Values were fit to a sigmoidal dose-response curve using nonlinear regression analysis. The goodness of the fits was assessed by r^2. (**B**) AG490 (AG) (30 μM) also prevents the production of ROS induced by $FeSO_4$ (Fe) at concentrations ranging from 1 to 50 μM. $^{***}P < 0.001$ against control; $^{\&\&\&}P < 0.001$ against Fe. (**C**) AG490 (30 μM) prevents the production of ROS induced by nitroprussiate (NP) at concentrations of 100 and 500 μM. $^{***}P < 0.001$ against control; $^{\&\&\&}P < 0.001$ against NP. (**D**) The oxyblot technique shows increased protein oxidation after H_2O_2, and reduction of this effect by AG490. Arrow indicates the band that was used for quantification. (**E**) Nitroprussiate (NP, 100 μM), but not H_2O_2 (300 μM) or $FeSO_4$ (25 μM), induces protein nitrosylation, as revealed by Western blot with an antibody against nitrotyrosine (NitroTyr), but AG490 (30 μM) does not prevent this effect. β-Tubulin is shown as a loading control. (**F H**) Jak2-Inhibitor II (Inh-II) (50 μM), either incubated for 30 min (F) or 16 h (G H), does not affect the production of DCF induced by H_2O_2 (300 μM) (F,G) or FeSO4 (25 μM) (H) in mixed glia cultures (n 18 obtained in 3 independent experiments). $^{***}P < 0.001$ against control.

glia, the level of Stat1 phosphorylation in pure astrocytes was lower (Fig. 6D). Again, the induction of pStat1 in astroglia was prevented by AG490 (Fig. 6E).

Antioxidant Properties of AG490 Were Independent of Its Inhibitory Action on Jak2

To ascertain the suspected antioxidant properties of AG490, we tested whether this compound was effective against lipid peroxidation by measuring its effect on the formation of TBARS generated in a microsomal preparation *in vitro* (see Methods). Various AG490 concentrations ranging from 1 to 16 μM were tested (n = 4 curves obtained in n = 2 independent experiments). Data were fit to a sigmoidal dose-response curve by nonlinear regression analysis (r^2 = 0.91, IC_{50} = 8.2 μM) (Fig. 7A). AG490 effectively decreased TBARS supporting the view that AG490 behaves as an antioxidant.

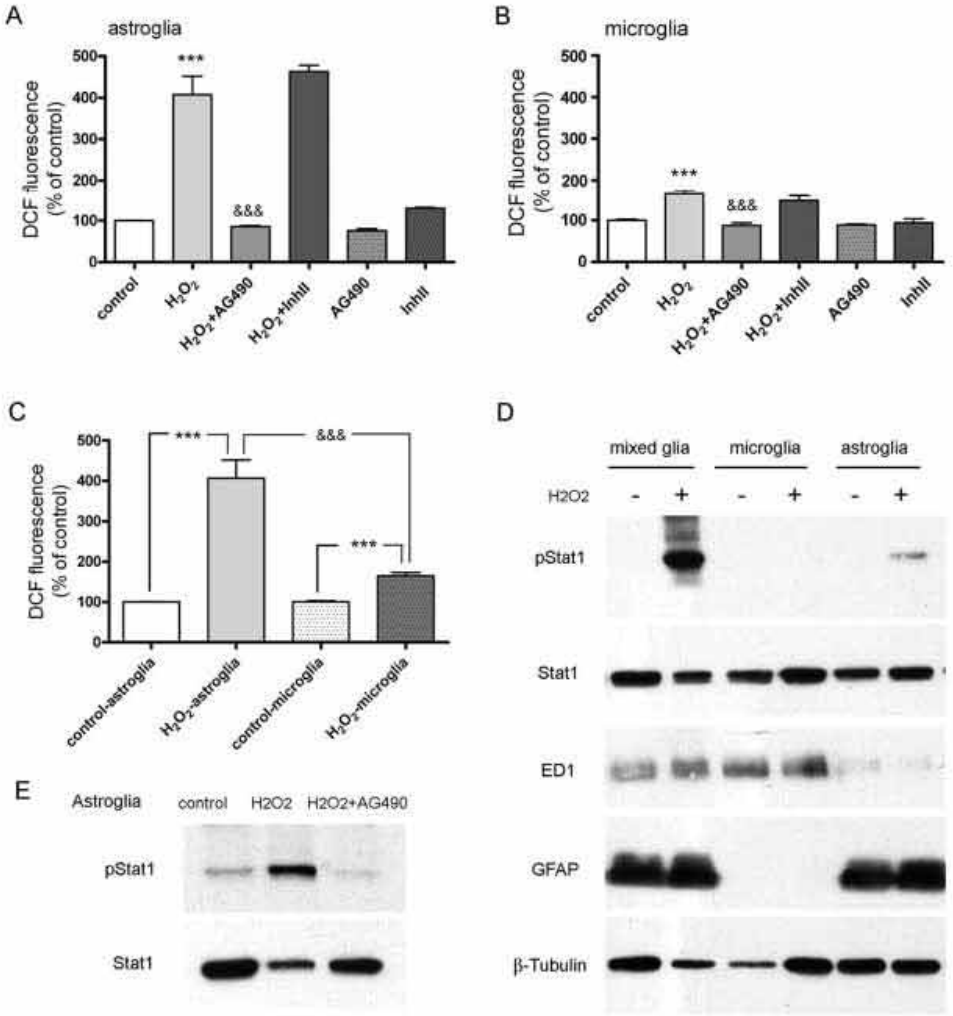

Fig. 6. H_2O_2 induces oxidative stress and pStat1 in astrocytes more than in microglia. (**A**) Purified astrocytes were exposed to 300 μM H_2O_2 for 1 h in the presence or absence of Jak2 inhibitors (30 μM AG490, 30 min preincubation) or Jak2-Inhibitor II (50 μM Inh-II, 30 min preincubation) and production of DCF was assessed. H_2O_2-induced DCF (***$P < 0.001$) and AG490, but nor Inh-II, significantly ($^{\&\&\&}P < 0.001$) prevented this effect. (**B**) Purified microglia were exposed to 300 μM H_2O_2 in the presence or absence of Jak2 inhibitors (30 μM AG490, 30 min preincubation) or Jak2-Inhibitor II (50 μM Inh-II, 16 h preincubation) and production of DCF was assessed. H_2O_2-induced DCF (***$P < 0.001$) and AG490, but nor Inh-II, significantly ($^{\&\&\&}P < 0.001$) prevented this effect. (**C**) The DCF increase induced by H_2O_2 against control (***$P < 0.001$) is higher in astrocytes than in microglia ($^{\&\&\&}P < 0.001$). (**D**) Mixed glia, microglia, and astroglia were exposed to H_2O_2 for 90 min and the expression of pStat1 was examined by Western blot. pStat1 was detected in mixed glia and, to a lesser extent, in astroglia, but not in microglia, in spite that all types of cell culture expressed Stat1. The same membrane was reprobed with ED1, as a marker of microglia, and with GFAP, as a marker of astroglia. (**E**) H_2O_2-induced pStat1 in astroglia was also prevented by AG490.

To further validate this finding, we transfected mixed glia and pure astrocytes with siRNA against Jak2 and 3 days later we found a significant reduction in basal Jak2 expression (Fig. 7B). Under depletion of Jak2 protein expression after siRNA treatment, AG490 was still capable of reducing H_2O_2-induced ROS in both, mixed glia (Fig. 7C), and astrocytes (Fig. 7D), thus further strengthening the view that AG490 had antioxidant properties independent of its inhibitory action on Jak2.

DISCUSSION

We previously reported (Gorina et al., 2005) that H_2O_2 increases the phosphorylation of Stat1 in cultured glia. Here we compared the effect of H_2O_2 on induction of oxidative stress and Stat1 phosphorylation with the effects of other pro-oxidant agents in mixed glia cultures. We tested the effect of $FeSO_4$, which generates peroxides and hydroxyl radicals (Burkitt and Gilbert, 1989, 1991), of NP, a NO donor that can react with superoxide to form the potent oxidant peroxynitrite (Squadrito and Pryor, 1998), and of the herbicide PQ, which mainly generates superoxide (Bus and Gibson, 1984). All these compounds increased the formation of ROS in astrocytes. The production of DCF increased with H_2O_2, $FeSO_4$, and NP, while a positive reaction with the DHE method was found with PQ and H_2O_2. The DCFH-DA technique is mainly sensitive to peroxides and NO, while the DHE technique is mainly sensitive to superoxide O_2^-

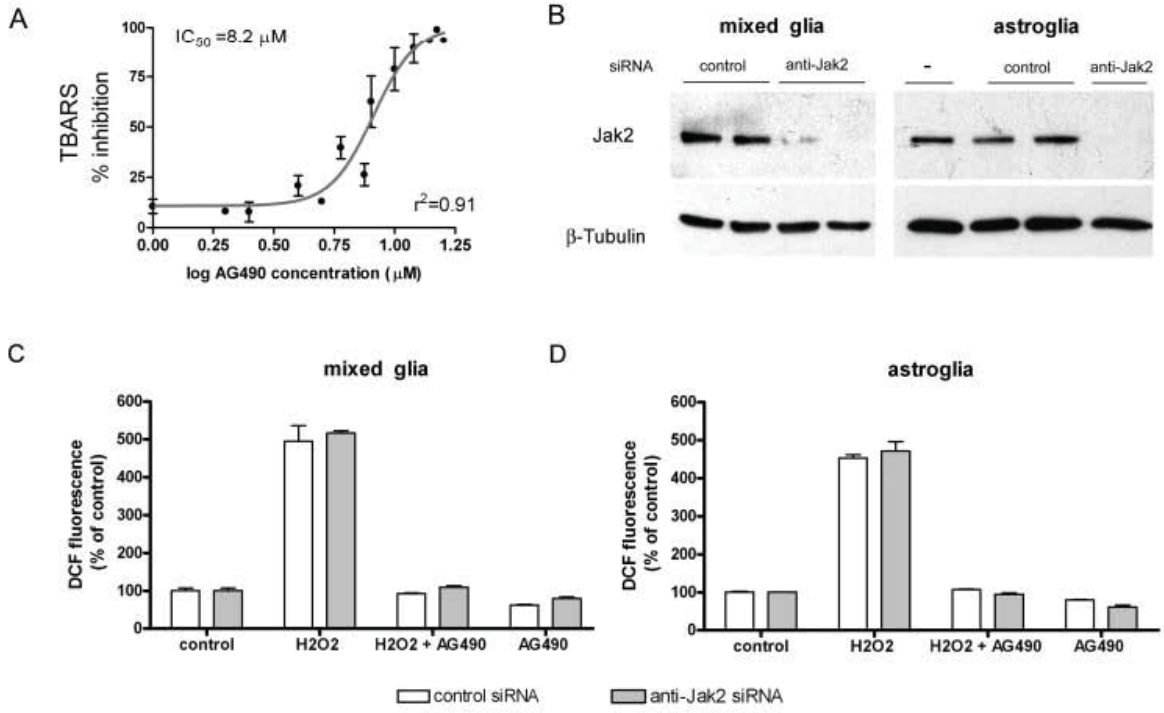

Fig. 7. AG490 has Jak2-independent antioxidant properties. (A) AG490 (1 16 μM dissolved in a final concentration of <1% DMSO) inhibits, in a concentration-dependent manner, the production of TBARS induced in an *in vitro* microsomal preparation. Values are expressed as the mean % inhibition ± SD of *n* 4 curves obtained in 2 independent experiments. The concentration of AG490 is expressed in the *x*-axis as the logarithm (log) of μM. Data were fit to a sigmoidal dose-response curve using nonlinear regression analysis (IC_{50} 8.2 μM). The goodness of the fit was assessed by r^2. (B) Treatment of astroglia with anti-Jak2 siRNA for 3 days reduces the level of Jak2 expression. β-Tubulin is shown as a loading control (C, D) AG490 (AG) (30 μM) prevents the generation of ROS (DCFH-DA technique) induced by H_2O_2 (300 μM) in mixed glia (C) and astrocytes (D) even after treatment with siRNA against Jak2 (as shown in B). Data (*n* 4) were obtained in 2 independent experiments. Values are expressed as the mean ± SEM. The increase in DCF induced by H_2O_2 (***$P < 0.001$ vs. control) was prevented by AG490 ($^{\&\&\&}P < 0.001$) in mixed glia and in astroglia. Two-way ANOVA by siRNA condition (control or anti-Jak2) and by drug treatment showed that the effect of AG490 was equal under both siRNA conditions, and thus regardless of Jak2 expression.

(Sharikabad et al., 2001). In spite of the pro-oxidant effects of all the compounds tested, only H_2O_2 caused strong Stat1 phosphorylation. This finding agrees with previous data showing selective activation of Stat proteins by peroxides (Gorina et al., 2005; Simon et al., 1998). $FeSO_4$ induced only a small increase in pStat1, in spite that it caused a high increase in the DCFH-DA reaction, while NP and PQ did not induce pStat1. These findings suggest that Stat1 activation is triggered mainly by peroxide rather than by ROS. Furthermore, the antioxidants trolox and PG effectively prevented ROS formation, but they did not prevent the activation of Stat1 after H_2O_2. PG is a synthetic phenolic antioxidant that has the capacity to remove hydroxyl radicals and superoxide radicals (Kahl and Hildebrandt, 1986; Sroka and Cisowski, 2003), whereas the Vitamin E analog, trolox, is a powerful scavenger of hydroxyl radical (Aruoma et al., 1990). In contrast to PG and trolox, NAC reduced the accumulation of pStat1 after H_2O_2 in mixed glia, in agreement with a previous report in other cells (Simon et al., 1998), in spite that NAC was unable to reduce the production of ROS in our cultures. NAC scavenges hydroxyl radical through a rapid reaction but it reacts more slowly with H_2O_2 (Vanderbis et al., 1996), which might explain the poor inhibitory action of NAC against H_2O_2-induced DCF production. In addition to these effects, NAC can be deacetylated to cysteine to become a precursor of cellular glutathione synthesis and thus stimulate the glutathione system (Gillissen and Nowak, 1998). However, it is currently unknown whether this latter capacity might be involved in the effect on NAC preventing H_2O_2-induced pStat1. Taken altogether, these results suggest that Stat1 activation was dissociated from the generation of ROS.

To elucidate whether the earlier responses in mixed glia were due to astrocytes or to microglia, we prepared purified cultures of astroglia and of microglia. H_2O_2 induced oxidative stress in all cultures but increases above basal were higher in astrocytes than in microglia. Regarding H_2O_2-induced Stat1 tyrosine phosphorylation, we only detected pStat1 in astrocytes but not in microglia. However, the level of pStat1 induced by H_2O_2 was lower in astroglia than in mixed glia. This suggests that the signalling reported here in mixed glia cultures is attributable to astrocytes, but that these cells behave in a slightly different manner depending on whether they are cultured in the presence or in the absence of microglia.

H_2O_2-induced pStat1 was prevented by Jak2 inhibitors, indicating that Jak2 was involved in this process, in agreement with previous results (Simon et al., 1998). In addition, we unexpectedly found that one of the Jak2 inhibitors used here, AG490, reduced the formation of ROS and protein oxidation induced by H_2O_2. Moreover, AG490 also prevented the formation of ROS induced by $FeSO_4$ and NP, and it attenuated protein oxidation. These findings led us to suspect that AG490 had antioxidant properties. To further investigate this possibility, we examined whether AG490 was capable of reducing lipid peroxidation in an *in vitro* system devoid of cells. The results of this study further supported that view. Additional independent evidence was obtained after inhibiting Jak2 expression with siRNA, as under this condition AG490 equally prevented ROS generation after H_2O_2 exposure. These results confirmed that AG490 has intrinsic antioxidant properties, which are independent of Jak2. This finding might be relevant to the biological effects of AG490 when it is used as a Jak2 inhibitor. The chemical structure of AG490 is compatible with antioxidant features as it shares some molecular similarities with the phenolic antioxidants PG and trolox. AG490 has two hydroxyl groups bonded to one aromatic ring in a catechol like disposition (Fig. 4A) that confer a strong radical scavenger activity to this compound (Sroka and Cisowski, 2003). Further work is needed to fully characterize the antioxidant properties of AG490.

A main issue remaining unsolved is the precise molecular mechanism underlying H_2O_2-induced increase in tyrosine phosphorylated Stat1. Although evidences suggest involvement of kinases, other processes affecting phosphatase activity deserve further attention. Also, the possible contribution of Stat1 activation to the powerful peroxide detoxifying properties of astrocytes needs to be examined. These issues are currently under investigation in our laboratory.

In brief, these results show that tyrosine phosphorylation of Stat1 is a specific effect of peroxide, that is dissociated from the generation of ROS, and that is predominant in mixed glia cultures and, to a lesser extent, in astrocytes. Also, the present findings unravel previously unrecognized Jak2-independent antioxidant properties of the Jak2 inhibitor AG490.

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Article nº 3

Astrocytes are very sensitive to develop innate immune responses to short interfering RNA

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ASTROCYTES ARE VERY SENSITIVE TO DEVELOP INNATE IMMUNE RESPONSES TO SHORT INTERFERING RNA

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ABSTRACT

Short interfering RNA (siRNA) inhibits the synthesis of specific proteins through RNA interference. However, in cells of the immune system, such as dendritic cells, siRNA can induce innate immune responses that are mediated by Toll-Like Receptors (TLRs). Here we sought to evaluate whether siRNA can induce such responses in astrocytes, as these cells are active players in cerebral innate immunity and express TLRs. We examined the effects of various siRNAs (6 silencing sequences targeting specific genes and 2 non-silencing control sequences). siRNA sequences induced variable degrees of silencing-independent non-specific effects, e.g. increased Stat1 expression and release of IL-6 and IP-10 in primary cultures of astroglia. These effects could be prevented through chemical modification of siRNA by nucleoside 2'-O-methylation in the sense strand, without impairing the specific gene silencing effect. Primary astroglia cultures contain non-negligible proportions of microglia. However, siRNA also induced non-specific responses in purified astroglia, but not in microglia or 3T3 cells. Microglia showed higher TLR7 mRNA expression than primary or purified astroglia and 3T3 cells, whereas TLR3 mRNA expression was higher in cultures containing astroglia than in microglia or 3T3 cells. Accordingly, the TLR3 agonist poly(I:C) (PIC) induced higher release of IFN-β in primary astroglia and purified astroglia than in microglia. As siRNA, PIC induced IP-10, Stat1, VCAM-1 and COX-2 and increased the expression of TLR3 mRNA. These results show that astroglia is particularly sensitive to develop innate immune responses against siRNA sequences, and suggest that this might be mediated, at least in part, by activation of TLR3.

Key words: Stat1, IP-10, IL-6, glia, microglia, TLR

Running title: Immune response to siRNA in astroglia

INTRODUCTION

Cells of the immune system have the ability to develop qualitatively different innate immune responses depending on the cell type, the nature of the pattern recognition receptor, and the tissue microenvironment (Colonna et al., 2006). In the central nervous system (CNS), stimulation of innate immune responses depends on the cell type and the environmental signal (Jack et al., 2005). Microglial cells are regarded as the CNS resident immune cells (Block and Hong, 2005). Nevertheless, astrocytes are also important mediators of cerebral immune and inflammatory reactions (Dong and Benveniste, 2001), and the view is growing on the importance of astrocytes in cerebral innate immune responses (Farina et al., 2007, Falsig et al., 2006; Scumpia et al., 2005).

The innate immune response is a defence mechanism triggered by infectious agents and other molecules. Host defence against virus infection involves the recognition of viral nucleic acids mediated by innate immune pattern recognition receptors, including membrane-bound Toll-like receptors (TLRs), which recognize double-stranded (ds) RNA, single-stranded (ss) RNA and dsDNA. TLRs can then activate antiviral gene programs (Doyle et al., 2003) mediated by induction of type I interferon and inflammatory cytokines (Kawai and Akira, 2006). Besides TLRs, cytoplasmic RNA helicases, such as Protein-Kinase R (PKR) and RIG-I, are important cytoplasmic sensors of viral infection that become activated by dsRNA (Jefferies and Fitzgerald, 2005).

Short interfering RNAs (siRNAs) are used in living cells and organisms to silence RNA coding for specific proteins through RNA interference (RNAi). Yet, it is now recognised that siRNAs can induce non-specific effects by activating the innate immune response (Sledz et al., 2003); particularly when they are combined with lipids, as for *in vitro* cell transfection (Hornung et al., 2005) and to avoid serum nuclease degradation *in vivo* (Judge et al., 2005, 2006). Living organisms and cultured dendritic cells and macrophages exposed to siRNAs show up-regulation of type-I interferon (IFN) and a subsequent increase in the expression of signal transduction and activator of transcription-1 (Stat1), an interferon-stimulated gene that mediates

innate immunity (Durbin et al., 1996), and they release various cytokines (Sledz et al., 2003; Karikó et al., 2004a). Although these unwanted effects do not seem to prevent the expected RNAi, they cause a huge drawback for the therapeutical use of siRNA, and they may confound the biological effect of RNAi.

Whether siRNA can induce immune responses in glial cells has not been investigated so far. Here we examined whether a variety of siRNAs can trigger immune responses in different types of mouse brain glia cultures and, for comparison purposes, we also used non-glial cells. We then tested whether the effects of siRNA could be prevented by 2'-O-methylation of uridine or guanosine nucleosides of the siRNA sense strand, and compared the effects of siRNA with those induced by the TLR3 agonist poly(I:C) (PIC).

MATERIALS AND METHODS

Cell cultures

Animal work was authorised by the Ethical Committee of the University of Barcelona and it was performed in agreement with the local regulations. Primary cultures of glial cells were prepared as reported (Saura et al., 2003). Cerebral cortices from 1- to 2-day-old Swiss CD1 mice (Charles-River, France) were used. Culture medium was Dulbecco's modified Eagle medium:F-12 nutrient (DFF) (1:1) (Gibco-BRL), supplemented with 10% foetal bovine serum (FBS) (Gibco-BRL), and 4 mL/L of 10,000U/mL penicillin/10,000µg/mL streptomycin (Gibco-BRL). All products and reagents, unless otherwise stated, were from Sigma-Aldrich. Cells were plated in 24-well plates (NUNC, Roskilde, Denmark) and maintained in DFF and 10% FBS with antibiotics, as above. Medium was replaced every 4-5 days and confluency was achieved after 10-12 days in vitro. These cell cultures contain astrocytes and around 25% of microglia (Gorina et al, 2007).

Enriched astroglia cultures were prepared following a reported procedure (Gorina et al., 2007). Mouse primary mixed glial cultures on 10-12 DIV were treated with 10µM of the antimitotic cytosine arabinoside (Ara-C, Sigma-Aldrich) for 4 days to eliminate dividing cells,

i.e. mostly microglia and progenitors, but not quiescent cells, i.e. mostly confluent astrocytes. The adherent cells were detached with trypsin 0.05%/EDTA 0.2mM and seeded at 6 x 10^4 cells/mL with culture medium (see above). Astrocytes were used two days after subculturing and they contained more than 96% astrocytes (Gorina et al., 2007).

Microglial cells were obtained by mild trypsinization with a method yielding high purity (>98%) (Saura et al, 2003; Gorina et al., 2007), and they were used one day after isolation.

Primary glial cultures were also obtained from homozygous Stat1 -/- mice (129S6/SvEv background) and from the corresponding controls (129S6/SvEv wild type, WT) (Taconic Farms, Germantown, NY, USA).

NIH3T3 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum and antibiotics, as above. For transfection, 5 × 10^4 NIH3T3 cells were plated in 24-well plates.

siRNA treatment

Cell cultures were exposed to siRNAs for several time periods ranging from 30 min to 6 days. The typical concentration of siRNA used was 100 nM, but a range of concentrations (from 0.01 to 100 nM) was tested. In addition, high siRNA concentration (3.3 μM) was used in one set of experiments designed to examine whether siRNA could induce TLR mRNA expression (see below). siRNA was mixed with oligofectamine™ (Invitrogen) 15 min at RT prior to cell transfection. The final concentration of oligofectamine in the culture medium was 4 μg/mL. Control cultures were treated with oligofectamine in the absence of siRNA. Two kinds of silencing siRNAs were used, either consisting of a population of multiple duplex sequences against Stat1 (#RM-1171), GAPDH (#RM-0057), and Jak2 (#RM-1164) (Superarray Bioscience Corporation), or consisting of one unique duplex sequence (Qiagen) against MAPK1 (ERK) (#1022564) or p21Cip1/Waf1 (p21-Q) (#1024837). In addition, we obtained the latter duplex sequence against p21 (antisense: C.G.A.A.G.U.C.A.A.A.G.U.U.C.C.A.C.C.G.dT.dT, sense: C.G.G.U.G.G.A.A.C.U.U.U.G.A.C.U.U.C.G.dT.dT) from a different source, as it was custom made by another supplier (p21-D1; Dharmacon). Also, a different commercially available

duplex sequence (p21-D2) against p21 was tested (ON-TARGET *plus*™ duplex J-0586636-05, Mouse CDKN1a, NM_007669, Dharmacon). According to the supplier, ON-TARGET*plus*™ modification enhances siRNA specificity and reduces off-target effects. Also, two different non-silencing siRNAs were used as controls for the RNAi effect. One of these (ns-m) was composed of a population of duplex sequences (#RM-1171 negative control, Superarray Bioscience Corporation), and the other (ns) consisted of one duplex sequence (#1022076, Qiagen). According to the manufacturers, the sequences of the non-silencing siRNAs had no homologies with the known rodent genes and they were used as negative controls.

siRNA modifications

The siRNA sequence against p21 (p21-Q and p21-D1) was modified by introducing 2'-O-methylation (m) in nucleosides of the sense sequence; either in all uridine nucleosides (C.G.G.mU.G.G.A.A.C.mU.mU.mU.G.A.C.mU.mU.C.G.dT.dT), or in all guanosine nucleosides (C.mG.mG.U.mG.mG.A.A.C.U.U.U.mG.A.C.U.U.C.mG.dT.dT), while keeping the antisense sequence unmodified (C.G.A.A.G.U.C.A.A.A.G.U.U.C.C.A.C.C.G.dT.dT). These siRNAs were custom made upon request (Dharmacon).

Other treatments

Cells were treated with the synthetic double-stranded RNA, polyinosinic-cytidylic acid (poly(I:C), PIC)) (Sigma) (50 or 100 μg/mL), and cells and media samples were obtained at several time points ranging from 4 to 24 h for Western blot and ELISA measurements. Exposure to chloroquine (50 μg/mL, Sigma) was initiated two hours prior to PIC or siRNA treatment.

Western blotting

Western blotting was performed as described (Gorina et al., 2005) with monoclonal antibodies against Stat1 and Stat3 (BD Transduction Laboratories) diluted 1:4,000; p21 (BD Pharmingen) diluted 1:2000; panERK (Cell Signaling) diluted 1:8,000; iNOS (BD Transduction

Laboratories) diluted 1:1,000; and phosphorylated eIF2α (peIF2α) (Epitopics) diluted 1:1000. The following polyclonal antibodies were used: a goat antibody against VCAM-1 (Santa Cruz Biotechnology Inc.) diluted 1:1000; and a rabbit antibody against COX-2 (Cayman Chemical Co) diluted 1: 1000. β-Tubulin diluted 1:50,000, or actin diluted 1:10,000 (Sigma-Aldrich) were used as loading controls. The optical density of the bands was measured by densitometric analysis (Kodak Digital Science 1D, Kodak). The ratio between band intensity of specific proteins and the corresponding loading control was calculated to correct for differences in protein gel loading. For each gel, these ratio values were expressed as percentage of the control samples run in the same gel. Then, the average of the values (percent of corresponding control) of various samples of the same treatment group (n=3 or more) that were run in different gels was calculated and statistical analysis was performed to test whether differences were statistically significant.

Immunocytochemistry

To verify that siRNA was entering the cells we incubated either pure cultures of astrocytes or pure microglia with a fluorescent rhodamine-labelled control non-targeting siRNA sequence (100 nM, #D-001600-01) for 24h and then examined the cells under the microscope. Double labelling was carried out with fluorescent-siRNA and a rabbit polyclonal antibody against the astroglial marker glial fibrillary acidic protein (GFAP) (#Z0334, Dako) diluted 1:1,000, or with a mouse monoclonal antibody against the microglial marker ED1 (#MCA341R, Serotec), diluted 1:100). The secondary antibodies were green-fluorescent Alexa Fluor 488 dye-labelled goat anti-rabbit IgG (#A-11070; Molecular probes) or goat anti-mouse IgG (#A-11017, Molecular Probes). After the immunoreaction, cells were stained with Hoecht to visualise the nuclei.

ELISA Assays

The concentrations of IP-10 (Quantikine murine IP-10/CRG-2/CXCL10, #MCX100, R&D Systems Europe, Ltd. Abingdon, UK), IP-9 (Quantikine murine CXCL9/MIG, #MCX900, R&D

Systems), IL-6 (#860.020.192, Diaclone SAS, Besançon, France) and IFN-β (#42400-1, R&D Systems) in the culture media were determined by ELISA.

Real-time detection of the expression of TLR3 and TLR7 mRNA

RNA was extracted using guanidinium thiocyanate (Chomczynski & Sacchi, 1987). Two μg of RNA were used for cDNA synthesis using the AMV First-Strand cDNA Synthesis Kit (# 12328-040, Invitrogen, Carlsbad, Ca, USA). Two μl of cDNA synthesis reaction were used in a 25 μl real-time PCR reaction with FAM-labelled Taqman® Gene Expression Assays for TLR3 (Mn00446577_g1), TLR7 (Mn00446590_m1), and β-actin (Mn00607939_s1) (Applied Biosystems, Foster City, CA, USA). The amplification conditions were: 10 min at 95°C followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C. C_T values were analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Livak & Schmittgen, 2001).

Data analyses

Results are the mean of 3 to 9 independent experiments. The effects of siRNA treatment were analysed with one-way ANOVA. Comparison for treatments and cell types was made by two-way ANOVA. Thereafter the effect of individual treatments was evaluated by post-hoc analysis with the Bonferroni's multiple comparisons test. Data not passing the normality test (D'Agostino & Pearson omnibus test) were analysed with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical analyses were performed using GraphPad Prism software.

RESULTS

siRNA reduced the expression of specific proteins, but not of Stat1

Primary astroglial cell cultures were exposed to several siRNA sequences to induce RNAi. siRNA was mixed with oligofectamine for transfection and cells not receiving siRNA were exposed to oligofectamine alone. We used non-silencing siRNA sequences as negative controls, Treatments with certain siRNAs successfully silenced targeted genes in these cultures by reducing the expression of the corresponding proteins, as illustrated with siRNA against p21 (Fig. 1A) and ERK (Fig. 1B). However, when we tried to silence the interferon (IFN)-responsive protein Stat1 with siRNA, we found an increase in Stat1 protein expression, instead of a reduction (Fig. 1C). Control non-silencing siRNA (ns), which did not target specific proteins, also increased Stat1 expression (Fig. 1C) suggesting that siRNA induced Stat1 expression in a non-specific manner in primary astroglia cultures. In addition, a tendency to increase p21 protein expression was also found after treatment with ns siRNA (Fig. 1A). Bhunia et al. (2002) reported that the JAK-STAT pathway was involved in p21 induction. For this reason we tested whether non-silencing siRNA was still capable of inducing the small increase in p21 protein in Stat1 deficient cultures. The results showed a similar increase in p21 in Stat1 deficient cells (not shown), and therefore we could not evidence a link between increases in Stat1 and p21 proteins induced by the non-silencing siRNA sequence.

siRNA induced Stat1 expression in primary glial cell cultures

Non-silencing (ns) siRNA that was used as negative control was capable of inducing the expression of Stat1 in primary astroglia cultures at different time points (Fig. 2A), but it did not affect Stat3 expression (Fig. 2B). The effect was observed from 12h to 6 days, with a maximum around 4 days (Fig. 2A). We then tested various siRNA sequences directed against specific targets (such as GAPDH, Jak2, Stat1, p21 and ERK) as well as other negative control non-silencing sequences (ns_m, ns) for their capacity to induce the expression of Stat1 in our cultures. We found that the sequences tested induced variable degrees of Stat1 protein expression (Fig. 2

C). This suggests that induction of Stat1 was a non-specific effect of siRNA, which was independent of its silencing effect, and that the intensity of the effect depended on the siRNA sequence. The observed Stat1 induction did not alter the capacity of siRNA to silence targeted genes that were not related with the immune response, as shown for p21 and ERK (Fig. 1A, B).

The concentration of 100 nM was chosen in this study as it induced a significant reduction of protein expression, as shown for p21 siRNA in Fig. 3A. siRNA-induced Stat1 expression was concentration-dependent; it was already statistically significant at 10 nM and increased at 100 nM, as shown for exposure to p21 siRNA in Fig 3B.

In an effort to elucidate whether Stat1 induction was caused by a given siRNA sequence or it might be due to any other possible feature of the siRNA product, we obtained the same siRNA sequence from different suppliers. This experiment was carried out for the p21 sequence used above (p21-Q) (presented in Fig. 1 and 2) that was custom made by a different supplier (p21-D1) (see Methods). Both siRNA products were capable of silencing p21 to a similar extent (Fig. 3C), and produced a similar degree of Stat1 induction (Fig. 3D). The result showed that this particular siRNA sequence against p21 triggered Stat1 induction. Finally, we also tested a different commercially available sequence against p21 (p21-D2). The latter also silenced p21 (Fig. 3C) while it did not induce Stat1 expression (Fig. 3D), again supporting the view that siRNA-induced Stat1 was dependent on the siRNA sequence and independent of the silencing effect.

Besides increased Stat1 expression, other non-specific effects of siRNA were detected at 1 and 4 days, including release of IFN-inducible protein 10 (IP-10), IL-6, and IP-9 (see below in Fig. 4). However, we did not find increases in eIF2α phosphorylation from 30 min to 4d after siRNA exposure (not shown), suggesting that the non-specific effects of siRNA were not mediated by activation of PKR.

Stat1 induction was prevented by selective 2'-O-methylation of certain nucleosides

It was reported (Judge et al., 2005) that the siRNA-induced immune response could be inhibited by 2'-O-methylation of certain nucleosides in the sense strand of the siRNA duplex.

Here we tested whether this strategy was effective in our primary astroglia culture system. We designed two modified siRNAs from the sequence of the siRNA against p21 by introducing 2'-O-methylation in the uridine or guanosine nucleosides of the sense strand. The modifications did not impair the capacity to silence p21 (Fig. 4A, B). In addition, 2'-O'methylation of the guanosine nucleosides effectively reduced Stat1 induction (Fig. 4C, D) and release of IL-6 (Fig. 4E), IP-9 (Fig. 4F) and IP-10 (Fig. 4G), whereas no effect was apparent upon modification of the uridine nucleosides (Fig. 4). The former modification prevented the release of IP-10 even in cultures of Stat1-deficient cells (Fig. 4H), indicating that it abrogated Stat1-dependent and Stat1-independent responses induced by siRNA. These findings show that the siRNA-induced immune response can be prevented by selective nucleoside modification.

Effect of siRNA in different cell types

The primary astroglial cultures used here contain mainly astrocytes but, to a lesser extent, they also contain non-negligible amounts of microglia. We then questioned whether residual microglia growing in the primary astroglial cultures, rather than the astrocytes, might be responsible for the non-specific effects of siRNA. We subcultured astroglia in order to obtain a higher purity in astrocytes, while the microglia were kept as a separate culture (see Methods). We tried several siRNAs in the different cultures (the negative control non-silencing is shown in Fig. 5A as a representative example). siRNA increased the expression of Stat1 in astroglia but not in microglia, which already showed comparatively higher basal levels of Stat1 than mixed glia and astroglia (Fig. 5 A, B). This shows that astroglial cells are particularly sensitive to activate a Stat1-mediated immune response after exposure to siRNA.

In addition to Stat1, the concentration of the chemokine IP-10 increased to different extents at 24 and 96 h after siRNA treatment in the medium of primary astroglia cultures and in purified astroglia, but it was not detected in microglia (Fig. 5C). Likewise, IL-6 was induced by certain siRNAs in primary astroglia cultures and in pure astroglia, but not in microglia (Fig. 5D). Another chemokine, IP-9, showed small increases after exposure to certain siRNAs in mixed astroglia cultures only (Fig. 5E). We also examined iNOS expression, which at the basal state

was already very high in microglial cultures and moderate in mixed glia, while it was not detected in purified astrocytes (Fig. 5F). siRNAs induced small increases of iNOS expression in primary astroglia cultures ($p<0.05$), but significant changes were not detected in pure microglia and no signal was found in pure astroglia (Fig. 5F). These results suggest that the behaviour of astrocytes was different depending on whether they were cultured in the presence or absence of microglia, and that primary astroglia cultures were more responsive to siRNA than secondary purified astroglia cultures.

In order to verify that the lack of response of microglia was not attributable to deficient siRNA transfection, we incubated either purified astroglia or purified microglia with a red fluorescent control siRNA sequence. Fig. 6 shows that siRNA entered astroglia and microglia, which were immunostained with GFAP and ED1, respectively.

We also tested whether siRNA was able to induce the above non-specific responses in an epithelial cell line (NHI3T3). However, in these cells we did not detect effects on Stat1 expression (Fig. 7A,B) nor in IP-9, IP-10 and IL-6 release (not shown), in spite of the fact that the expected silencing effect of siRNA was observed (Fig. 7C, D).

Expression of TLR3 and TLR7 and effect of the TLR3 agonist poly(I:C) (PIC) in different cell cultures

The highest expression of TLR7 mRNA was found in purified microglia whereas a comparatively lower expression was found in mixed astroglia and in purified astroglia (Fig. 8A). The inverse situation was found for TLR3 mRNA, as the highest expression was found in primary astroglia cultures, followed by cultures of pure astroglia, and to a much lower extent in pure microglia (Fig. 8B). Altogether, these results suggest that in astroglia TLR3 might mediate, al least in part, the immune effects of siRNA. In 3T3 cells, expression of TLR7 (Fig. 8A) and TLR3 (Fig 8B) mRNA was low, in agreement with the lack of immune response of these cells to siRNA treatment. We also tested whether treatment with siRNA or with the TLR3 agonist PIC modified the level of TLR3 and TLR7 mRNA expression. Incubation for 24h with either PIC (50 μg/mL) or with a high dose of siRNA (3.3 μM, i.e. approximately 50 μg/mL) increased

the expression of TLR3 mRNA, but this effect was not observed with the siRNA dose used for silencing gene expression (100 nM) (Fig. 8C). However, neither siRNA nor PIC increased the expression of TLR7 mRNA at these doses (not shown).

We then tested the effect of PIC on release of IFN-β in the different glia cultures. PIC induced a much higher IFN-β release in primary astroglia cultures and in pure astroglia, than in microglia (Fig. 8D). This effect was in accordance with the expression of TLR3 mRNA observed above in the different cell types.

Besides the above effect of PIC, this TLR3 agonist increased the expression of Stat1, VCAM-1, and Cox-2 (Fig 8E), in agreement with previous reports (Scumpia et al., 2005; Krasowska-Zoladek et al., 2007; Park et al., 2006). siRNA treatment also induced VCAM-1 and Cox-2 expression, as PIC did. These effects of PIC and siRNA were abolished by pre-treatment with the endosomal acidification inhibitor chloroquine, suggesting that internalization to mature endosomes might be involved in the observed responses (Fig. 8F).

DISCUSSION

Here we report that 1) siRNA can induce a non-specific innate immune response associated with induction of Stat1 expression and release of cytokines and chemokines in astroglia; 2) this effect does not prevent silencing of specific targets; 3) the intensity of the response varies depending on the siRNA sequence; 4) siRNA modification by 2'-O-methylation of certain nucleosides of the sense sequence can abrogate this response while keeping the RNAi effect; and 5) these non-specific effects of siRNA in astroglia might be mediated, at least in part, by TLR3.

A variety of siRNA sequences induced Stat1 expression and release of cytokines and chemokines, and we also evidenced a tendency to increase in p21 protein expression by certain siRNAs. The JAK-STAT pathway can be involved in p21 induction (Bhunia et al., 2002), however here we could not find an association between siRNA-induced Stat1 and p21 by examining p21 expression in Stat1 deficient cells. Also, siRNA-induced release of IP10 was

observed in Stat1 deficient cells. Unless there were unknown compensatory mechanisms in Stat1-deficient cells, these findings suggest that, besides Stat1, other pathways might be affected by siRNA treatment in a non-specific manner.

We characterised non-specific effects of siRNAs in cultures of different cell types by studying the induction of Stat1, and release of proinflammatory cytokines and chemokines. Primary cultures of astroglia were particularly prone to show Stat1 induction, release of IP-10, IL-6, and IP-9, and increase in iNOS expression after exposure to siRNA. In purified astrocytes, we also observed induction of Stat1, IP-10 and IL-6, but not of IP-9 or iNOS, but, we did not detect increases in the expression of these molecules in microglia or epithelial 3T3 cells. Therefore, astrocytes emerged as cells responsive to siRNA that can generate an innate immune response against it. The different response of glia depending on whether astroglia and microglia were cultured separated or combined shows that the capacity of the cells to respond to immunostimulatory agents may depend on particular culture conditions. This suggests that the cellular phenotype of astroglia is altered by the presence of microglia, and vice versa.

RNA helicases, such as PKR, are important cytoplasmic sensors of viral infection that become activated by dsRNA (Jefferies and Fitzgerald, 2005). After viral infection, PKR is involved in IFN induction (Diebold et al., 2003), and phosphorylates certain factors involved in translation, such as eukaryotic initiation factor 2α (eIF2α) (Srivastava et al., 1998). In several cell lines, siRNA has been reported to activate PKR (Sledz et al., 2003). However, in astroglia, we did not detect phosphorylation of eIF2α after exposure to siRNA, suggesting that this process is not responsible for the immune response induced by siRNA in these cells. In plasmacytoid dendritic cells, siRNA induces IFN-α through TLR7 (Hornung et al., 2005), which is known to mediate the recognition of single-stranded RNA (Lund et al., 2004). Signaling through TLR3 might also be involved in the immune responses to siRNA (Karikó et al., 2004a, b). TLR3 mediates cellular responses to dsRNA (Alexopoulou et al., 2001) and induces downstream selective activation of IFN regulatory factor 3 (IRF3) (Doyle et al., 2002). Astrocytes express TLRs (Farina et al., 2005; Jack et al., 2005; Konat et al., 2006) and respond to certain viruses and to

the TLR3 ligand PIC (Scumpia et al., 2005; Carpentier et al., 2007; Krasowska-Zoladek et al., 2007; Park et al., 2006; Rivieccio et al., 2006; Vincent et al., 2007). We found that our astroglia cultures were comparatively richer in TLR3 mRNA than in TLR7 mRNA, while we found the reverse situation for microglia. In agreement with higher TLR3 expression in astroglia than in microglia, the TLR3 agonist PIC induced much higher IFN-β release in cultures containing astroglia than in pure microglia. Also, PIC induced a variety of other effects in astroglia that were similar to those induced by siRNA. The effects of both agents were attenuated by the endosomal acidification inhibitor chloroquine, suggesting that intracellular TLR3 might contribute to mediate non-specific effects of siRNA in astroglia. However, previous findings (Bsibsi et al., 2002) have shown preferential expression of TLR3 in the astroglia membrane. Therefore, we cannot exclude the participation of membrane TLR3 in siRNA-induced immune responses. Pro-inflammatory cytokines, TLR3 or TLR4 agonists, and oxidative stress induce TLR expression in human astrocytes (Bsibsi et al., 2006). In agreement with these observations, we found that treatment with a very high dose of siRNA (3.3 μM, which is approximately equivalent to 50 μg/mL) or with the TLR3 agonist PIC (50 μg/mL) increased TLR3 mRNA expression in our primary astroglia cultures, but this effect was not observed for TLR7 mRNA; again pointing to TLR3 as a possible mediator of siRNA-induced non-specific effects in astroglia.

The effects of siRNAs in our cells occurred regardless of whether the sequences were silencing or not, and were independent on the RNAi effect. Certain motifs have been attributed immunostimulatory properties (Judge et al., 2005; Hornung et al., 2005). In agreement with this, the induction of the immune response studied here in glia was sequence-specific, as its magnitude was variable depending on the siRNA sequences, as previously reported in other cells (Hornung et al., 2005; Judge et al., 2005; Sioud 2005). We carried out two different modifications on the siRNA sequence against p21 aiming to attenuate this non-specific response. We designed 2'-O-methylation of either uridine or guanosine nucleosides of the sense chain, since this strategy was reported to abrogate siRNA-induced immune responses (Judge et

al., 2006; Sioud 2007). Our results showed that introduction of 2'-O-methyl in all guanosine nucleosides of the sense sequence of the double stranded siRNA duplex prevented Stat1 and iNOS induction, and IP-10, IP-9 and IL-6 release, while the silencing effect was not impaired. This suggests that nucleoside 2'-O-methylation can be used to abrogate immune responses of siRNA in astrocytes. Therefore, the use of precise siRNA sequences and siRNA modifications intended to avoid non-specific immune responses is important to carry studies of gene silencing in astrocytes.

In conclusion, this study shows that astrocytes are sensitive to develop non-specific innate immune responses to siRNA sequences, and that these responses can be prevented by certain siRNA modifications. In addition, the results suggest that intracellular TLR3 might contribute to mediate non-specific effects of siRNA in astroglia.

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FIGURE LEGENDS

Figure 1. Specific siRNA sequences reduce the level of expression of the targeted proteins. Primary glia cell cultures were exposed to oligofectamine alone (-) as a control, or in combination with various siRNAs (100 nM). The expected RNA interference effect of specific silencing siRNA sequences is obtained in primary glial cultures, as evidenced by Western blot for p21 (A, B) and ERK (C, D) proteins after treatment with p21 siRNA (using the p21-Q siRNA sequence, see Methods) and ERK siRNA, respectively. However, siRNA against interferon-responsive Stat1 increases the expression of this protein (E, F). Control non-silencing siRNA sequences (ns) does not reduce protein expression (A-D), but rather causes a massive increase in Stat1 (E,F) and a non-significant trend to increase in p21 (A,B). (E). Data were obtained in at least 4 independent experiments per siRNA sequence. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control (-).

Figure 2. siRNA sequences induce the expression of Stat1 in primary cultures of glia. Cells were exposed to oligofectamine alone (-) as a control, or in combination with various siRNAs (100 nM). A-B) Control non-silencing siRNA (ns) induces significant expression of Stat1 (A), but not Stat3 (B), at the stated time points, as assessed by Western blot. C) Five different silencing siRNAs (against Stat1, GAPDH (GAP), Jak2, p21-Q, and ERK) and two non-silencing siRNAs (ns and ns_m) were tested for Stat1 expression at day 4. The extent of Stat1 induction varies depending on each siRNA. n indicates the number of samples obtained in 3-6 independent experiments per siRNA treatment. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus control (-).

Figure 3. Stat1 protein-induction increases as a function of the siRNA concentration and it is dependent on the siRNA sequence, but independent of the silencing effect. A-B) Primary glia cultures were incubated with 1 to 100 nM siRNA against p21 (p21-Q) for 4 days. siRNA significantly silences p21 expression at the dose of 100 nM (A), and it increases Stat1

expression, in a concentration-dependent manner (B). Samples were obtained in 3-6 independent experiments. * $p<0.05$, *** $p<0.001$ vs control (0 nM siRNA in the presence of vehicle). C-D) Primary glia cultures were incubated with different siRNAs against p21 (see Methods) (100 nM), or with vehicle (-). Sequences p21-Q and p21-D1 were identical but obtained from different suppliers. Sequence p21-D2 was different from the former. All sequences have a silencing effect against p21 (C), but only the first sequence (p21-Q and p21-D1 siRNAs) induces Stat1 expression whereas the second sequence (p21-D2) does not (D).

Figure 4. O'-methylation of certain nucleosides of the siRNA sense strand abrogates Stat1, chemokine and cytokine induction, without altering the silencing effect. A, B) Expression of p21 is specifically attenuated after treatment with either non-modified p21 siRNA, or with p21 siRNA methylated at uridine- (p21-U) or guanosine- (p21-G) nucleosides, but it is not reduced after treatment with non-silencing control siRNA (ns) or with unrelated siRNA sequences, such as siRNA against ERK (n=4). O'-methylation of G nucleosides, but not of U nucleosides, abrogates siRNA-induced Stat1 (n=4) (C, D) and iNOS (n=3) expression (D), and IP-9 (E), IL-6 (F) and IP-10 (n=6) release (G). siRNA-induced IP-10 release is also reduced by G-modification in Stat1-KO mice (n=3) (H). siRNA concentration was 100 nM. Data correspond to the 4-day time point, but Stat1 and IP-10 were also examined at 24h and the same effect was found. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus oligofectamine vehicle as control (-); $^{\&\&\&}$ $p<0.001$, $^{\&}$ $p<0.05$ versus p21 siRNA.

Figure 5. siRNA induces Stat1 expression and cytokine and chemokine release depending on the type of glia culture. A) Stat1 expression above basal is induced by siRNA in primary cultures of astroglia (primary), and in secondary cultures enriched in astrocytes (astroglia), but not in purified microglia cultures. B) Quantification of the signal (n=3-6) shows statistically significant increases. C, D) siRNA induces release of IP-10 (n=3) (C) and IL-6 (n=4) (D) in primary astroglia cultures and in astrocytes, but not in microglia. E) siRNA also induces a small increase in IP-9 release in primary astroglia only (n=2). F) Basal iNOS expression is

detected in microglia and, to a lesser extent, in primary astroglia cultures, but not in purified astroglia. A small induction of iNOS expression is apparent after siRNA in primary astroglia (n=3). Illustrated data correspond to the 4-day time point. siRNA concentration was 100 nM. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus the oligofectamine vehicle (-).

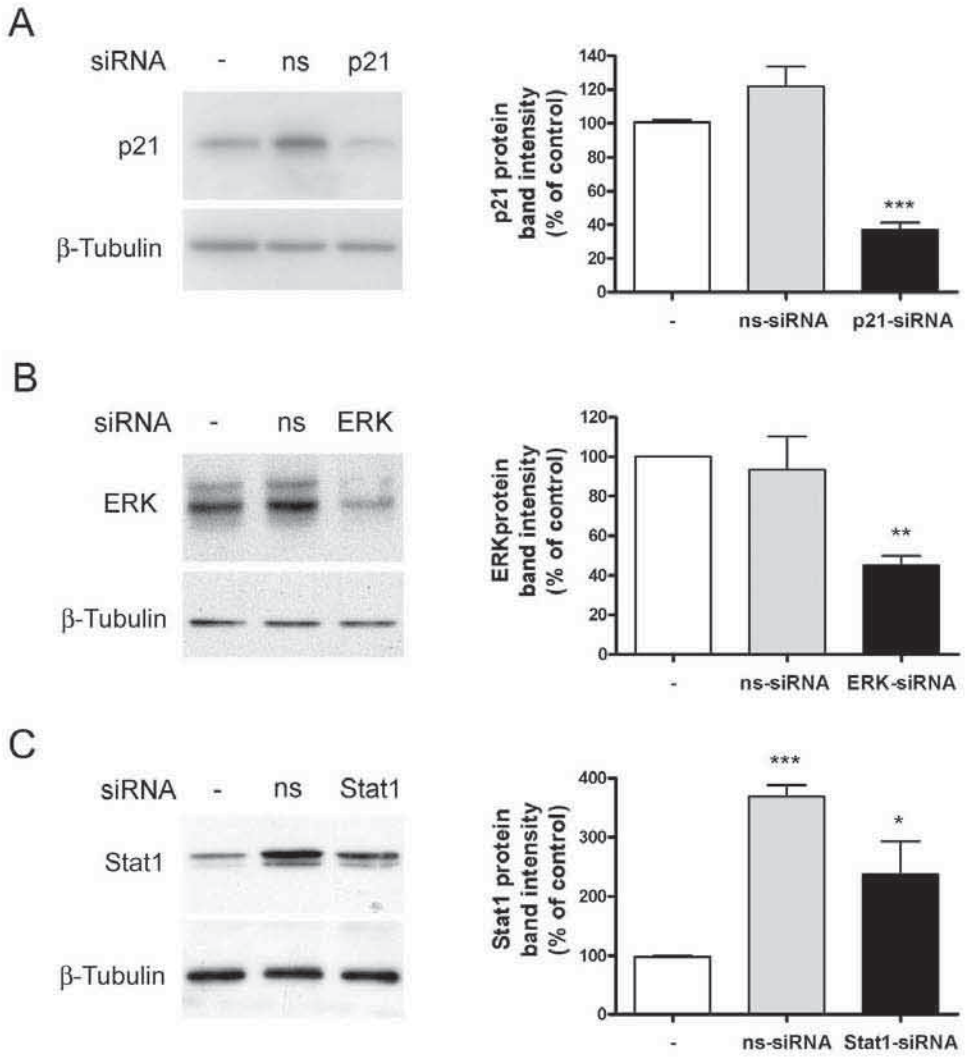
Figure 6. siRNA is internalized into astrocytes and microglia. Purified astroglia (A, B, C) and microglia (D, E, F) cells were treated with 100 nM fluorescent siRNA (red) for 24h. Cells are immunostained (green) with GFAP (A) or ED1 (D) to evidence astroglia and microglia, respectively. Fluorescent siRNA in astroglia (B) and microglia (E) cultures. Merged images (C, F) illustrate siRNA cell transfection. The nucleus of the cells is shown in blue after Hoecht staining (E, F). Bar scale: 25 μm.

Figure 7. siRNA does not increase Stat1 expression in NIH-3T3 cells. A,B) No induction of Stat1 is observed after siRNA (100 nM) treatment in epithelial 3T3 cells (n=4). C, D) However, these cells showed silencing responses after the siRNA treatment, as shown by reduced p21 protein expression after treatment with anti-p21 siRNA (n=5). * $p<0.05$ versus vehicle (oligofectamine) (-).

Figure 8. TLR7 and TLR3 mRNA expression in the different cell cultures, and effect of the TLR3 agonist PIC. A) TLR7 mRNA expression (n=4-5) and B) TLR3 mRNA expression (n=2-3) in primary astroglia cultures (primary), purified astroglia cultures (astroglia), purified microglia (microglia) and 3T3 cells show higher expression of TLR7 mRNA in microglia than in cultures containing astroglia and 3T3 cells. The reverse situation is found for TLR3 mRNA, and the highest expression is found in primary astroglia cultures. Comparatively, low TLR3 and TLR7 mRNA expression is found in 3T3 cells. Results are expressed as relative mRNA levels in relation to the value obtained in pure astrocytes (* $p<0.05$, ** $p<0.01$). C) siRNA (100 nM, or 3.3 μM, which is approximately equivalent to 50 μg/mL) or PIC (50 μg/mL) treatment increases the level of TLR3 mRNA expression at 24h

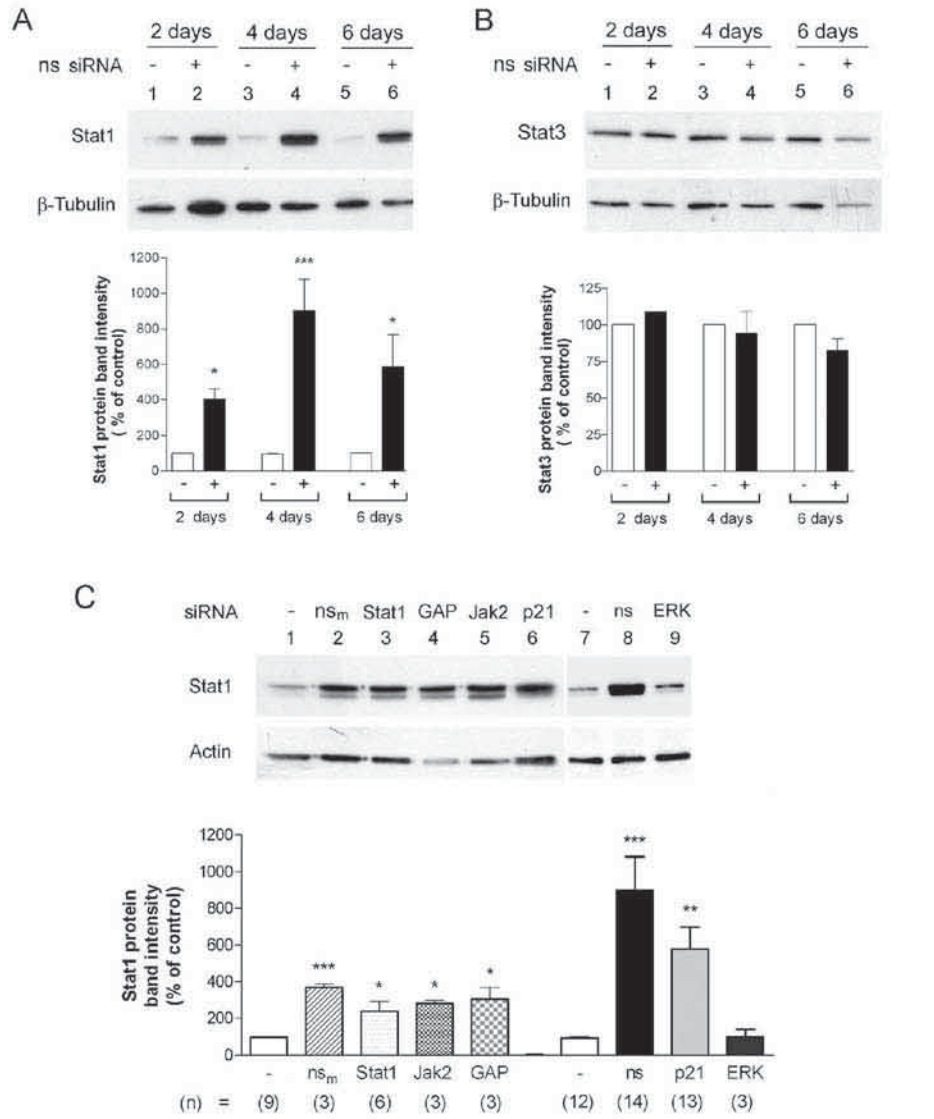
(n=3-4) in primary astroglia cultures. D) PIC (100 μg/mL) induces high IFN-β release at 24h in primary astroglia (n=3) and purified astroglia, but the induction is comparatively very low in microglia. E) PIC (100 μg/mL) and certain siRNAs (100 nM) induce the expression of Stat1, Cox-2 and VCAM-1 at 24h (n=3). The effects of unmodified siRNA against p21 is prevented by O-methylation of Guanosine (G), but not Uridine (U) nucleosides (n=3). F) Chloroquine (50 μg/mL) attenuates the effects of PIC (100 μg/mL) and siRNA (100 nM) (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 1



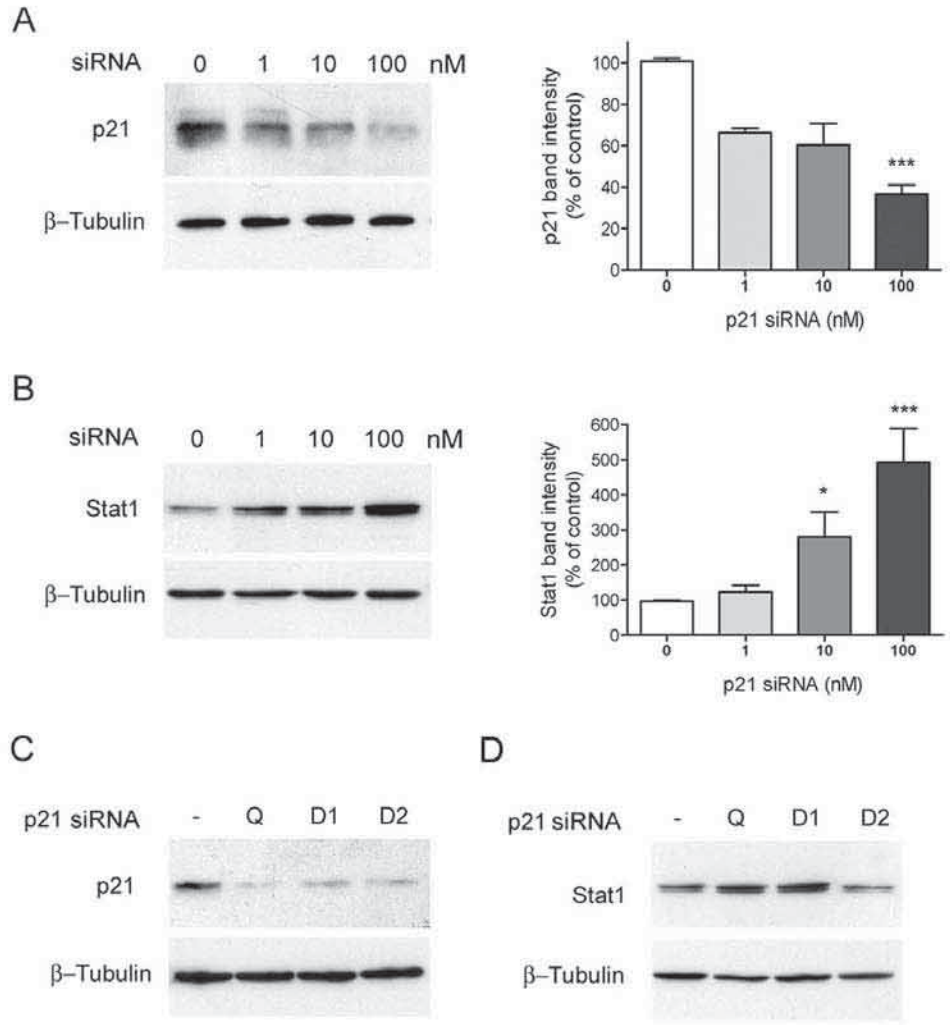
Specific siRNA sequences reduce the level of expression of the targeted proteins
119x134mm (300 x 300 DPI)

Figure 2



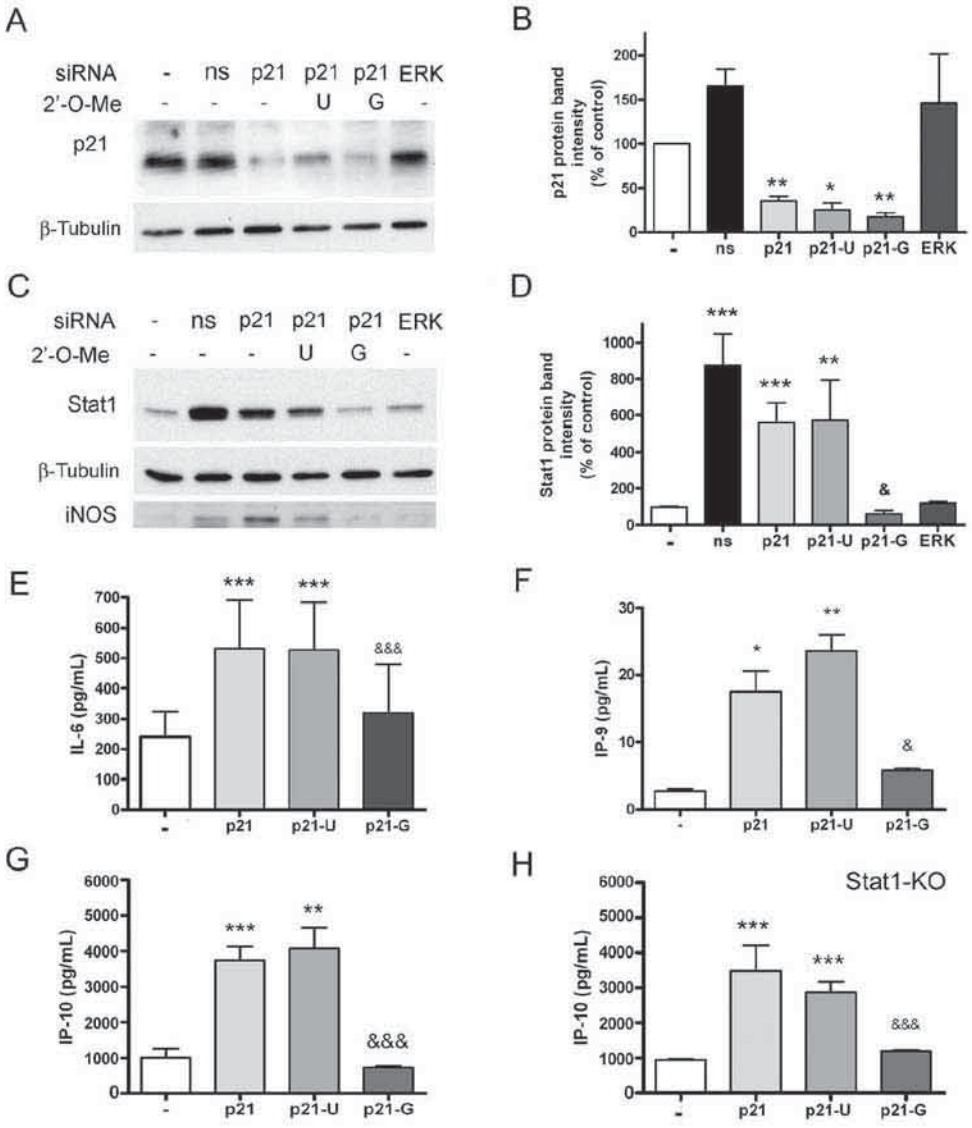
siRNA sequences induce the expression of Stat1 in primary cultures of glia
140x188mm (300 x 300 DPI)

Figure 3



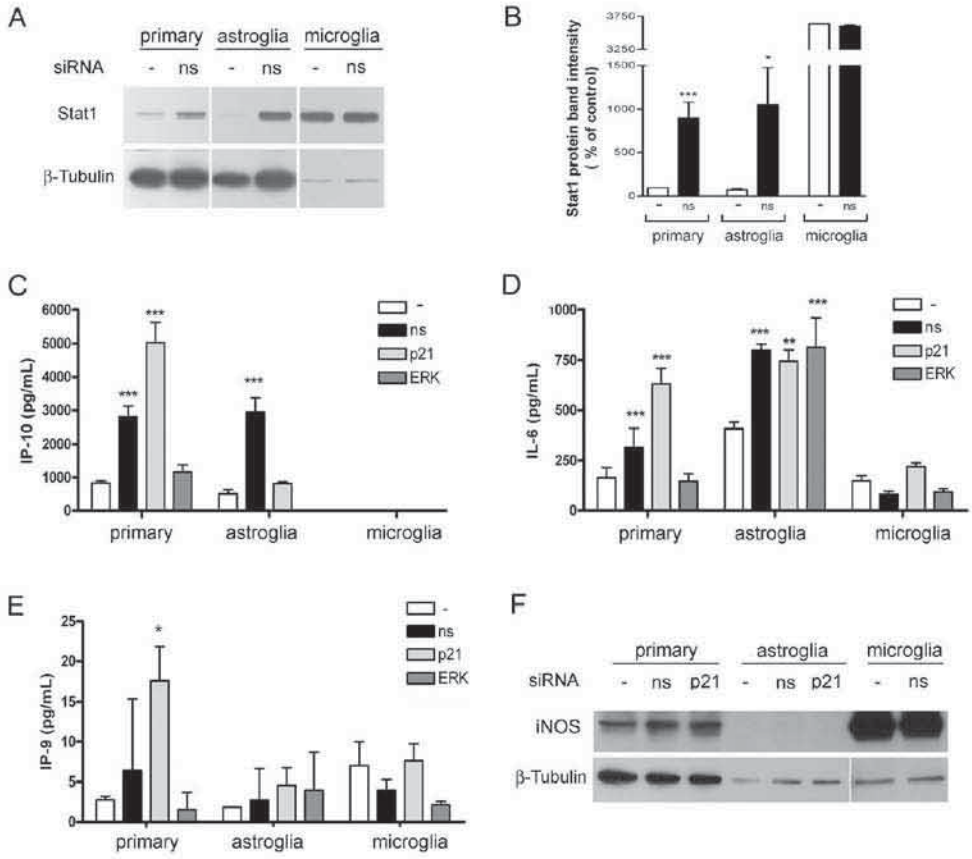
Stat1 protein-induction increases as a function of the siRNA concentration and it is dependent on the siRNA sequence, but independent of the silencing effect.
119x138mm (300 x 300 DPI)

Figure 4



O'-methylation of certain nucleosides of the siRNA sense strand abrogates Stat1, chemokine and cytokine induction, without altering the silencing effect
140x169mm (300 x 300 DPI)

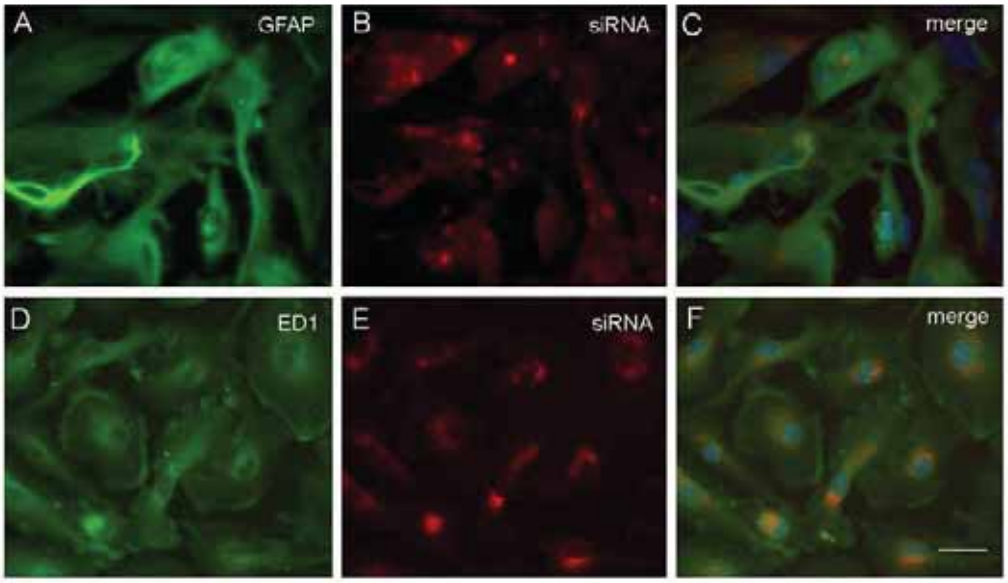
Figure 5



siRNA induces Stat1 expression and cytokine and chemokine release depending on the type of glia culture

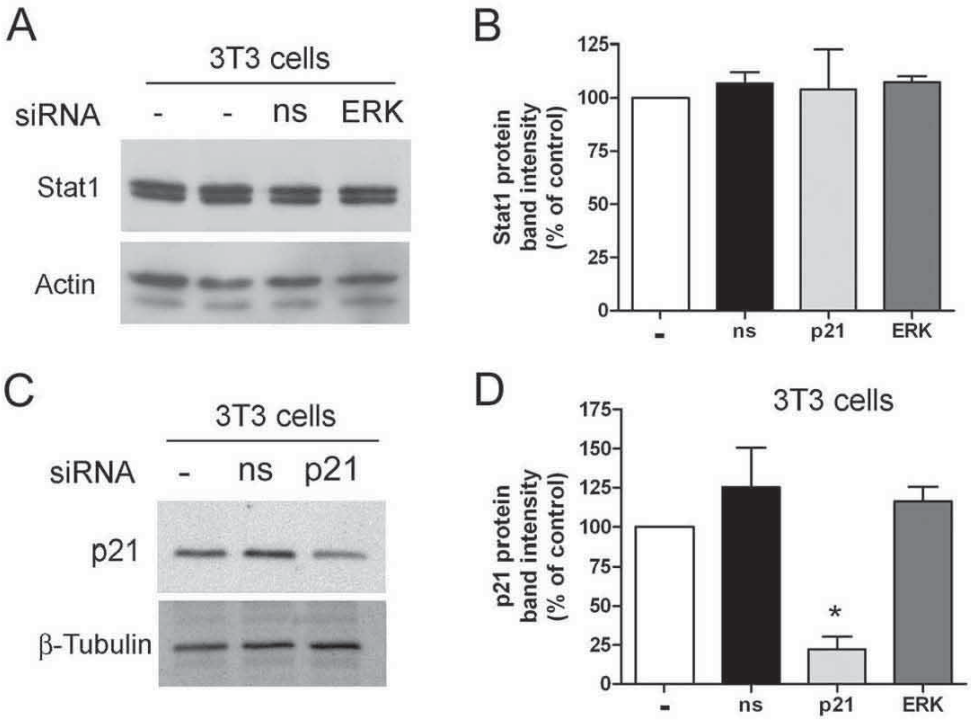
160x149mm (300 x 300 DPI)

Figure 6



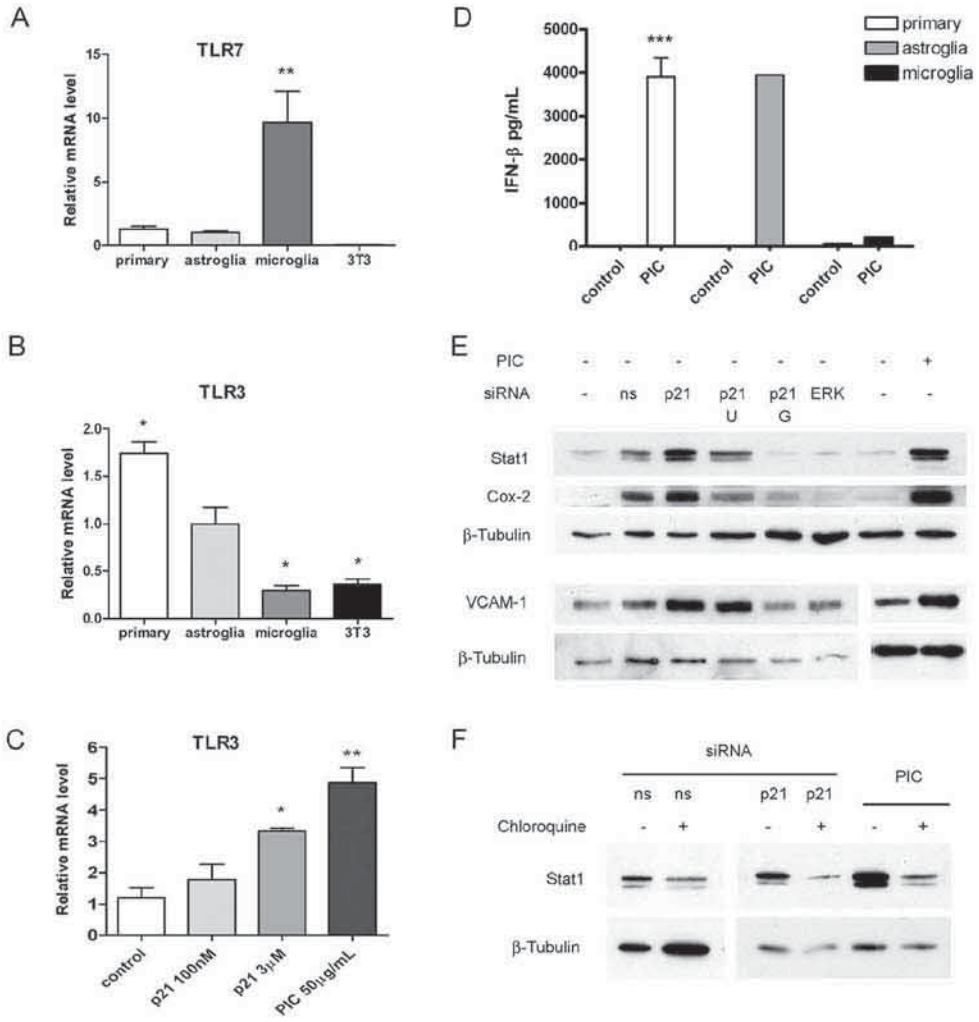
siRNA is internalized into astrocytes and microglia
140x90mm (300 x 300 DPI)

Figure 7



siRNA does not increase Stat1 expression in NIH-3T3 cells
94x78mm (300 x 300 DPI)

Figure 8



TLR7 and TLR3 mRNA expression in the different cell cultures, and effect of the TLR3 agonist PIC

140x150mm (300 x 300 DPI)

Article nº 4

Signalling pathways mediating inflammatory responses in brain ischaemia

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Signalling pathways mediating inflammatory responses in brain ischaemia

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Abstract

Stroke causes neuronal necrosis and generates inflammation. Pro-inflammatory molecules intervene in this process by triggering glial cell activation and leucocyte infiltration to the injured tissue. Cytokines are major mediators of the inflammatory response. Pro-inflammatory and anti-inflammatory cytokines are released in the ischaemic brain. Anti-inflammatory cytokines, such as interleukin-10, promote cell survival, whereas pro-inflammatory cytokines, such as TNFα (tumour necrosis factor α), can induce cell death. However, deleterious effects of certain cytokines can turn to beneficial actions, depending on particular features such as the concentration, time point and the very intricate network of intracellular signals that become activated and interact. A key player in the intracellular response to cytokines is the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway that induces alterations in the pattern of gene transcription. These changes are associated either with cell death or survival depending, among other things, on the specific proteins involved. STAT1 activation is related to cell death, whereas STAT3 activation is often associated with survival. Yet, it is clear that STAT activation must be tightly controlled, and for this reason the function of JAK/STAT modulators, such as SOCS (suppressors of cytokine signalling) and PIAS (protein inhibitor of activated STAT), and phosphatases is most relevant. Besides local effects in the ischaemic brain, cytokines are released to the circulation and affect the immune system. Unbalanced pro-inflammatory and anti-inflammatory plasma cytokine concentrations favouring an 'anti-inflammatory' state can decrease the immune response. Robust evidence now supports that stroke can induce an immunodepression syndrome, increasing the risk of infection. The contribution of individual cytokines and their intracellular signalling pathways to this response needs to be further investigated.

Introduction

Severe ischaemia causes necrosis accompanied by a strong inflammatory reaction that is believed to contribute to brain injury. Inflammation has therefore been considered a target for therapeutic intervention. Yet, ischaemia is not an intrinsic inflammatory disease, in spite of the fact that inflammatory factors in the endothelium might contribute to the aetiology of atherosclerosis, a risk factor for stroke. Inflammation in stroke is more complex as the brain exerts a master control on the local and remote responses to brain injury. Subsequently, both local and systemic pro-inflammatory and anti-inflammatory signals are activated, some derived from brain and some probably built up peripherally in response to brain damage. Key players in this response are cytokines. The main intracellular signalling pathways activated by cytokines include the NF-κB (nuclear factor κB) and the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathways. Both pathways share the ability to act as 'latent' transcription factors, as they are already present in the cytoplasm in an inactive form and can rapidly respond to extracellular signals and become activated, entering the nucleus and inducing gene transcription. Here, we will focus on the JAK/STAT pathway.

Key words: anti-inflammatory cytokine, immune system, ischaemia, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, stroke, suppressor of cytokine signalling (SOCS).

Abbreviations used: IFN, interferon; JAK, Janus kinase; IL, interleukin; NF-κB, nuclear factor κB; STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated STAT; SOCS, suppressors of cytokine signalling; Th1, T helper type 1; TNF, tumour necrosis factor.

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Pro-inflammatory and anti-inflammatory cytokines

Cytokines are soluble polypeptides that exert control on the function of different cell types, including regulation of growth and differentiation [1]. Cytokines are released to the cell environment and to the bloodstream to induce local and remote responses. They mainly act on membrane receptors that activate intracellular signalling pathways, which induce changes in the pattern of gene transcription as a cellular response to the altered milieu. The expression of cytokines is low under physiological conditions, but it can be highly up-regulated under pathological conditions, particularly those involving inflammation. Important features of cytokines are pleiotropy and redundancy [1], resulting in activation of similar pathways by different molecules and activation of multiple pathways by a single molecule.

Cytokines increase in the plasma of acute stroke patients, and the levels of certain cytokines may have prognostic value.

Indeed, increased IL-6 (interleukin-6) plasma concentrations were associated with early neurological worsening [2,3], and high IL-6 predicted early neurological deterioration and poor functional outcome in lacunar infarction [4]. In contrast, other findings showed a significant inverse correlation between plasma IL-6, neurological impairment and infarct growth, suggesting that plasma IL-6 might also signal neuroprotective effects in patients with ischaemic brain injury [5]. Experimental evidence also supports that certain pro-inflammatory cytokines might have beneficial properties in the ischaemic brain. IL-6 was protective in a model of permanent focal ischaemia in the rat [6], and blockade of IL-6 signalling aggravated ischaemic damage in mice [7]. High TNFα (tumour necrosis factor α) plasma levels were strongly related to poor outcome in stroke patients [4,5], and TNFα blockade reduced brain infarct volume and cerebral oedema after transient focal ischaemia in rats [8], and prevented damage and inflammation in mice [9]. However, in organotypic hippocampal cultures exposed to ischaemic conditions, pre-treatment with TNFα was protective against neuronal death, while treatment after ischaemia was detrimental and it increased oxidative stress [10]. Furthermore, an appropriately timed increase in TNFα induced ischaemic preconditioning in experimental studies [11,12], and it was associated with ischaemic tolerance in stroke patients [13]. Thus TNFα has a wide range of effects on brain ischaemia [11]. Therefore the role of individual cytokines is more complex than previously anticipated; their concentration and time of activation, and the cross-talk between different signals are essential for the cellular and pathological outcome.

Anti-inflammatory cytokines, such as IL-10, are associated with beneficial effects in the injured brain. Indeed, IL-10 knockout mice showed larger infarctions than wild-type mice after permanent focal ischaemia [14], while administration of IL-10 to the lateral ventricle [15] or treatment with adenoviral vectors encoding human IL-10 [16] reduced infarct volume and inflammation after permanent focal ischaemia in rats. In the clinic, poor outcome and neurological worsening were predicted by low IL-10 plasma levels [17]. Yet, IL-10 has been associated with a higher susceptibility to infection, suggesting that it may contribute to attenuate the capacity of mononuclear phagocytes to initiate inflammatory and adaptive immune responses [18]. Therefore excessive anti-inflammatory signals, such as IL-10, might have an effect on the immune system by displacing the Th1 (T helper type 1)/Th2 balance towards a Th2 predominant response [19], which can increase the susceptibility to infection. Also, IL-10 is produced by Th2 cells, and it inhibits Th1-cell function [20]. Reduced immune responses can be promoted by acute ischaemic brain injury, mainly through the hypothalamic–pituitary–adrenal axis and the peripheral nervous system [21,22]. Furthermore, focal cerebral ischaemia promotes the release of cytokines and chemokines by the peripheral immune system in mice [23]. Robust experimental evidence now supports the proposal that brain ischaemia induces an immunodepression syndrome facilitating infection [21–24], and the sympathetic nervous system has been identified as a key mediator in this process [21], although further studies at the bedside are necessary. Infection after stroke is associated with higher mortality [25], and we addressed whether post-stroke infection could be prevented by prophylactic treatment with antibiotics in the ESPIAS (Early Systemic Prophylaxis of Infection After Stroke) trial, which gave negative results [26]. A currently ongoing trial on preventive antibacterial short-term therapy in patients with acute ischaemic infarction, PANTHERIS (http://www.charite.de/ch/neuro/forschung/teams/experimentell/pantheris.htm), might help to unravel this intricate question. We also found that stroke-associated infection is a marker of the severity of stroke without an independent outcome effect when it is promptly treated [27]. More recently, we examined the patterns of pro-inflammatory and anti-inflammatory cytokines in the circulation and their relationship with the appearance of infection in patients with acute stroke. IL-10 was increased, and the ratio TNFα/IL-10 was decreased very early after clinical onset in patients at greater risk of infection [27a], supporting the view that an excessive anti-inflammatory cytokine profile was a major predisposing factor.

JAK/STAT: a main signalling pathway mediating cellular responses to cytokines

JAKs are coupled with the intracellular domain of several cytokine membrane receptors, and can become phosphorylated as a result of cytokine receptor binding. Subsequently, JAKs can phosphorylate members of the STAT family, which then form homo- or hetero-dimers, translocate to the nucleus and bind to specific DNA consensus sequences to promote selective transcriptional activation [28]. JAKs and STATs are normally expressed in brain [29–31], and their expression increases after focal ischaemia in the rat, particularly in reactive astrocytes and microglia cells [29,31], indicating that JAK/STAT is an important mediator of inflammatory responses in brain ischaemia. A strong increase in the expression of STAT3 [29] and STAT1 [30] occurs in reactive microglia/macrophages and is coincidental with the time point when this reaction becomes very strong (i.e. from 4 days post-ischaemia). However, increases in the expression of cytokines are detected much earlier after brain ischaemia (hours) [32], suggesting that activation of basal JAK/STAT takes place long before increased expression of these proteins is induced. Activated STAT3 was found in neurons after focal ischaemia [33,34], but its function is controversial as different studies associated it with survival [33], while others related it with cell death [34]. STAT1 was also found phosphorylated in the lesion core and in the periphery [35], and STAT1 knockout mice were more resistant to ischaemic brain damage [36]. Taken together, these results show activation of STAT proteins after ischaemia and suggest a contribution of the JAK/STAT pathway to the cell fate. However, the primary stimuli responsible for JAK/STAT activation in the ischaemic brain have not yet been identified.

We undertook *in vitro* studies in primary cultures of glial cells to examine the ability of various stimuli to activate

STATs. Anti-inflammatory cytokines such as IL-10 induced STAT3 activation, but not STAT1 activation, while pro-inflammatory signals such as IFN-γ (interferon-γ) induced STAT1 activation, but not STAT3 activation, and both STAT1 and STAT3 were activated by IL-6 [37]. JAK2 was involved in the reported activation of STATs after IL-6 and IFN-γ but not after IL-10, as evidenced using the JAK2 inhibitor tyrphostin (AG490) [37]. Besides direct activation of JAK/STAT by certain cytokines, several pro-inflammatory stimuli, such as the bacterial lipopolysaccharide protein, also trigger STAT1 activation. Yet, in this case, this is a delayed effect mediated by *de novo* protein synthesis, as it was prevented by cycloheximide and it was abrogated by NF-κB inhibitors (R. Gorina and A.M. Planas, unpublished work), again indicating a close interaction between the different intracellular signalling pathways.

STATs can also become activated by oxidative stress, which can be generated in the ischaemic brain and contribute to ischaemic brain damage. In astrocytes, treatment with H_2O_2 increases tyrosine phosphorylated STAT1 and STAT3, and both tyrosine phosphorylations are mediated by JAK2 [37]. As a result of this, STATs translocate to the nucleus and activate the DNA GAS (gamma-activated site), an effect that was abrogated by inhibition of JAK2 (R. Gorina and A.M. Planas, unpublished work). More important, this inhibitor prevented H_2O_2-induced cell death in astrocytes and in neuroblastoma cells, showing that JAK2 signalling was involved in this process [37]. The JAK2 inhibitor repressed STAT1 phosphorylation and nuclear translocation, and, in spite of the fact that it also reduced STAT3 phosphorylation, it did not prevent its nuclear translocation, suggesting that its protective effect was attributable to strong inhibition of H_2O_2-induced STAT1 activation (R. Gorina and A.M. Planas, unpublished work). Preliminary results in astrocytes obtained from STAT1-deficient mice give further support to the deleterious effect of STAT1 activation on cell viability, as these cells were more resistant than wild-type astrocytes to H_2O_2-induced cell death (R. Gorina and A.M. Planas, unpublished work). Altogether, these results comply with the view that JAK2/STAT1 activation promotes cell death [38], and agree with the results found *in vivo* in brain ischaemia [36]. In contrast, activation of STAT3 is induced by anti-inflammatory signals and may have pro-survival effects [39]. Therefore STAT1 and STAT3 appear to have opposite effects [40], and the specific balance between them may trigger selective patterns of gene transcription. Besides STAT1 and STAT3, other members of the STAT family are expressed in the brain and play various roles in cytokine signalling, as proved with the use of genetically modified animals [41], but whether they are involved in ischaemic brain damage remains to be elucidated.

Moreover, the JAK/STAT signalling pathway is involved in regulation of the immune response [42]. Signalling through JAK/STAT participates in the generation of Th1 and Th2 cells. IL-12 activates a pattern of JAKs and STATs that triggers the expression of Th1-specific proteins, such as IFN-γ, while IL-4 activates a distinct pattern of JAK/STATs leading to the synthesis of Th2-type cytokines [43]. These pathways cross-talk, exert self-positive feedback loops, and regulate each other at the transcriptional level [43]. The possible involvement of the JAK/STAT pathway in the altered systemic cytokine response that we observed in stroke patients susceptible to infection needs to be investigated.

Regulation of STAT signalling

The local cytokine environment causes different degrees of STAT activation, and the activities of certain phosphatases and of negative modulators, such as SOCS (suppressors of cytokine signalling) and PIAS (protein inhibitor of activated STAT), exert an important regulatory control on STAT activity [44]. The action of STAT signalling may be very transient, as its activation can induce the expression of SOCS proteins and exert a control on STAT phosphorylation [44]. Persistent activation of STATs may be due to a failure of the system to induce the synthesis of SOCS, which might be prevented under conditions of protein synthesis inhibition, as it occurs in brain ischaemia [45]. Increased expression of SOCS3 mRNA was found after transient MCA (middle cerebral artery) occlusion, and antisense knockdown of SOCS3 expression exacerbated infarct volume, indicating that SOCS3 might have beneficial effects [46]. In agreement with this view, SOCS3 has been reported to inhibit inflammation and apoptosis [47]. However, SOCS-3 overexpression leads to neuroblastoma cell death [39]. These findings indicate that controlled negative modulation of STAT activation is necessary for cell survival, and that STAT activation must be well regulated to avoid negative effects of this signalling, including inflammation. SOCS3 is also involved in the regulation of immune responses through its modulator effect on cytokine signalling, and due to its predominant expression in Th2 cells [48]. SOCS3 transgenic mice showed increased Th2 responses, and dominant-negative mutant SOCS3 transgenic mice, as well as mice with a heterozygous deletion of Socs3, had decreased Th2 development [48]. SOCS3-deficient CD4+ T-cells produced more transforming growth factor-β1 and IL-10, but less IL-4 than control T-cells, and mice lacking Socs3 in T-cells showed reduced immune responses [49]. Also, SOCS3 signalling has been associated with susceptibility to infection [18].

PIASs act as nuclear transcriptional co-repressors that modulate several transcription factors by functioning as SUMO-1 (small ubiquitin-related modifier-1) ligases and inhibit STAT function [44]. Again, several lines of evidence support an *in vivo* function for PIAS in the regulation of innate immune responses [50]. Hence, the molecular mechanisms underlying the regulation of STAT activity are very complex and still not fully understood, while there is no doubt about its major role in the regulation of inflammatory and immune responses to cytokines.

Conclusions

Cytokines are involved in the post-ischaemic inflammatory response that takes place locally in the injured tissue, but

also systemically in the circulation. Several lines of evidence suggest that cytokines might be cellular mediators, not only in brain inflammation, but also in the immunodepression syndrome that has been associated with stroke. A major pathway signalling cytokine receptor activation is JAK/STAT. This pathway involves different protein members of the JAK/STAT family that trigger selective patterns of gene transcription governing the cellular response to the cytokine challenge. In the brain, STAT1 activation is associated with detrimental effects in terms of cell viability, while STAT3 is rather regarded as a pro-survival factor. Nonetheless, tight control of its activation is necessary, as evidenced by studies in mice genetically modified for the expression of SOCS. The evidence is also growing on the involvement of JAK/STAT and its negative regulators in mediating immune responses. Further studies are needed to better understand the origin and function of circulating cytokines after stroke, and to elucidate whether anti-inflammatory signals that are believed to be beneficial locally in the ischaemic brain are linked to depression of the immune system and to increased risk of infection in stroke patients.

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