

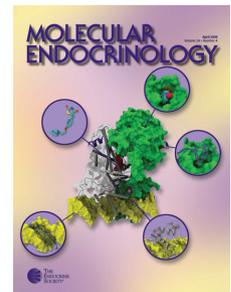
Endocrinology

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Life and Health Sciences Research Institute (ICVS) (F.M., A.M.F., J.C.S., N.S., M.C.-N., J.A.P.), School of Health Sciences, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal; and Program in Neurogenetics (G.C., D.G.), Department of Neurology, David Geffen School of Medicine-University of California Los Angeles, Los Angeles, California 90095

Iron is essential for normal cellular homeostasis but in excess promotes free radical formation and is detrimental. Therefore, iron metabolism is tightly regulated. Here, we show that mechanisms regulating systemic iron metabolism may also control iron release into the brain at the blood-choroid plexus-cerebrospinal fluid (CSF) barrier. Intraperitoneal administration of lipopolysaccharide (LPS) in mice triggers a transient transcription of the gene encoding for hepcidin, a key regulator of iron homeostasis, in the choroid plexus, which correlated with increased detection of pro-hepcidin in the CSF. Similarly, the expression of several other iron-related genes is influenced in the choroid plexus by the inflammatory stimulus. Using primary cultures of rat choroid plexus epithelial cells, we show that this response is triggered not only directly by LPS but also by molecules whose expression increases in the blood in response to inflammation, such as IL-6. Intracellular conveyors of these signaling molecules include signal transducer and activator of transcription 3, which becomes phosphorylated, and SMAD family member 4, whose mRNA levels increase soon after LPS administration. This novel role for the choroid plexus-CSF barrier in regulating iron metabolism may be particularly relevant to restrict iron availability for microorganism growth, and in neurodegenerative diseases in which an inflammatory underlying component has been reported. (*Endocrinology* 150: 2822–2828, 2009)

Iron is a required element for normal cellular homeostasis, participating in processes that range from cell proliferation to oxygen transport and energy metabolism (1, 2). Although iron deficiency leads to conditions such as anemia, iron in excess, by promoting free radical formation and oxidative stress, is deleterious for the organism. Thus, iron homeostasis has to be tightly regulated. One of its major regulators is hepcidin, a liver-derived peptide hormone that modulates iron release from the intestine into the plasma (3–6), the rate-limiting process in iron homeostasis (7). In addition, hepcidin also regulates iron efflux from hepatocytes and macrophages into the bloodstream. Iron is taken up from the diet by enterocytes and is released into the bloodstream through the iron exporter ferroportin. In conditions of iron sufficiency, hepcidin mediates ferroportin internalization and deg-

radation by the enterocyte (8). As a consequence, iron is kept within the enterocyte, which results in lower iron release into the circulation. It has been shown that increased hepcidin levels are associated with anemia (7, 9), whereas decreased levels are associated with iron-overload diseases (7). Once in the blood, iron is mostly bound to plasma proteins, particularly to transferrin, and is delivered to cells mainly through transferrin receptor 1-mediated endocytosis (10, 11). Within cells, iron can be stored bound to ferritin (12).

Although the mechanisms regulating iron homeostasis are relatively well described in the periphery, less information is available on brain iron metabolism. It has been proposed that iron uptake by the brain depends on transferrin-mediated endocytosis by endothelial cells of the blood-brain barrier (10, 13).

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Abbreviations: BMP, Bone morphogenetic protein; Cp, ceruloplasmin; CSF, cerebrospinal fluid; *Fth1*, ferritin heavy chain 1; *Ftl1*, ferritin light chain 1; *Hamp*, hepcidin; *Hfe*, hemochromatosis; *Hfe2*, hemochromatosis type 2 (juvenile); *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Il6*, IL-6; LPS, lipopolysaccharide; qRT-PCR, quantitative real-time PCR; *Slc40a1*, ferroportin; SMAD4, SMAD family member 4; STAT3, signal transducer and activator of transcription 3; *Tfr1*, transferrin receptor type 1; *Tfr2*, transferrin receptor type 2; TIBC, total iron binding capacity; *Tlr4*, toll-like receptor 4; UIBC, unsaturated iron-binding capacity.

Iron would then be likely released through ferroportin (14) into the interstitial fluid of the brain parenchyma to be taken up by neurons and glia cells. Iron transfer into the brain also occurs at the choroid plexus (15), and into the cerebrospinal fluid (CSF), because transferrin (16), transferrin receptor type 1 (17), and ferroportin (18) are present in the choroid plexus epithelial cells.

Iron homeostasis is challenged in several conditions. Among these are infectious diseases. Because microorganisms need iron for survival and proliferation, the host promptly responds by decreasing iron availability. This is accomplished by changes in the levels of several iron-related proteins, such as ferritin and ceruloplasmin (19). However, most microorganisms secrete siderophores, molecules with higher affinity for iron than the major plasma iron-carrier proteins (20). In response, the host secretes lipocalin 2 (21, 22), a protein that has the ability to sequester iron-loaded siderophores (23). Of relevance, it has been recently shown that lipocalin 2 is quickly synthesized and secreted by the choroid plexus into the CSF upon an inflammatory stimulus induced in the periphery (24). This observation suggested that limiting iron availability to microorganisms is part of the strategy set by the choroid plexus to fight potential infections of the central nervous system. Taken together, these observations prompted us to further investigate iron metabolism in the choroid plexus in response to inflammation. The data presented here highlight a novel function for the choroid plexus in iron homeostasis. We suggest that the choroid plexus has, for the brain, a role similar to that played by both the liver and the intestine in iron homeostasis in the periphery.

Materials and Methods

Animals and lipopolysaccharide (LPS) injection

In vivo experiments were conducted using 8- to 9-wk-old C57BL/6 mice and 8- to 9-wk-old Wistar rats (Charles River, Barcelona, Spain), in accordance with the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. Animals were maintained under 12-h light, 12-h dark cycles at 22–24 C and 55% humidity, and fed with regular rodent's chow and tap water *ad libitum*. To reduce the stress-induced changes in the hypothalamus-pituitary axis associated with the injection, animals were handled for 1 wk before the beginning of the experiments. Animals were given LPS (*Escherichia coli*, serotype O26:B6; Sigma-Aldrich Corp., St. Louis, MO) (5 μ g/g body weight) ip; control animals were injected with vehicle (0.9% NaCl). For primary culture studies, rat choroid plexus epithelial cells were obtained from neonate (postnatal d 3 or 4) Wistar rats born from control females.

One, 3, 6, 12, 24, or 72 h after LPS injection, mice were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg), transcardially perfused with cold saline and killed. For the mRNA studies, choroid plexuses were rapidly removed under conventional light microscopy (SZX7; Olympus, Hamburg, Germany), frozen in dry ice, and stored at -80 C. At least five pools (from three animals each) of choroid plexus were prepared for each time point.

Rats were similarly anesthetized 3, 6, 12, and 24 h after LPS injection. CSF was collected from the cisterna magna, and choroid plexus removed and frozen as described previously. An aliquot of each CSF sample was used to verify the absence of blood contamination and the remainder immediately frozen until use. Blood was collected, centrifuged, and serum was kept at -80 C until use. The serum was used for two purposes: stimulation of primary cultures of rat choroid plexus epithelial cells (serum obtained 3 and 6 h after LPS injection); and determination of he-

matological iron parameters (serum obtained 6, 12, and 24 h after LPS treatment).

Primary cultures of rat choroid plexus epithelial cells

Epithelial cells from rat choroid plexus were prepared as described previously by Strazielle and Ghersi-Egea (25), with minor modifications. Briefly, choroid plexuses from neonates were dissected under conventional light microscopy (SZX7). The tissue was rinsed twice in PBS (without Ca^{2+} and Mg^{2+}), followed by a 25-min digestion with 0.1 mg/ml pronase (Sigma-Aldrich) at 37 C. Predigested tissue was recovered by sedimentation and briefly shaken in 0.025% trypsin (Invitrogen Corp., Carlsbad, CA) containing 12.5 μ g/ml deoxyribonuclease I (Roche Diagnostics, Basel, Switzerland). The supernatant was then withdrawn and kept on ice with 10% fetal bovine serum (Invitrogen). This step was repeated five times. Cells were pelleted by centrifugation and resuspended in culture media consisting of Ham's F-12 and DMEM (1:1) (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine (Invitrogen), 50 μ g/ml gentamycin (Sigma-Aldrich), 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml insulin-transferrin sodium selenite (ITS, Sigma-Aldrich), 10 ng/ml epidermal growth factor (Sigma-Aldrich), 2 μ g/ml hydrocortisone (Sigma-Aldrich), and 5 ng/ml basic fibroblast growth factor (Invitrogen). For further epithelial cells enrichment, cells were incubated on plastic dishes for 2 h at 37 C. Because epithelial cells do not attach to the plastic dish, the supernatant containing mostly these cells was collected and placed for seeding on laminin (Boehringer-Ingelheim GmbH, Ingelheim, Germany) coated transwells (Corning, Lowell, MA). Experiments were performed after the formation of confluent cell monolayers, approximately after 7 d in culture. Once established, the upper chamber represents the fluid in contact with the apical side of epithelial cells (the CSF side), whereas the lower chamber represents the basolateral side (the blood side). Choroid plexus epithelial cells were stimulated in the basolateral side, for 3 or 6 h, with IL-6 (2.8 ng/ml), LPS (1 μ g/ml), or serum obtained from rats previously injected with LPS or with saline.

Gene expression measurements

Total RNA was isolated from choroid plexus using TRIzol reagent (Invitrogen). Five hundred nanograms of total RNA were amplified using the Superscript RNA amplification system (Invitrogen) according to the manufacturer's instructions. For the primary cultures of choroid plexus epithelial cells, total RNA was collected and extracted using a Micro Scale RNA Isolation Kit (Ambion, Inc., Austin, TX). RNA was reverse transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR (qRT-PCR) analysis was used to measure the mRNA transcripts of the following genes (gene abbreviations and gene names are specified in accordance with the human genome organization (HUGO) Gene Nomenclature Committee available at <http://www.genenames.org>): ceruloplasmin (*Cp*), ferritin heavy chain 1 (*Fth1*), ferritin light chain 1 (*Ftl1*), ferroportin (*Slc40a1*), hepcidin (*Hamp*), hemochromatosis (*Hfe*), hemochromatosis type 2 (juvenile) (*Hfe2*, alias for *Hju*), IL-6 (*Il6*), SMAD family member 4 (*Smad4*), signal transducer and activator of transcription 3 (*Stat3*), toll-like receptor 4 (*Tlr4*), transferrin receptor type 1 (*Tfrc*, alias for *Tfr1*), and transferrin receptor type 2 (*Tfr2*). The reference gene, hypoxanthine guanine phosphoribosyl transferase (*Hprt*), was used as an internal standard for normalization because we have previously shown that its expression is not influenced by the experimental conditions (24). The oligonucleotide primers for the transcripts analyzed were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA; http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Ref. 43) on the basis of the GenBank sequences: NM_001042611, NM_010239, NM_010240, NM_016917, NM_032541, NM_031168.1, NM_010424, NM_027126, NM_008540, NM_011486, NM_021297, NM_011638, NM_015799, and NM_013556. The qRT-PCRs, using equal amounts of total RNA from each sample, were performed on a LightCycler instrument (Roche Diagnostics) using the QuantiTect SYBR Green RT-PCR reagent kit (QIAGEN,

Hamburg, Germany). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primers used.

Analysis of (Tyr705)-STAT3 protein expression

For protein extraction, mouse choroid plexuses were homogenized in suspension buffer [NaCl 0.1 M; Tris (pH 8), 0.01 M; EDTA (pH 8), 0.001 M; and one tablet of complete protease inhibitor cocktail for 50 ml buffer; Roche Diagnostics], followed by sonication for 30 sec in 2× Laemmli buffer [sodium dodecyl sulfate 4%; Tris (pH 8), 0.12 M; glycerol 20%; and dithiothreitol 0.2 M]. Proteins were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were separated by SDS-PAGE (50 μg/lane) and transferred into a nitrocellulose membrane. Membranes were then stained with Ponceau S (Sigma-Aldrich) to confirm transfer efficiency; blocked with 5% skim milk in PBS, and probed with antiphospho (Tyr705)-STAT3 antibody (diluted to 1:1000; Cell Signaling Technology, Inc., Danvers, MA) at 4 C overnight. Membranes were washed and incubated with goat antirabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted to 1:10,000. Each blot was developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to x-ray film. Finally, the membranes were stripped with a 2% sodium dodecyl sulfate, 100 mM β-mercaptoethanol solution warmed to 50 C for 30 min, thoroughly washed, blocked and reblotted with mouse anti-α-tubulin (Santa Cruz Biotechnology), and diluted to 1:5000 to normalize for protein load.

Hepcidin prohormone measurements

The quantification of the hepcidin prohormone in the CSF was done using the DRG hepcidin prohormone ELISA kit (DRG Instruments GmbH, Marburg, Germany). This kit is optimized for measuring pro-hepcidin in serum and urine, for which there is a reported intraassay variation less than 5% and an interassay variation less than 10%. Because no reports were available on the CSF levels, we first checked in a set of CSF samples collected from rats 3, 6, and 12 h after LPS, and concluded that pro-hepcidin could be measured using a total volume of 50 μl. This volume was used in the present study, and the assay procedures were performed following the manufacturer's instructions.

Serum iron concentration and unsaturated iron-binding capacity (UIBC)

Serum rat iron concentration and serum UIBC were determined using a commercial kit (BIOLABO, Maizy, France). UIBC was used to calculate total iron binding capacity (TIBC) as follows: TIBC = serum iron + UIBC. Because most of circulating iron is bound to transferrin, it is well accepted to consider the transferrin saturation as the ratio between serum iron concentration and TIBC.

Statistical analysis

Values are reported as mean ± SE. Statistical significance was determined using the nonparametric Mann-Whitney *U* test, with differences considered significant at *P* < 0.05.

Results

Peripheral administration of LPS alters the expression of genes involved in iron metabolism in the mouse choroid plexus

Hamp expression, which was rarely detectable, by qRT-PCR in basal conditions, became consistently detectable after LPS injection, from 1–12 h, reaching the maximum expression at 3 h (Fig. 1A), and returning almost to basal levels at 24 h. Similar induction was observed in rat choroid plexus (data not shown).

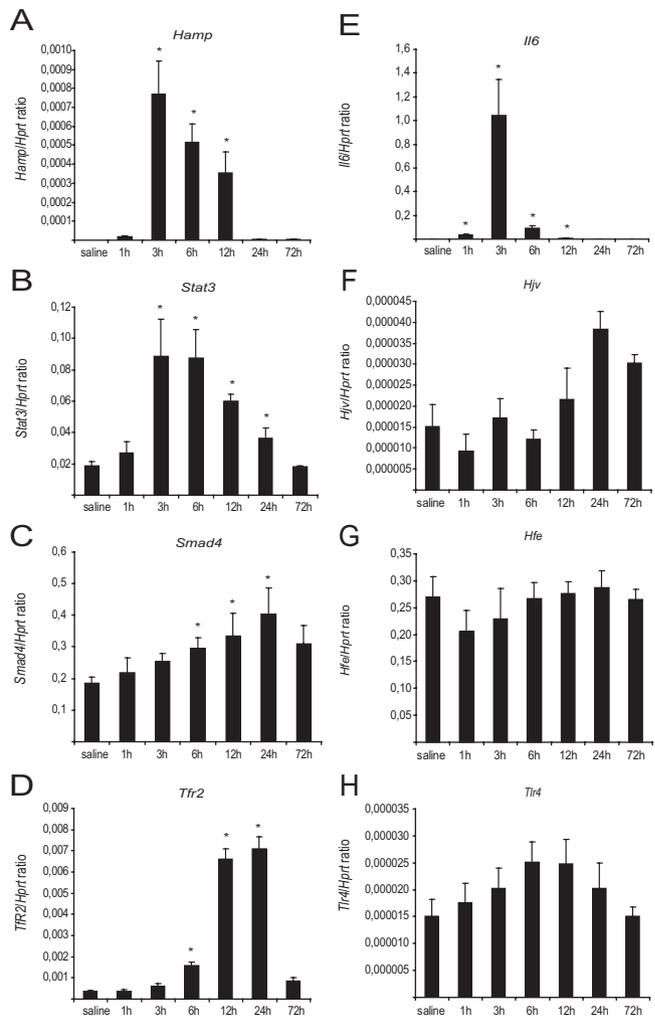


FIG. 1. The expression levels of mouse genes encoding for hepcidin and for potential modulators of *Hamp* expression are increased in response to LPS. A, *Hamp* expression was strongly induced by peripheral LPS administration, reaching a maximum level at 3 h and returning to basal levels after 24 h. The genes encoding for the upstream regulators of *Hamp* expression *Stat3* (B), *Smad4* (C), *Tfr2* (D), and *Il6* (E) were also induced in the choroid plexus after LPS administration, whereas those encoding for *Hjv* (F), *Hfe* (G), and *Tlr4* (H) were not altered. Results are expressed as the ratio between the gene of interest and that of the reference gene *Hprt*. Data are represented as mean ± SE; n = 5 pools (from three animals each) of the choroid plexus for each time point. *, *P* < 0.05 vs. saline.

In accordance with the up-regulation of the *Hamp* gene in the choroid plexus, the ability to detect hepcidin prohormone in the CSF increased upon LPS injection. Although in basal conditions we could only detect pro-hepcidin in one of five rat CSF samples, this number increased to two of six at 3 h, three of six at 6 h, and returned to one of five at 12 h.

We next looked for the expression of genes encoding for transcription factors described in the liver to modulate *Hamp* expression in response to inflammation, namely that of *Stat3* and *Smad4*. The expression of *Stat3* (Fig. 1B) increased as soon as 3 h after LPS administration, and that of *Smad4* (Fig. 1C) at 6 h, returning to basal levels at 72 h. The increased expression of *Stat3* corresponded to the expected activation of STAT3 because it became phosphorylated from 1 h until 6 h after LPS administration (Fig. 2).



FIG. 2. Peripheral LPS injection induces a rapid and transient phosphorylation of STAT3. Proteins extracted from mouse choroid plexus collected 1–24 h after LPS administration were separated by SDS-PAGE (50 μ g/lane) and probed with antiphospho (Tyr705)-STAT3. α -Tubulin was used to normalize for protein load.

SMAD4 and STAT3 participate in several signaling transduction pathways. In the context of iron metabolism, and particularly to what has been described regarding *Hamp* expression in the periphery, three pathways are of particular relevance: those mediated by LPS, IL-6, and the bone morphogenetic protein (BMP)/SMAD pathway. Although activation of these pathways does not necessarily require increased expression of receptor proteins, we next investigated some of their components. The expression of *Tfr2* (Fig. 1D) increased from 6–24 h after LPS administration, whereas no statistically significant differences were observed for *Hju* (Fig. 1F) and *Hfe* (Fig. 1G).

A key modulator of the liver acute-phase response is IL-6, whose levels increase in the blood in response to inflammation. Similarly, we observed a rapid and transient up-regulation of *Il6* expression in the choroid plexus, which peaked at 3 h after LPS injection and returned to basal levels at 12 h (Fig. 1E). Because the response to LPS is mediated by the TLR4, which is present in the choroid plexus in basal conditions, we measured the expression of *Tlr4* and found that it was not altered by the inflammatory stimulus (Fig. 1H).

Other iron-related genes are influenced by inflammation in the liver (19). As an attempt to characterize better the overall alterations in the choroid plexus iron metabolism, we next investigated the expression levels of genes encoding for key iron-related proteins. Although no statistically significant differences were observed for the expression of *Tfr1* (Fig. 3A), *Slc40a1* (Fig. 3B), and *Ftl1* (Fig. 3C), the latter seems to present a trend toward increase and *Tfr1* toward decrease. The expression of *Fth1* (Fig. 3D) and *Cp* (Fig. 3E) were significantly up-regulated by peripheral inflammation.

Triggering effectors of the choroid plexus response

The choroid plexus, located within the cerebral ventricles, is composed of a vascularized stroma surrounded by a tight layer of epithelial cells (26). Therefore, two aspects of the choroid plexus response to peripheral inflammation should be considered: 1) in what extent the response is mediated specifically by the choroid plexus epithelial cells, which is particularly relevant because these cells contribute to the composition of the CSF; and 2) because LPS administration in the periphery induces an acute-phase response that results in an increase in the blood concentration of several immune modulators and proteins (e.g. such as IL-6), it is of relevance to identify which are the triggering effectors of the choroid plexus response. To address these questions, we used primary cultures of rat choroid plexus epithelial cells. We chose the rat for two reasons: 1) the amount of tissue is considerably greater when compared with the mouse, and 2) as a second animal model for determining the choroid plexus response.

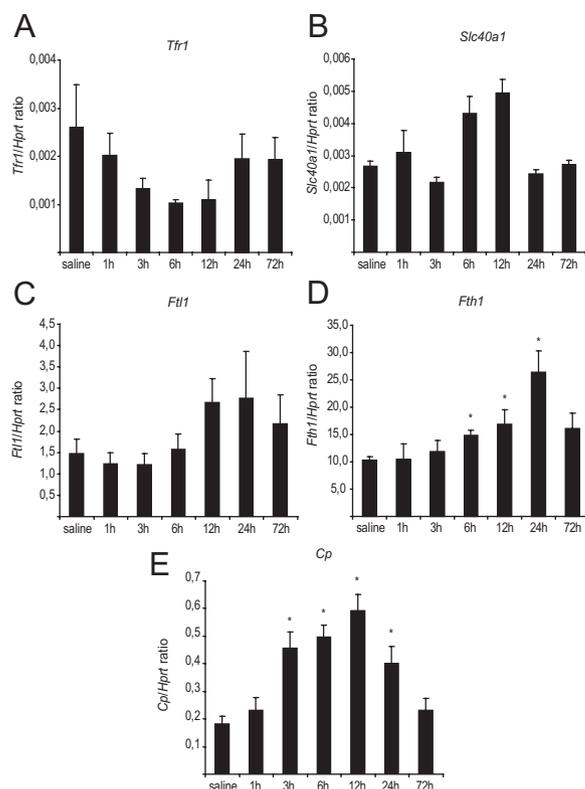


FIG. 3. Expression levels of the genes encoding for iron-related proteins in mouse choroid plexus after LPS administration. No statistically significant alterations were observed in the expression levels of *Tfr1* (A), *Slc40a1* (B), and *Ftl1* (C). *Fth1* and *Cp* mRNA levels were increased in response to LPS (D and E, respectively). Results are expressed as the ratio between the gene of interest and that of the reference gene *Hprt*. Data are represented as mean \pm SE; $n = 5$ pools of the choroid plexus (from three animals each) for each time point. *, $P < 0.05$ vs. saline.

When rat cells were stimulated in the basolateral side with serum obtained from rats previously injected with LPS (serum collected 3 and 6 h after LPS injection), an induction of the *Hamp* gene was observed (Fig. 4A). To investigate further which molecules within the serum were responsible for the observed effects, cells were incubated with either LPS or IL-6 alone. As shown in Fig. 4B, both LPS and IL-6 were able to contribute directly to the altered *Hamp* expression pattern. The concentration of IL-6 was chosen considering the levels of IL-6 present in the mouse serum 1 h after the administration of LPS (data not shown). The concentration of LPS used was based on its effects in inducing *Hamp* expression in human monocyte cell cultures (27). Similarly, serum (Fig. 4C) and LPS or IL-6 alone (Fig. 4D) were able to induce the expression of the gene encoding for ceruloplasmin.

These observations show that epithelial cells of the choroid plexus respond to the inflammatory stimulus, and that at least LPS and IL-6 are triggering modulators of such response.

Serum iron concentration and UIBC

To characterize whether the LPS stimulus was inducing the appropriate response in iron metabolism in the periphery, we measured serum iron levels and serum transferrin saturation. As expected (28), LPS administration induced a decrease in total serum iron levels, TIBC and transferrin saturation (data not shown). A similar attempt was made with respect to the CSF, but up to a volume of 100 μ l pools, the concentration was below detection.

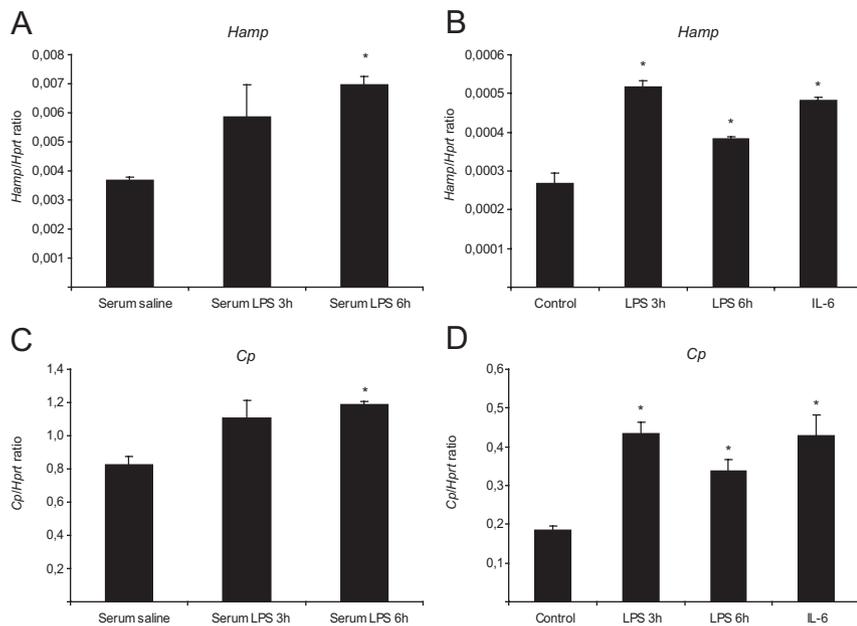


FIG. 4. Expression levels of *Hamp* in rat primary choroid plexus epithelial cells after stimulation with LPS, IL-6 or serum obtained from rats previously injected with LPS. Six hours of stimulation in the basolateral side of choroid plexus epithelial cells with LPS or IL-6 alone (B), or with serum collected from rats 3 and 6 h after *in vivo* administration of LPS (A), increased the expression of *Hamp*. Similarly, serum (C) and LPS or IL-6 alone (D) were able to induce the expression of the gene encoding for ceruloplasmin (*Cp*). Results are expressed as the ratio between the gene of interest and that of the reference gene *Hprt*. Data are represented as mean \pm SE. The result presented is representative of five independent experiments. *, $P < 0.05$ vs. serum saline or control.

Discussion

This study shows that the expression of iron-related genes in the choroid plexus is influenced by inflammatory stimulus induced in the periphery. This response seems to be quick and transient, and highlights the choroid plexus as a site of regional iron homeostasis. It includes not only hepcidin, the key hormone in iron metabolism, but also iron-related proteins and signaling-transduction molecules. Altogether, and because the choroid plexus gene expression profile will ultimately influence the composition of the CSF, this response may be transmitted to neuronal and glia cells within the brain parenchyma.

We first observed that the gene encoding for hepcidin, rarely detected in basal conditions, was strongly up-regulated in the choroid plexus promptly after LPS administration. This correlated with the increased secretion of pro-hepcidin into the CSF and may indicate increased levels of hepcidin in the CSF (even though to date, no clear relation between pro-hepcidin and hepcidin has been established) (29). Because the choroid plexus is composed not only of epithelial cells but also of a stroma containing endothelial cells of blood vessels and eventually immune cells, we next asked if epithelial cells of the choroid plexus were responsible for up-regulating the expression of genes encoding for hepcidin and other iron-related genes. Stimulation of the basolateral membrane of rat epithelial choroid plexus primary cells with LPS (to mimic the environment to which the choroid plexus is exposed *in vivo*) triggered the up-regulation of *Hamp* and of *Cp* expression. The direct effect of LPS is likely mediated by interaction with the cognate receptor TLR4, present in the basolateral mem-

brane of choroid plexus epithelial cells (30) and whose expression we found unaltered by LPS.

LPS is known to induce an acute-phase response in the liver and in other organs, and these react by secreting proteins into the bloodstream (19), such as ILs that can also activate the choroid plexus response. Among these is IL-6, previously shown to regulate *Hamp* expression in the liver, upon interaction with the cognate cellular receptor, and through the STAT3 signaling transduction pathway (31–35). The same mechanism seems to be operational in the choroid plexus. In accordance, choroid plexus epithelial cells respond to both LPS and IL-6 applied to the basolateral side of the cells by inducing the expression of *Hamp* and *Cp*. Supporting the IL-6-mediated signaling transduction pathway, increased phosphorylation of STAT3 was observed in the choroid plexus collected from mice upon administration of LPS. One interesting aspect to refer is the observation that when LPS or IL-6 were applied to the culture media in contact with the apical membrane of the choroid plexus epithelial cells, no effect was observed in the expres-

sion of *Hamp*. Therefore, and at least with respect to LPS and IL-6, the cognate receptors seem to be located in the basolateral (facing the blood) membrane.

Other pathways involved in controlling the expression of iron-related genes have been the focus of intense research (36). Patients with mutations in *Hfe*, *Tfr2*, or *Hju* show iron deposition that is similar and consistent with elevated iron absorption, suggesting that these molecules may contribute to the regulation of *Hamp* expression. In the present study, we did not find alterations in the expression levels of *Hfe* or *Hju*. However, there was a strong increase in the expression of *Tfr2* after LPS stimulation. Interestingly, all these molecules belong to the BMP signaling complex that is the most powerful mechanism known to activate hepcidin transcription through activation of the transcription factor SMAD4 (35). Of notice, we show here that the expression of *Smad4* is likewise induced by LPS. Further support for the involvement of TFR2-mediated effect on *Hamp* regulation comes from the observation that *Tfr2*-mutant mice have down-regulated expression of *Hamp* (37). In addition, the TFR2 and the IL-6-mediated pathways seem to cross talk since Wang *et al.* (38) reported that the liver-specific disruption of *Smad4* results in a markedly decreased *Hamp* expression when stimulated with IL-6. Similarly, a recent study using human hepatocyte cell cultures showed that mutations in the BMP-response element strongly impair *Hamp* activation in response to IL-6 (35). Therefore, the effect of IL-6 in the choroid plexus may, as well, be mediated by both SMAD4 and STAT3 signaling.

The expression of *Il6* itself was also strongly up-regulated soon after the LPS stimulus, which fits with the increased con-

centration of IL-6 in the CSF. Because molecules within the CSF may ultimately equilibrate with the interstitial fluid of the brain parenchyma, this may be important in the regulation of *Hamp* expression observed in brain regions such as the cortex in response to inflammation (28).

Another relevant issue regards the direct action of hepcidin in mediating ferroportin internalization and degradation, a well-characterized mechanism to reduce iron release into the bloodstream (5, 8). Of notice, the relation between ferroportin internalization and degradation and the levels of ferroportin gene (*Slc40a1*) expression seems to be tissue specific: whereas synthetic hepcidin has reduced *Slc40a1* expression in spleen macrophages, it produced no effect in duodenum (39), which seems also to be the case in the choroid plexus because we observed no altered *Slc40a1* expression.

Regardless of the precise mechanisms involved, the overall response of the choroid plexus to peripheral inflammation includes changes in the expression of genes implicated in iron metabolism, thus suggesting a role for the choroid plexus in the regulation of brain iron homeostasis. We propose that, upon exposure to peripheral inflammatory stimuli, these modifications lead to a reduction in iron availability in the CSF (Fig. 5). The increase in hepcidin levels induced by the inflammatory

stimulus may increase ferroportin internalization, which, as a consequence, can decrease ferroportin-mediated iron release into the CSF, due to the specific localization of ferroportin on the apical membrane of the choroid plexus epithelial cell (18). The up-regulation of FTH1 may promote storage of iron inside the epithelial cells of the choroid plexus. In addition, increased secretion of ceruloplasmin may facilitate iron binding to transferrin and internalization by cells of the brain parenchyma. Of notice and as previously shown (24), lipocalin 2 is also synthesized and secreted into the CSF in response to LPS. Collectively, the present and earlier results (24) show that the choroid plexus responds to inflammatory stimuli by restricting iron availability in the CSF.

The presented ability of the choroid plexus to regulate iron metabolism in conditions of peripheral inflammation might be of relevance for diseases such as Alzheimer's and multiple sclerosis. The observations that an inflammatory component may underlie such diseases (40), that iron accumulates with age (41), and that ceruloplasmin-deficient mice exhibit increased iron accumulation and demyelination when exposed to LPS (42), warrant that additional studies to clarify the regional role of the choroid plexus in iron metabolism are necessary and of great opportunity.

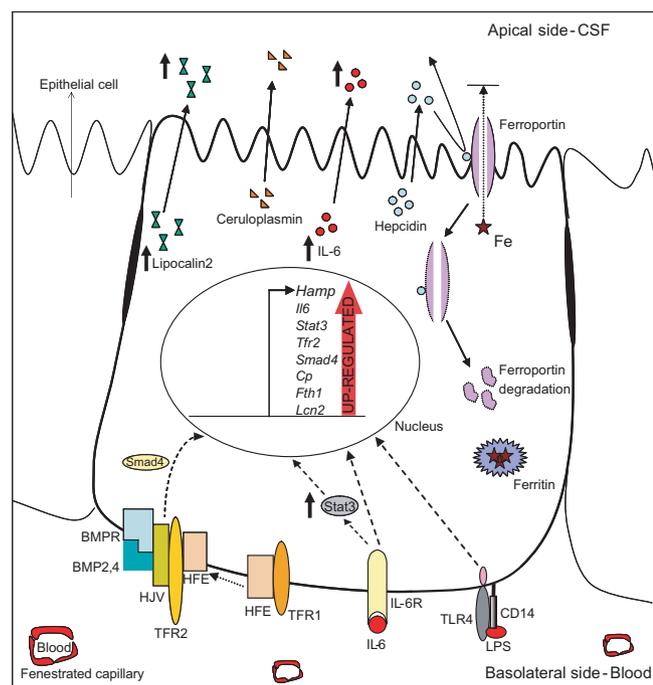


FIG. 5. Proposed model for iron metabolism in the choroid plexus after peripheral LPS injection. LPS and ILs whose concentration increases in the blood in response to LPS, such as IL-6, interact with their cognate receptors, in the basolateral membrane of the choroid plexus epithelial cell. Signaling to the nucleus through STAT3 and SMAD4, and possibly other transcription factors, induces the expression of *Hamp* and of other iron-related genes. Although activation of STAT3 is likely to occur through IL-6, that of SMAD4 is more likely to be mediated through the BMP/SMAD. Hepcidin may then be secreted into the CSF and bind, in the apical side, to ferroportin and induce its internalization and degradation, therefore, preventing iron release into the CSF. The increased expression of the gene encoding for ferritin suggests the possibility that in response to inflammation, the choroid plexus iron storage capacity is increased. Likewise, increased secretion of ceruloplasmin and lipocalin 2 (*Lcn2*) may contribute to a decrease iron content in the CSF. *Black wide arrows* indicate increased gene expression levels or protein concentration.

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Address all correspondence and requests for reprints to: Joana Almeida Palha, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal. E-mail: japalha@ecsau.de.uminho.pt.

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