Proteomic Analysis of Astrocytic Secretion in the Mouse. Comparison with the Cerebrospinal Fluid Proteome


UPR CNRS 2580, 141 rue de la Cardonille, 34094 Montpellier Cedex 5, France.
Phone: +33 4 67 14 29 83 Fax: +33 4 67 14 29 10.

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Corresponding author: Dr Philippe Marin, CNRS UPR 2580, CCIPE, 141, rue de la Cardonille, 34094 Montpellier Cedex 05, France. Phone: +33 4 67 14 29 83; Fax: +33 4 67 14 29 10; email: marin@montp.inserm.fr

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SUMMARY

Astrocytes, the most abundant cell type in the central nervous system, are intimately associated with synapses. They play a pivotal role in neuronal survival and the brain inflammatory response. Some astrocytic functions are mediated by the secretion of polypeptides. Using a proteomic approach, we have identified more than 30 proteins released by cultured astrocytes. These include proteases and protease inhibitors, carrier proteins and antioxidant proteins. Exposing astrocytes to brefeldin A, which selectively blocks secretory vesicle assembly, suppressed the release of some of these proteins. This indicates that astrocytes secrete these proteins by a classical vesicular mechanism and others by an alternative pathway. Astrocytes isolated from different brain regions secreted a similar pattern of proteins. However, the secretion of some of them, including metalloproteinase inhibitors and apolipoprotein E, was region-specific. In addition, proinflammatory treatments modified the profile of astrocytic protein secretion. Finally, more than two thirds of the proteins identified in the astrocyte-conditioned medium were detectable in the mouse cerebrospinal fluid, suggesting that astrocytes contribute to the cerebrospinal fluid protein content. In conclusion, this study provides the first unbiased characterization of the major proteins released by astrocytes, which may play a crucial role in the modulation of neuronal survival and function.
INTRODUCTION

Glial cells represent the largest cell population in the central nervous system (CNS)\(^1\). They are divided into three categories: astrocytes, the most abundant glial cell type, oligodendrocytes, the central equivalent of Schwann cells, and microglial cells, which share features with immune cells. For decades, astrocytes were essentially considered to be passive elements providing a structural support for neurons and contributing to the blood brain barrier by wrapping processes around CNS microvessels. Several physiological properties related to CNS homeostasis (clearance and metabolism of neurotransmitters, regulation of extracellular pH and K\(^+\) level) have also been attributed to astrocytes, which thereby contribute to the maintenance of an ideal environment for neuronal cell function (1).

Many recent studies have established that astrocytes, which are intimately associated with synapses, are active integrators and regulators of neuronal activity and synaptic transmission (2-6). These astrocytic functions are mediated, at least in part, by the release of various substances including amino acids and polypeptides. Indeed, glutamate released from synaptic terminals not only binds to glutamate receptors on the post-synaptic neurons but also activates AMPA receptors on the surrounding astrocytes. This activation induces a rapid increase in intracellular Ca\(^{2+}\) and a Ca\(^{2+}\)-dependent release of glutamate from astrocytes that in turn activates post-synaptic glutamate receptors on neighboring neurons, thereby enhancing excitatory synaptic transmission (7,8). A similar modulatory effect involving perisynaptic astrocytes has been demonstrated at inhibitory synapses (9). A new concept of tripartite synapses based on neuronal-astrocytic cooperation for the processing of information has recently emerged from these findings (10). A recent study has demonstrated that perisynaptic glial cells of a molluscan cholinergic synapse respond to acetylcholine by releasing a nicotinic receptor-like protein into the synaptic cleft. This protein can capture acetylcholine released into the synapse, thereby inhibiting cholinergic synaptic transmission (11). Although such a mechanism remains to be established in the mammalian CNS, astrocytic secretion of proteins may be one of the critical determinants involved in the modulation of synaptic transmission.
To date, protein secretion by astrocytes has only been investigated in a piecemeal fashion, focusing on one or two protein species (12-15).

The present study was carried out to identify the major proteins secreted by murine astrocytes in primary culture, using a proteomic approach based on Matrix-Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF) mass spectrometry. The release of proteins by astrocytes was examined in cultures originating from different brain areas and exposed to pro-inflammatory treatments. Finally, the pattern of proteins secreted by astrocytes was compared with the cerebrospinal fluid (CSF) proteome.
EXPERIMENTAL PROCEDURES

Materials

Swiss mice were obtained from Janvier (Le Genest-St-Isle, France). Brefeldin A (BFA) was purchased from Tebu (Le Perray en Yvelines, France). Lipopolysaccharide (LPS, type 055:B5), fluorescein isothiocyanate (FITC)-conjugated isolectine B4 and the monoclonal anti-MAP2 antibody (clone AP20) were from Sigma (Saint Quentin Fallavier, France), recombinant murine interleukin 1-β (IL1-β) and recombinant murine tumor necrosis factor-α (TNF-α) from R&D Europe, Ltd. (Abingdon, UK). The polyclonal anti-cystatin C antibody, the polyclonal anti-β2-microglobulin antibody and the polyclonal anti-GFAP antibody were from Dako (Glostrup, Denmark).

Astrocyte cultures

Primary cultures of astrocytes from various brain regions were prepared as previously described (16). The brain structures from 18 day-old Swiss mouse embryos were mechanically dissociated in PBS, supplemented with 33 mM glucose (PBS-glucose). Cells were seeded (0.5 \(10^6\) cells/ml) in either 100-mm (15 ml/dish) or 30-mm (3 ml/dish) culture dishes, previously coated with poly-L-ornithine (1.5 µg/ml, Mw = 40,000). The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 nutrient, supplemented with glucose (30 mM), glutamine (2 mM), NaHCO₃ (13 mM), HEPES buffer (5 mM, pH 7.4), penicillin-streptomycin (100 IU/ml-100 µg/ml, respectively) and 10% Nu-serum (Beckton Dickinson Biosciences, Le Pont de Claix, France). The culture medium was changed every three days and cells were maintained for 21 days at 37°C in a humidified atmosphere containing 5% CO₂. At this stage, cultures were highly enriched in astrocytes. Indeed, more than 98% of the cells were stained by an indirect immunofluorescence technique using a rabbit anti-GFAP antibody (9 fields containing 350 cells originating from 3 independent cultures counted). Fluorescent staining of cultures using FITC-conjugated isolectine B4, a specific marker of microglial and endothelial cells in developing and adult
CNS (17), indicated that they contained less than 0.2% of microglial and/or endothelial cells. Cultures were also devoid of neurons because no immunostaining was observed using a mouse anti-MAP2 antibody. The remaining cells could be not fully differentiated astrocytes, which are unlabeled by GFAP antibodies (18).

Preparation of astrocyte-conditioned media and cell extracts

Cells, grown in 100-mm culture dishes, were washed six times with the serum-free culture medium. This washing procedure efficiently eliminated all serum proteins, because we did not detect any trace of bovine albumin, the major serum protein, in the astrocyte-conditioned medium. Cells were then covered with a minimal volume of the serum-free medium (6 ml per dish) for 18 h at 37°C and 5 % CO₂ in the presence of the indicated treatments. Astrocyte-conditioned media were harvested and centrifuged successively at 200 x g (5 min), 1,000 x g (10 min) and 20,000 x g (25 min) to remove non-adherent cells and debris. Samples (about 40 µg protein/dish) were precipitated for 2 h with 10% ice-cold trichloroacetic acid (TCA).

The cells were scraped off in 5 ml PBS-glucose and centrifuged at 200 x g for 5 min. They were then resuspended in ice-cold lysis buffer containing Tris-HCl (50 mM, pH 7.4), EDTA (1 mM), sodium dodecyl sulfate (SDS, 1%) and a cocktail of protease inhibitors (Roche), and homogenized 20 times with a glass-Teflon homogenizer. Samples were centrifuged for 30 min at 10,000 x g and the supernatants (whole cell extracts) were precipitated with 10% TCA. Protein concentration in conditioned media and cell extracts was determined with the bicinchoninic acid method (19).

Preparation of cerebrospinal fluid

The cerebrospinal fluid (CSF) was collected from the cisterna magna (sub-cerebellar cisterna) of 2 month-old male Swiss mice previously anaesthetized with an intra-peritoneal injection of pentobarbital (60 µg/g body weight). The CSF samples were centrifuged successively at 200 x g (5 min), 1,000 x g (10 min) and 20,000 x g (25 min) to eliminate cells and other insoluble material. The final supernatants were precipitated with 10% TCA.
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High resolution two-dimensional gel electrophoresis

TCA precipitates were washed three times with diethyl ether and resuspended in 350 µl isoelectrofocusing medium containing urea (7 M), thiourea (2 M), CHAPS (4%), ampholines (preblended, pI 3.5-9.5, 8 mg/ml, Amersham Biosciences, Upsalla, Sweeden), dithiothreitol (DTT, 100 mM), tergitol NP7 (0.2%, Sigma) and traces of bromophenol blue (20). Proteins (30 µg/gel) were first separated according to their isoelectric point along linear immobilized pH-gradient (IPG) strips (pH 3-10, 18 cm long) using the IPGphor apparatus (Amersham Biosciences). Sample loading for the first dimension was performed by passive in-gel re-swelling. After the first dimension, the IPG strips were equilibrated for 10 min in a buffer containing urea (6M), Tris-HCl (50 mM, pH 6.8), glycerol (30%), SDS (2%), DTT (10 mg/ml) and bromophenol blue, and then for 15 min in the same buffer containing 15 mg/ml iodoacetamide instead of DTT. For the second dimension, the strips were loaded onto vertical 12.5% SDS polyacrylamide gels, unless otherwise indicated. The gels were silver stained according to the procedure of Shevchenko et al. (21).

Image acquisition and two-dimensional gel spot pattern analysis

Gels to be compared were always processed and stained in parallel. Gels were scanned using a computer-assisted densitometer. Spot detection, gel alignment and spot quantification were performed using the Image Master 2D Elite software (Amersham Biosciences). Quantification of proteins was expressed as volumes of spots. To correct for variability resulting from silver staining, results were expressed as relative volumes of all spots in each gel. Data are the means of values from four gels originating from experiments performed on different sets of cultured astrocytes.

MALDI-TOF mass spectrometry and protein identification

Proteins of interest were excised and digested in gel using trypsin (sequencing grade,
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Promega, Charbonnières, France), as previously described (21). Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 µl formic acid (2%), desalted using Zip Tips C18 (Millipore, Bedford, MA), eluted with 10 µl acetonitrile-trifluoroacetic acid (TFA), (80-0.1%) and concentrated to 2 µl. Aliquots (0.5 µl) were mixed with the same volume of α-cyano-4-hydroxy-trans-cinnamic acid (Sigma, 10 mg/ml in acetonitrile-TFA, 50-0.1%) and loaded on the target of a BIFLEX III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Analysis was performed in reflectron mode with an accelerating voltage of 20 kV and a delayed extraction of 400 ns. Spectra were analyzed using the XTOF software (Bruker-Franzen Analytik) and autoproteolysis products of trypsin (mol wt: 842.51, 1045.56, 2211.10) were used as internal calibrates. Identification of proteins was performed using both Mascot and PeptIdent softwares (available online at http://www.matrixscience.com and http://www.expasy.org/tools/peptident.html, respectively). A mass deviation of 100 ppm was allowed for data base interrogation, but the mass accuracy of our analyses was usually better than 50 ppm. Coverage of the full-length protein exceeding 15% was considered to be sufficient unless there were some obvious conflicts between the experimental molecular weight or isoelectric point, and those of the identified protein. Matching peptides with one missed cleavage were considered as pertinent only when there were two consecutive basic residues or when arginine and lysine residues were followed by a proline or acidic residues inside the peptide amino acid sequence (22,23).

Immunoblotting

Proteins, resolved on one- or two-dimensional (1-D or 2-D) gels, were transferred electrophoretically onto nitrocellulose membranes (Hybond-C, Amersham Biosciences). Membranes were incubated overnight with primary antibodies. Immunoreactivity was detected with an enhanced chemiluminescence method (Renaissance Plus, NEN DuPont, Boston, MA).
Measurement of astrocyte viability

The viability of astrocyte cultures exposed to pro-inflammatory treatments was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (24). Cells were incubated with MTT (60 µg/ml, directly added to the serum-free medium) for 45 min at 37°C. The blue formazan derivative was solubilized in dimethyl sulfoxide and optical density was read at 560 nm. Lack of membrane integrity of astrocytes was also estimated by measuring propidium iodide (PI) incorporation (3 µg/ml, 10 min at room temperature). Cells were then fixed with 4% paraformaldehyde in PBS (30 min at 4°C) and nuclei were stained with Hoechst 33258 (1 µg/ml for 10 min at room temperature). They were then washed successively with PBS and distilled water and mounted in Mowiol under coverslips. Nuclear DNA staining was examined by digital fluorescence imaging microscopy (Axiophot 2 microscope, Zeiss). Necrosis was estimated by counting PI-positive nuclei versus total nuclei (stained with Hoechst 33258) in at least 9 different fields (about 350 cells per field) originating from three independent cultures.
RESULTS

Protein release by mouse striatal astrocytes through a brefeldin A-sensitive pathway

For a first insight into the composition of the proteins secreted by astrocytes, the medium conditioned by confluent striatal astrocytes (18 h at 37°C) was concentrated and proteins (30 µg per gel) were resolved by 2-D electrophoresis. Numerous protein spots were detectable on silver-stained 2-D gels (Fig. 1). To specifically identify proteins that were released from astrocytes through the vesicular pathway, the extracellular protein pattern was compared to that from astrocytes treated during the secretion period with BFA (1 µg/ml). BFA selectively blocks secretory vesicle assembly and, thus, the secretion of proteins through the classical vesicular pathway. Several spots or groups of spots detected in 2-D gels of conditioned media of control cells were absent in gels obtained from BFA-treated cells (Figs. 1 and 2). These spots were undetectable in 2-D gels of whole cell extracts (30 µg per gel, not shown). Exposing astrocytes to higher BFA concentrations (up to 10 µg/ml) did not decrease the expression of additional proteins in culture supernatants (data not shown). All proteins secreted by astrocytes through a BFA-sensitive mechanism were identified by MALDI-TOF mass spectrometry (see Table 1). These proteins include: 1) proteases (complement C3 α and β chains and carboxypeptidase H), 2) protease inhibitors (the matrix metalloproteinase inhibitor-2 (TIM-2) and cystatin C (CYSC)), 3) carrier proteins (ceruloplasmin, transcobalamin II, apolipoprotein E, insulin-like growth factor-binding protein-2 (IBP-2)), and 4) proteins with other functions (β2-microglobulin (B2MG), a cell surface component of class I major histocompatibility molecules, secreted protein and rich in cystein (SPARC), a protein that binds to several resident proteins of the extracellular matrix, and pentraxin-related protein 3, a prototypic long pentraxin that may be involved in the CNS immunity against pathogens and tissue damage). The identification of two of these proteins, CYSC and B2MG, was confirmed by 2-D immunoblotting (Figs. 2B and C). Note that several CYSC isoformes showing marked differences in apparent isoelectric points (pHi 7 to 9) and molecular weights (12-18 kDa) are released by striatal astrocytes (Figs. 2A and B). Western blot analysis also confirmed that both CYSC and B2MG were highly enriched in the astrocyte-conditioned
medium, compared with the total cell lysates and that their accumulation in the extracellular medium was inhibited by BFA (Figs. 2B and C).

**Protein release by striatal astrocytes through a brefeldin A-insensitive pathway**

A differential analysis of 2-D protein profiles of astrocyte-conditioned media and total cell extracts, performed with the Image Master software, revealed an enrichment of an additional group of proteins in the extracellular medium (Fig. 3). Eighteen proteins, each representing 1-4% of total extracellular proteins, were 2.5-35 fold more abundant in the extracellular medium, compared with the total cell extract (Fig. 3). This suggests that these proteins are released by astrocytes through a secretion process. Moreover, their enrichment in the extracellular medium does not result from cell disruption during the washing and harvesting procedures, as indicated by the lack of PI incorporation in cell nuclei (data not shown). The accumulation of these proteins in astrocyte-conditioned medium was not altered by BFA, thus ruling out a secretion through the classical vesicular pathway. In agreement with this observation, the amino acid sequences of these proteins lack obvious signal peptide. These proteins include: 1) endozepine, a 10-kDa polypeptide called diazepam binding inhibitor (DBI) which generates, through proteolytic cleavage, several biologically active peptides interacting with central or peripheral benzodiazepine receptors (25); 2) phosphatidylethanolamine binding protein (PEBP), a multifunctional protein (26); 3) cyclophilin A, an intracellular protein that binds the immunosuppressive agent cyclosporin A and possesses peptidyl-prolyl cis-trans isomerase activity (27), and 4) a set of antioxidant proteins that may be involved in the defense against oxidative stress. These antioxidant proteins include Cu/Zn superoxide dismutase, glutathione S-transferase class-mu (GST1) and several thiol-dependent antioxidant proteins such as thioredoxin peroxidases 1 and 2 (these proteins have recently been renamed peroxiredoxins 2 and 1, respectively), and antioxidant protein-2 (AOP2). AOP2 also belongs to the thioredoxin peroxidase family, but this protein has only one conserved cysteine residue instead of two. AOP2 was therefore called 1-Cys peroxiredoxin (28). An extracellular location has been proposed for some of these proteins.
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Finally, several cytosolic proteins including enzymes and brain-enriched 14-3-3 proteins were also found in the medium conditioned by striatal astrocytes. Altogether, these results suggest that astrocytes can secrete substantial amounts of various proteins through a mechanism independent of the classical vesicular pathway.

Regional specificity of astrocytic protein release

To examine the regional specificity of astrocytic protein release, we compared the pattern of proteins in the medium conditioned by striatal astrocytes with the pattern of proteins released by cultures of astrocytes isolated from other CNS regions. A similar profile of extracellular proteins was found in the medium conditioned by astrocytes from the striatum, the cerebral cortex, the hippocampus and the cerebellum, with a few remarkable differences, which are depicted in Fig. 4. Striatal astrocytes released larger amounts of apolipoprotein E than did astrocytes originating from the other brain regions examined. In contrast, striatal astrocytes secreted the lowest amounts of IBP-2. A regional specificity was also observed for the release of metalloproteinase inhibitors. Indeed, TIM-2 was only detectable in the supernatant of striatal astrocytes, whereas TIM-1 was specifically released by astrocytes from the cerebellum. AOP2, a protein which is released by a BFA-insensitive pathway, was greatly enriched in the conditioned medium of striatal, cortical and hippocampal astrocytes (about 2% of total extracellular protein content), compared with that measured in the supernatant of cerebellar cultures (0.28 ± 0.07 % of total extracellular protein content, n = 4) (Fig 4).

Effects of pro-inflammatory treatments on astrocytic protein release

It is well documented that astrocytes play a pivotal role in the type and extent of CNS immune and inflammatory response, in part by their ability to release various cytokines and chemokines in response to inflammatory stimuli (31). We thus examined the effects of pro-inflammatory treatments on the pattern of proteins secreted by cultured striatal astrocytes. Treating cells for 18 h with either LPS (1 µg/ml) or two pro-inflammatory cytokines, IL1-β
(200 pg/ml) and TNF-α (10 ng/ml), respectively, increased the amount of four protein species (B2MG, ceruloplasmin, complement C3 α and β chains) in the medium conditioned by striatal astrocytes (Fig. 5A). In contrast, their intracellular level remained unchanged (data not shown). Surprisingly, the astrocytic secretion of pentraxin related protein-3 (PTX3), a protein known to be highly induced by IL1-β or TNF-α in several cell types (32-34), was not increased by the proinflammatory treatments performed in the present study (data not shown). Two additional proteins appeared in the supernatant of cells exposed to these treatments (Fig. 5A). The first one is chitinase-3 like protein-1 (C3L1), a glycoprotein of the chitinase protein family which is thought to contribute to the capacity of cells to respond to changes in their environment (35) and is increased in the serum of patients with inflammatory diseases such as osteoarthritis and rheumatoid arthritis (36). The second one is neutrophil gelatinase-associated lipocalin (NGAL), a protein which was originally identified to be associated with 92-kDa gelatinase/matrix metalloproteinase 9 (MMP9) and secreted in specific granules from activated neutrophils (37). Lipocalins are a family of mainly extracellular proteins involved in the binding, transport and presentation of small lipophilic molecules, including retinoids, steroids and fatty acids (38). NGAL was one of the major proteins identified in the medium conditioned by striatal astrocytes treated with LPS or IL1-β (the NGAL isoformes represent 10.4 ± 2.5 and 6.6 ± 1.7% of the total amount of extracellular proteins in cultures treated with LPS and IL-1β, respectively). We have verified that pro-inflammatory treatments did not significantly decrease cell viability, as assessed by the MTT assay (93 ± 5, 97 ± 2 and 97 ± 3 % of cell survival measured in cultures treated with LPS, IL1-β and TNF-α, respectively, n = 3). Moreover, these treatments did not alter membrane integrity of astrocytes, a process that could be responsible for the increased expression of some proteins in culture supernatant, as indicated by the lack of PI incorporation in cell nuclei (less than 0.1% of PI positive cells in cultures exposed to sham or pro-inflammatory treatments).

Next, we have examined whether astrocytes isolated from other brain regions exhibited differences in reactivity to proinflammatory treatments. We focused on LPS, because this treatment was the most efficient in stimulating the secretion of the aforementioned proteins in
striatal astrocyte cultures, when compared with the other proinflammatory treatments used. Treating astrocytes isolated from the cerebral cortex, the hippocampus and the cerebellum with LPS increased the secretion of B2MG, ceruloplasmin, complement C3 (α and β chains) and NGAL in a same extent as that observed in striatal cultures (Fig. 5B). LPS also markedly stimulated the secretion of C3L1 in cortical cultures. However, a much lower expression of C3L1 was detected in supernatants of cerebellar astrocytes exposed to LPS (Fig. 5B). C3L1 was already detectable in the supernatant of untreated astrocytes from the hippocampus and its secretion was strongly stimulated by LPS (Fig. 5B).

Detection in the cerebrospinal fluid of proteins released by astrocytes

Several proteins identified in the medium conditioned by astrocytes, such as B2MG, complement C3 α and β chains, apolipoprotein E and cystatin C, are known as major protein constituents in the human CSF (39). This suggests that astrocytes may be an important source of proteins in the CSF. To our knowledge, no proteomic map of mouse CSF is available. To determine which proteins released by astrocytes are present in the CSF, a proteomic analysis of mouse CSF proteins was performed. The mouse CSF 2-D gel map was compared with the 2-D gel map of proteins released by astrocytes (Fig. 6). As observed in human CSF (40), the mouse CSF showed an enrichment in plasma proteins such as albumin, apolipoprotein A1, transferrin, α₁-antitrypsin, α₂-macroglobulin, hemopexin and transthyretin. In addition, more than two thirds of the proteins identified in the astrocyte-conditioned medium were also detectable in the mouse CSF (Fig. 6). They include proteins secreted through a BFA-sensitive pathway such as complement chains and ceruloplasmin, and proteins released through a BFA-insensitive mechanism, including PEBP, 14-3-3 proteins, antioxidant proteins (thioredoxin peroxidases 1 and 2, AOP2 and superoxide dismutase) and several cytosolic enzymes.
DISCUSSION

In the present study, using a proteomic approach based on MALDI-TOF mass spectrometry, we identified more than 30 proteins that accumulate in the conditioned medium of cultured astrocytes (see Fig. 7). The astrocytic secretion of several of these proteins, including complement C3, apolipoprotein E, ceruloplasmin, transcobalamin II and DBI has already been described (12-15). However, we also identified a set of proteins that have never been reported to be secreted by glial cells. Among these are a series of proteins that influence cell interactions with the extracellular matrix (SPARC and protease inhibitors), antioxidant proteins and several cytosolic proteins.

Our procedure based on silver staining allowed the detection of proteins secreted in relatively low amounts (a few ng) by astrocytic cultures. Silver staining also allows full use of the sensitivity of our MALDI-TOF mass spectrometer (femtomol range). However, despite its relatively high sensitivity, our approach only provides insight into a restrictive population of proteins, presumably the major proteins secreted by astrocytes. Indeed, we detected no growth factor nor cytokine, which are known to be produced by astrocytes, even in cultures exposed to pro-inflammatory treatments.

The comparative analysis between proteins released by astrocyte cultures and the CSF proteome indicated that more than two thirds of the proteins identified in the astrocyte-conditioned medium were also detected in the CSF. If the profile of protein secreted by astrocytes in vitro is reflective of proteins released by astrocytes in situ, our results suggest that astrocytes constitute one of the major source of CSF proteins. However, we must point out that the cultured astrocytes used in the present study were isolated from mouse embryos, whereas the CSF was from 2-month-old animals. Moreover, other cell types can contribute to the CSF protein content. A previous study has shown that cultured leptomeningeal cells secrete various CSF proteins (41). Many CSF proteins, such as 14-3-3 proteins, can also be released by neurons, in particular following neurotoxic processes. In this regard, the isoform pattern of 14-3-3 proteins in the human CSF has been considered as diagnostic marker of Creutzfeldt-Jakob disease (42).
The comparative analysis of proteins secreted by astrocytes isolated from various CNS regions showed a similar profile of protein secretion, even though a regional specificity was observed for the release of a few protein species. These disparities in secretion profiles may reflect regional differences \textit{in vivo}, although one cannot exclude that they result from the proliferation of a small subset of the isolated cells in our cultures.

Our results indicate that astrocytes can secrete proteins from the biosynthetic pathway by exocytosis of secretory vesicles. This conclusion is supported by 1) the inhibition of the secretion of several proteins by BFA, a specific inhibitor of exocytotic vesicle assembly, and 2) the accumulation of these proteins in the extracellular medium compared with their low amounts in whole cell extracts. We also provide evidence that an alternative pathway contributes to the astrocytic secretion of proteins. Indeed, a differential 2-D gel analysis of astrocyte-conditioned medium against whole-cell extract indicated an extracellular enrichment of several proteins lacking any classical signal sequence for transport into the endoplasmic reticulum (18 proteins showing an enrichment \( \geq 2.5 \) fold in cell-conditioned medium versus whole cell extract were identified). Moreover, the accumulation of these proteins in the extracellular medium was not inhibited by BFA. Several observations further support that this accumulation results from an active secretion process: 1) some of these proteins have already been identified to be secretion products of various cell types. In this regard, cyclophilin A was detected in the conditioned medium of immune cells stimulated by LPS as well as the synovial fluid of patients with rheumatoid arthritis (27,43). Similarly, the immunodetection of PEBP on the cell surface and the presence of recombinant His-tagged PEBP in the supernatant of transfected cells indicate that the localization of this protein is not restricted to the cytosol or the inner leaflet of the plasma membrane (26). 2) Most of these proteins have already been found in biological fluids and were identified as major protein components of the mouse CSF in the present study.

In addition to the classical exocytotic pathway, the endocytic pathway is an alternative mechanism that may account for the secretion of cytosolic proteins in the extracellular medium (44). This secretion process results from the fusion of late multivesicular endosomes
with the plasma membrane and the release of intraluminal vesicles in the extracellular medium. These vesicles, dubbed exosomes, are generated by inward budding from the limiting membrane into the lumen of endosomes. This creates a membrane-enclosed compartment in which the lumen is topologically equivalent to the cytosol (44). To date, the release of exosomes has been described for various cell types including reticulocytes, B- and T-lymphocytes, macrophages, dendritic cells and intestinal epithelial cells (44-46). These cells are mainly of hematopoietic origin and/or antigen-presenting cells. Although astrocytes cannot function as fully competent antigen-presenting cells, they were the first CNS cell type shown to express major histocompatibility complex antigens and some co-stimulatory molecules. Moreover, the overall protein composition of exosomes of dendritic and epithelial cells, as determined by proteomic analyses (45,46), showed many similarities with the protein pattern of the astrocyte-conditioned medium. These observations suggest that the accumulation of some cytosolic proteins in astrocyte-conditioned medium may result from their release through the exosomal secretory pathway. Another unconventional secretion process, which requires specific export and/or internalization sequences, has been involved in the release homeoproteins such as Engrailed 2, HIV-transactivator protein, and other "messenger proteins" (47). This secretion process is probably not involved in the astrocytic release of proteins lacking signal peptides. Indeed, none of them bear strong sequence similarities to either the leucine-rich nuclear export sequence of Engrailed 2 or the translocation sequence identified in Antennapedia and the HIV transactivator protein.

With regard to function, antioxidant proteins constitute the largest group of proteins identified in astrocyte-conditioned medium. These proteins may contribute to the well-documented neuroprotective effect of astrocytes against oxidative stress (48). GST class-mu, the unique GST identified in the astrocyte-conditioned medium, shows a high detoxifying activity toward 4-hydroxy-2-nonenal which is the major hydroxyalkenal formed during the peroxidation of polyunsaturated fatty acid and is highly cytotoxic to neuronal cells (49,50). Astrocytes also release 3 peroxiredoxins (peroxiredoxin 1 and 2, AOP2), which belong to a family of enzymes that reduce hydroperoxides and might play a major role in the clearance of low
concentrations of \( \text{H}_2\text{O}_2 \), such as those measured in the CNS (51). All peroxiredoxins except AOP2 share two conserved reactive cystein residues in the active site and use thioredoxin as a physiological electron donor (28). A recent study has demonstrated that AOP2, which contains only one of the conserved cystein residues, binds to cyclophilin A and that this protein supports its peroxidase activity as an immediate electron donor (51). This suggests that cyclophilin A released by astrocytes also contributes to extracellular peroxidase activity. Finally, ceruloplasmin, which is essential for brain iron metabolism, may also participate in the antioxidant function of astrocytes (52).

Proteases and protease inhibitors constitute another major group of proteins secreted by astrocytes that may modulate vulnerability to neurotoxicity. In this regard, both beneficial and detrimental effects on neuronal survival have been attributed to extracellular proteases, including matrix metalloproteinases (MMPs) (53-55). Our study suggests that astrocytes are an important source of MMP inhibitors rather than MMPs. We also provide evidence that astrocytes release substantial amounts of the multifunctional protein PEBP, which was recently identified as a major inhibitor of serine proteases including thrombin, neuropsin and trypsin (26).

Extracellular proteases also remodel pericellular microenvironment, primarily through the cleavage of extracellular matrix components. They participate in structural changes in neuronal architecture and in the pattern and number of neuronal connections associated with long-lasting forms of synaptic plasticity (56,57). SPARC is another major protein constitutively released by astrocytes that may contribute to synaptic plasticity (58). Finally, the astrocytic secretion of the octadecaneuropeptide DBI, a negative allosteric modulator of \( \text{GABA}_\lambda \) receptor channels, may participate in the regulation of GABAergic synaptic transmission (25,59).

In conclusion, the present study provides the first unbiased characterization of the major proteins secreted by astrocytes \textit{in vitro}. If astrocytes \textit{in situ} produce the same protein species, the secretion of proteins may be one critical mechanism by which astrocytes modulate neuronal survival and function.
REFERENCES

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**FOOTNOTES**

1 The abbreviations used are: AOP2, antioxidant protein 2; BFA, brefeldin A; B2MG, beta-2-microglobulin; C3L1, chitinase-3 like protein-1; CNS, central nervous system; CSF, cerebrospinal fluid; CYSC, cystatin C; DBI, diazepam binding inhibitor; GST1, glutathione-S-transferase class-Mu; IBP2, insulin-like growth factor binding protein 2; IL1-β, recombinant murine interleukin 1-β; IPG, immobilized pH-gradient; LPS, lipopolysaccharide; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization-Time-of-Flight; MMP, matrix metalloproteinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NGAL, neutrophil gelatinase-associated lipocalin protein; PEBP, phosphatidylethanolamine-binding protein; PI, propidium iodide; PTX3, pentraxin-related protein chain 1; SPARC, secreted protein acid and rich in cystein; TFA, trifluoroacetic acid; TIM1, and 2, metalloproteinase inhibitor 1 and 2; TNF-α, recombinant murine tumor necrosis factor-α.
FIGURE LEGENDS

Figure 1. **Two-dimensional analysis of proteins released by cultured striatal astrocytes in the absence or presence of brefeldin A**

Confluent striatal astrocytes were exposed for 18 h in serum-free culture medium in the absence (Control) or presence of 1 µg/ml brefeldin A (BFA). Astrocyte-conditioned media were harvested and proteins (30 µg protein per gel) were separated according to their isoelectric point on immobilized pH-gradients (IEF), and then by standard SDS-PAGE (10% gels). **A:** A representative silver-stained 2-D gel (untreated cells, Control) is illustrated. The position of protein spots that were not detectable in the medium conditioned by astrocytes exposed to BFA is indicated. **B:** Areas of interest of 2-D gels showing the inhibition of the secretion of the corresponding proteins by BFA are depicted. The abbreviations used are defined in Table 1.

Figure 2. **Two-dimensional analysis of cystatin C and β2-microglobulin secretion by striatal astrocytes**

Striatal astrocytes were incubated for 18 h in serum-free culture medium in the absence (Control) or presence of 1 µg/ml BFA. Astrocyte-conditioned media were harvested. Proteins (30 µg/gel) were resolved on 15% 2-D gels and were either silver stained (**A**) or transferred electrophoretically onto nitrocellulose membranes (**B** and **C**, left panels). Arrows and arrowheads indicate the position of spots that were identified as CYSC and B2MG by MALDI-TOF mass spectrometry, respectively. Extracellular and whole cell extract proteins (30 µg protein/lane) were also analyzed by 1-D immunoblotting (**B** and **C**, right panels). Immunoblotting was performed with a polyclonal anti-CYSC antibody and a polyclonal anti-B2MG antibody (1/5,000 dilution each).
Proteomic analysis of astrocytic secretion

Figure 3. **Differential analysis of 2-D protein patterns of astrocyte-conditioned media and total cell extracts: identification of proteins secreted through a brefeldin A-insensitive pathway**

Proteins of astrocyte-conditioned medium and whole cell extract (30 μg each) were resolved on 2-D gels and stained with silver. The quantification of proteins (spot volume relative to the volume of all spots) was performed with the Image Master software. Spots representing more than 0.5% of the volume of all spots were analyzed. A filtering of spots showing an enrichment ≥ 2.5 fold in the extracellular medium versus cell extract was performed. For each protein, the ratio between the content in the extracellular medium and in whole cell extract is indicated above the bars. The values obtained for two proteins (HSP90 and β-actin) showing no enrichment in astrocyte-conditioned medium versus cell extract are also illustrated. The abbreviations used are defined in Table 1. Data are the means ± SEM of values obtained from four gels.

Figure 4. **Differential pattern of proteins secreted by astrocytes originating from various brain regions**

Media conditioned by confluent astrocytes from the striatum, the cerebral cortex, the hippocampus and the cerebellum (18 h in serum-free culture medium) were harvested. Proteins (30 μg per gel) were resolved on 2-D gels and stained with silver. **A** Area of gels showing different amounts of protein spots are depicted. Arrowheads indicate the spots corresponding to the indicated protein. The abbreviations used are defined in Table 1. The data are representative of four gels performed on different sets of cultured astrocytes. **B** The quantification of protein spots was performed as indicated in the legend to Figure 3. ND: non detectable. * p<0.01 versus other brain regions.
Figure 5. Effects of pro-inflammatory treatments on the profile of proteins secreted by astrocytes

A Confluent striatal astrocytes were incubated for 18 h in serum-free culture medium in the absence or presence of either LPS (1 µg/ml), IL-1-β (200 pg/ml) or TNF-α (10 ng/ml). B Astrocytes isolated from either the cerebral cortex, the hippocampus or the cerebellum of mouse embryos were incubated in the absence or presence of LPS. Astrocyte-conditioned media were harvested. Proteins (30 µg per gel) were resolved on 2-D gels. Area of gels showing a different level of extracellular proteins in response to the indicated treatments are illustrated. The abbreviations used are defined in Table 1. The data are representative of four gels performed on different sets of cultured astrocytes.

Figure 6. Protein 2-D gel maps of astrocyte-conditioned medium and cerebrospinal spinal fluid

Proteins of the medium conditioned by cultured striatal astrocytes exposed to LPS (1 µg/ml) (A) and of the CSF from the mouse (B) (30 µg protein in each gel) were separated according to their isoelectric point on immobilized pH-gradients 3-10, and then by standard SDS-PAGE (12.5% gels). In A, arrows indicate the position of proteins secreted through a BFA-sensitive mechanism. Hatched arrows indicate the theoretical position of spots corresponding to TIM-1, which were only detectable in the medium conditioned by cerebellar astrocytes. Arrowheads show the position of proteins released by astrocytes through a BFA-insensitive mechanism. In B, arrows indicate the position of proteins identified in astrocyte-conditioned media and arrowheads that of additional major CSF proteins identified in the present study. The images illustrated are representative gels stained with silver. A2MG, alpha-2-microglobulin; CAIII, carbonic anhydrase III; CKM, creatine kinase M; ENOA, alpha enolase; FABP, fatty acid-binding protein; KNG, kininogen precursor; PVA, parvalbumin. The other abbreviations used are defined in Table 1.
Figure 7. **Schematic summary of astrocytic protein secretion**

The major proteins secreted by mouse astrocytes and identified by MALDI-TOF mass spectrometry are listed according to their secretion pathway. The abbreviations used are defined in Table 1.
Proteomic analysis of astrocytic secretion

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<th>Protein</th>
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Protein parameters of astrocytic section

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Proteomic analysis of astrocytic secretion
Proteomic analysis of astrocytic secretion

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Proteomic analysis of astrocytic section

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Proteomic analysis of astrocytic secretion

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<td>HSP90 and β-actin (Additional)</td>
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Table 1: Proteins identified in the conditioned medium of cultured astrocytes from various brain regions. When several spots are identified as a single protein, the sequence coverage resulting from the analysis of the major spot is indicated. When several spots are identified as a single protein, the scores of two additional proteins (HSP90 and β-actin) which are illustrated in medium conditioned by astrocytes versus whole cell extract. The scores of two additional proteins (HSP90 and β-actin) which are more than 2-fold enriched in the conditioned medium compared to the whole cell extract are also indicated. The proteins listed are 1) proteins whose release by astrocytes is inhibited by BFA and 2) proteins which are more than 2.5-fold enriched in the conditioned medium compared to the whole cell extract.
Figure 1

A

Control

IEF

SDS-PAGE

Mw (kDa)

3

200

116

97.4

66.3

55.4

36.5

31

21.5

B

Control

BFA

CERU (1)

CO3A (2)

CO3B (3)

CBPH (4)

SPARC (5)

Transcobalamin II (6)

PTX3 (7)

IBP2 (8)

APOE (9)

TIM-2 (10)
Figure 3
Figure 5
Figure 6
Figure 7
Proteomic analysis of astrocytic secretion in the mouse. Comparison with the cerebrospinal fluid proteome
Mireille Lafon-Cazal, Oumeya Adjali, Nathalie Galéotti, Joël Poncet, Patrick Jouin, Vincent Homburger, Joël Bockaert and Philippe Marin

J. Biol. Chem. published online April 22, 2003

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