Bacterial lipopolysaccharide induces type 2 deiodinase in cultured rat astrocytes

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Abstract

In the brain, 3,5,3'-triiodothyronine, which binds to the thyroid hormone receptor with high affinity, is locally generated from thyroxine by type 2 iodothyronine deiodinase (D2) expressed mainly in astrocytes and tanycytes. We have investigated the effects of bacterial lipopolysaccharide (LPS) on D2 in cultured rat astrocytes. LPS induced D2 activity with a lag-time of 4–8 h and a maximum at 24 h. LPS also promoted D2 mRNA accumulation. Glucocorticoids enhanced both the basal and LPS-stimulated D2 activity and mRNA accumulation. These glucocorticoid effects were blocked by the glucocorticoid receptor antagonist RU486. Our results obtained with different specific signaling pathway inhibitors indicated that D2 induction by LPS required ERK and p38-MAPK signaling pathways. NF-κB inhibitor

sulfasalazine blocked the effects of LPS on both D2 activity and mRNA accumulation. Hence, D2 induction by LPS appeared to implicate NF-κB pathway in astrocytes. NF-κB responsiveness of the rat *dio2* gene was studied in astrocytes with *dio2* 5'-flanking region promoter assays. The long form of the *dio2* promoter was transactivated by NF-κB. CCAAT/enhancer-binding protein β, which is upregulated by LPS in astrocytes, increased the transcriptional activity of the *dio2* promoter in its long or truncated forms containing CCAATs. Our observations, which demonstrate D2 induction by LPS in astrocytes and specify some characteristics of D2 induction mechanism, support the possible implication of brain D2 in adaptative responses to an infectious stress.

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Introduction

Type 2 iodothyronine deiodinase (D2) catalyzes the 5'-deiodination of thyroxine (T₄) into the active form of thyroid hormones, 3,5,3'-triiodothyronine (T₃; Bianco *et al.* 2002, Gereben *et al.* 2008). Notably, in the brain most T₃ is produced locally by D2 (Crantz *et al.* 1982), which is primarily expressed in astrocytes and tanycytes that line the third ventricle in the mediobasal hypothalamus (Guadano-Ferraz *et al.* 1997, Tu *et al.* 1997). Bacterial lipopolysaccharide (LPS) rapidly and markedly increases D2 activity and mRNA in the mediobasal hypothalamus (Fekete *et al.* 2004), independently of the LPS-induced fall in serum thyroid hormone levels (Fekete *et al.* 2005). This D2 induction by LPS in tanycytes and the resulting local increase in T₃ production could be implicated in the suppression of the hypothalamic–pituitary–thyroid axis during peripheral bacterial infection (Fekete *et al.* 2004, 2005). Because the hypothalamic–pituitary–adrenal axis is markedly upregulated during infection (Tilders *et al.* 1994), the role of corticosterone has been studied in D2 induction by LPS in tanycytes *in vivo* (Sanchez *et al.* 2008). Administering high doses of

corticosterone fails to mimic the LPS-induced increase in *dio2* gene expression in the mediobasal hypothalamus (Sanchez *et al.* 2008), indicating that mechanisms other than adrenal activation are involved. On the other hand, i.p. injection of LPS in rats increased D2 activity in the cerebral cortex (Fekete *et al.* 2004). D2 induction in astrocytes has been also observed after traumatic brain injury and brain focal ischemia (Zou *et al.* 1998, Margail *et al.* 2005). These brain injuries are associated with acute inflammation (Lucas *et al.* 2006). D2 in rat cultured astrocytes is regulated by multiple factors including selenium, thyroid hormone, cAMP, phorbol ester tetradecanoylphorbol-13-acetate (TPA), growth factors, glucocorticoids, TSH, hypoxia, and oxidant stress (Courtin *et al.* 2005, Lamirand *et al.* 2007, 2008). However, LPS effects on D2 have not yet been tested in primary cultured astrocytes although LPS increases D2 activity in a mesothelioma cell line and in H4 glioma cells (Zeold *et al.* 2006, Freitas *et al.* 2010).

NF-κB is a transcription factor which is classically involved in LPS signaling pathways (Chen & Greene 2004). An NF-κB inhibitor, i.e. sulfasalazine, partially inhibits LPS-induced D2 activity in human mesothelioma cells (Zeold *et al.* 2006). In HC11 and HEK-293 cells, p65 NF-κB markedly increased

expression of both rat and human *dio2* genes as analyzed by promoter assays (Fekete *et al.* 2004). Human *dio2* promoter was more sensitive to p65 NF- κ B than the rat *dio2* promoter (150- vs 3-fold increase) in HC11 cells. Recent findings have underlined the existence of an NF- κ B-binding site with transactivation potency of the p65 subunit of NF- κ B in the promoter of the human *dio2* gene in HC11 cells (Zeold *et al.* 2006). Other cytokine mediators, such as signal transducer and activator of transcription-3 (STAT3) or -5 (STAT5), did not induce transcription of the *dio2* gene (Zeold *et al.* 2006). Other transcription factors, such as CCAAT/enhancer-binding proteins (C/EBPs), have not been studied although C/EBPs are implicated in activation of LPS-inducible gene expression in different cells including astrocytes (Sweet & Hume 1996, Kelicen & Tindberg 2004).

We examined the effect of LPS on D2 activity and its mRNA accumulation in primary cultures of rat astrocytes in order to explore the regulation of D2 by LPS in astrocytes. Induction of D2 by LPS in astrocytes was studied in the presence or absence of glucocorticoids. We examined D2 induction by LPS in the presence of inhibitors of p38-MAPK and ERK pathways that are activated by LPS in astrocytes (Bhat *et al.* 1998). We also studied the effects of inhibitor of NF- κ B sulfasalazine. Finally, we tested the ability of the transcription factors NF- κ B and C/EBPs to act on the promoter of the rat *dio2* gene in astrocytes.

Materials and Methods

Materials and animals

T₄, T₃, dithiothreitol (DTT), N-acetyl cysteine (NAC), SB203580, cortisol, dexamethasone, RU486, LPS purified by phenol extraction from *Escherichia coli* strain 055:B5, sulfasalazine, L-NAME, and antibiotics were obtained from Sigma Aldrich Co. U0126 was purchased from Biomol (Plymouth Meeting, PA, USA). Sodium selenite was purchased from Merck. Exgen500 was obtained from Euromedex (Souffelweyersheim, France). [¹²⁵I]T₃ (3 mCi/ μ g) and [¹²⁵I]T₄ (1.5 mCi/ μ g) were purchased from Amersham International. Sprague–Dawley rats were purchased from Iffa–Credo (L'Albresle, France). FCS and culture media were obtained from Life Technologies. Culture dishes were obtained from Nunclon (Roskilde, Denmark).

Cell culture conditions

Brains were removed from 2-day-old Sprague–Dawley rats and cleaned of meninges and blood vessels. The two cerebral hemispheres were then dissociated to form a cell suspension by a passage through a nylon mesh (82 μ m pore size) into 40 ml DMEM supplemented with 6 g/l glucose, 2.4 g/l sodium bicarbonate, antibiotics (100 U/ml penicillin, 100 μ g/l streptomycin, and 0.25 μ g/l amphotericin B), and 10% FCS (DMEM/FCS). Aliquots (3 ml) of this cell

suspension were placed in 60 mm Petri dishes and the cultures were maintained at 37 °C under 5% CO₂, 95% air, and more than 90% humidity. The medium was changed every 2–3 days until cells reached confluency at ~10 days. At this stage, the DMEM/FCS was removed, and the cells were washed with a chemically defined medium that consisted of a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 4.5 g/l glucose, 1.8 g/l sodium bicarbonate, and the antibiotics listed above (DMEM/F12). The cells were then cultured for 3 additional days in DMEM/F12 supplemented with 30 nM sodium selenite, 10 μ g/ml insulin, and 10 μ g/ml transferrin followed by 1 additional day in DMEM/F12 supplemented with 30 nM sodium selenite, 10 μ g/ml transferrin, and eventually 1 μ M of cortisol. Under these conditions, more than 90% of the cells contained immunoreactive glial fibrillary acidic protein, a specific marker of astrocytes (Esfandiari *et al.* 1994, Pallud *et al.* 1999). Astrocytes were treated with the test agents for the times and the concentrations indicated in each experiment.

Plasmids

Reporter construct rdio2#1 (Gereben *et al.* 2001) corresponds to the chloramphenicol acetyl-transferase (CAT) reporter vector, pOCAT2, containing the rat *dio2* promoter (~3.8 kb *rdio2* 5'-FR and ~600 bp rD2 5'-UTR). The two truncated rdio2#3 and rdio2#4 constructs contain respectively 658 or 83 bp 5' to the *rdio2* Transcription Start Site (TSS) and the same portion of the 5'-UTR sequence (Gereben *et al.* 2001). These plasmids were gifts from P R Larsen. We also constructed, using SacI and BamHI restriction enzymes, a short reporter construct, rdio2#5 corresponding to 37 bp 5' upstream of the 5'-UTR sequence; p50 and p65 subunits of NF- κ B subcloned in the pcDNA3.1 expression vector and C/EBP α , C/EBP β , and C/EBP δ subcloned in the expression vector pREP4 were obtained from M Raymondjean (Antonio *et al.* 2002). Liver activating protein (LAP) or C/EBP β , liver inhibiting protein (LIP), and C/EBP-homologous protein (CHOP) were subcloned in the expression vector pREP4 and were gifts from M Pomerance (Pomerance *et al.* 2005).

Transient transfection, luciferase, and CAT assays

Aliquots (2 ml) of the astrocyte suspension were placed into six-well plate and the cultures were maintained at 37 °C under 5% CO₂, 95% air, and more than 90% humidity. The medium was changed the day after and on day 6. Transient transfection was performed on day 7 with Exgen500 (Euromedex) according to the manufacturer's instructions. Cells were cotransfected with 700 ng/well of rdio2#1, rdio2#3, rdio2#4, or rdio2#5, 200 ng/well of pGL2-SV40-luciferase expression vector, and 100 ng/well of the expression plasmids. These amounts were defined by previous dose–response studies for each plasmid. In experiments with pREP4-LIP, cells were also cotransfected with 500 ng/well

of empty pREP4 vector or pREP4-LIP. In experiments with pREP4-CHOP, cells were also cotransfected with 100 ng/well of empty pREP4 vector or pREP4-CHOP. One day after transfection, cells were incubated with DMEM containing 10% charcoal-treated FCS. Transfected cells were used 48 h after transfection. Luciferase activity was used to normalize the transfection efficiency in cultured dishes. The luciferase assay was assessed using a lumat luminometer (Berthold Technologies, Bad Wildbad, Germany) as previously described (Massaad *et al.* 2000a). The CAT activity was determined by using the two-phase assay (Massaad *et al.* 2000b). Each construct was transfected at least three times.

D2 mRNA quantification by real-time PCR

Total RNA was extracted using GenElute mammalian total RNA miniprep kit (Sigma Aldrich Co.) and was quantified by the absorption at 260 nm. Subsequently, cDNA was synthesized by reverse transcription using the M-MLV reverse transcriptase system (Invitrogen Co.). The cDNAs were amplified in a real-time PCR using the Taqman gene expression assay with the designing primers for D2 and 18S rRNA as an endogenous control (Applied Biosystems, Foster City, CA, USA). The generated D2 and 18S cDNA levels were measured using the ABI Prism 7000 sequence detection system and the relative standard curve method. Briefly, all genes were quantified from a standard curve representing a five-point serial dilution of mixed experimental and control cDNA, which were analyzed and used as calibrators of the quantification of the product generated in the exponential phase of the amplification curve. Both standards and samples were run in duplicate. Typically, the equivalent cDNA of 20 ng RNA was used for the real-time PCR of each sample. R^2 was >0.99 for all standard curves.

D2 and type 3 iodothyronine deiodinase assays

At the time of harvesting, the medium was aspirated, and the cells were rinsed with 3 ml ice-cold PBS on ice. Culture dishes containing the cells were then frozen at -80°C for later processing and analysis. This involved placing the plates on ice, scraping the content of each dish into 0.4 ml sample buffer (20 mM HEPES, 2 mM DTT, and 0.25 M sucrose, pH 7.4), and disrupting the cells by sonication. D2 activity was measured by incubating aliquots of the cell sonicate in an 80 μl final volume of 20 mM HEPES, pH 7.4, containing 20 mM DTT, 50 nM T_3 , and 1 nM $[^{125}\text{I}]\text{T}_4$ for 20–60 min at 37°C . Addition of 50 nM T_3 prevented 5-deiodination of both $[^{125}\text{I}]\text{T}_4$ and D2-generated $[^{125}\text{I}]\text{T}_3$ into $[^{125}\text{I}]\text{T}_{3,3',5'}$ -triiodothyronine (reverse T_3) and $[^{125}\text{I}]\text{T}_{3,3'}$ -diiodothyronine ($3,3'\text{-T}_2$) respectively, according to the previous *in vitro* studies on type 3 iodothyronine deiodinase (D3) activity in cultured astrocytes (Esfandiari *et al.* 1992). Reactions were stopped by adding 10 μl of 10 M NH_4OH containing 10 μM iodide, 10 μM T_3 , and 10 μM T_4 . The $[^{125}\text{I}]\text{T}_3$ produced was separated from $[^{125}\text{I}]\text{T}_4$ by descending paper chromatography

(Courtin *et al.* 1986). Then, the radioactive products were counted for determination of D2 activity, expressed as femtomoles of T_3/min per mg protein. Kinetic analysis was performed in sonicates of LPS-treated cells using 0.05–2 nM $[^{125}\text{I}]\text{T}_4$ as substrate and 20 mM DTT as cofactor. For D3 activity assays, homogenates were incubated at 37°C for 60 min in a final volume of 80 μl containing 20 mM HEPES buffer (pH 7.4), 20 mM DTT, and 5 nM $[^{125}\text{I}]\text{T}_3$. Reactions were stopped by adding 10 μl of 10 M NH_4OH containing 10 μM T_3 and 10 μM T_4 . The $[^{125}\text{I}]\text{T}_{3,3'}$ - T_2 produced was separated from $[^{125}\text{I}]\text{T}_3$ by descending paper chromatography (Courtin *et al.* 1986). Then, the radioactive products were counted for determination of D3 activity, expressed as femtomoles of $3,3'\text{-T}_2/\text{min}$ per mg protein. For D2 and D3 assays, deiodination was linear with respect to both protein concentration and incubation time and the quantity of protein assayed was adjusted to ensure that $<30\%$ of the substrate was consumed.

Protein determination

The protein content of cell sonicates was determined by the method of Bradford (Bradford 1976) using BSA as standard.

Statistical analysis

Statistical differences between the groups were determined using a commercially available program (GraphPad Prism 3.0; GraphPad, Inc., San Diego, CA, USA). Data were processed using one-way or two-way ANOVA, followed by Bonferroni *post hoc* test. Student's *t*-test was also used. Statistical significance was noted at $P < 0.05$.

Results

Effects of LPS on D2 activity and mRNA expression

Primary cultures of rat astrocytes, which were maintained for 2 days in a chemically defined medium containing cortisol as commonly used (Morrison & de Vellis 1981, Pallud *et al.* 1999), were treated with LPS. We found that the LPS stimulation of D2 activity was time- and dose-dependent (Fig. 1A and B). The effect of 1 $\mu\text{g}/\text{ml}$ LPS was visible after 4 h (significantly different using a *t*-test, $P < 0.001$) and reached a maximum at 24 h. This delayed action of LPS suggests an indirect effect. D2 activity was increased after a 10 h treatment with LPS from 0.1 $\mu\text{g}/\text{ml}$ (significantly different using a *t*-test, $P < 0.001$) and was maximal for 1 $\mu\text{g}/\text{ml}$, the concentration that we usually use. The LPS-induced D2 activity was not inhibited by 1 mM PTU and had a Michaelis constant (K_m) for T_4 around 1 nM (not shown). These enzymatic properties of D2 activity induced by LPS were characteristic of D2 (Bianco *et al.* 2002), as previously reported for other inducers in cultured astrocytes (Courtin *et al.* 1989, 2005). We also found that in the same

Figure 1 LPS increases D2 activity without affecting D3 activity. Astrocytes were cultured in the presence of 1 µM cortisol for the last 24 h. (A) Astrocytes were treated with 1 µg/ml LPS for different times before harvesting. D2 (black bars) and D3 (white bars) activities were measured as described in section 'Materials and Methods'. (B) Astrocytes were treated with several concentrations of LPS for 10 h before harvesting. Data are the means ± s.d. of results obtained from three dishes (one-way ANOVA analysis; LPS-treated versus the controls without treatment, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

culture medium containing cortisol, LPS increased D2 mRNA accumulation (Fig. 2B) indicating a pretranslational step of D2 regulation by LPS.

Both the basal level of D2 activity (0.4–2 femtomoles of T₃/min × mg protein) and its LPS stimulation after 12 h (8- to 30-fold) varied from one cell preparation to another as previously reported (Courtin *et al.* 1989). These variations could also be explained by the different efficiencies of LPS batches or their stabilities at 4 °C.

D3 activity, which also regulates cerebral T₃ concentration by catalyzing the 5-deiodination of thyroid hormones into inactive metabolites (Bianco *et al.* 2002, Courtin *et al.* 2005), was not induced by 1 µg/ml LPS in cultured astrocytes (Fig. 1A).

Role of glucocorticoids in LPS action

Glucocorticoids are powerful anti-inflammatory agents that suppress many effects of LPS in various systems including astrocytes (Kozuka *et al.* 2007). We therefore studied the effects of LPS treatment for 12 h on D2 activity (Fig. 2A) and D2 mRNA (Fig. 2B) in astrocytes cultured in the absence or presence of 1 µM cortisol. LPS increased D2 activity and D2 mRNA both in the absence and presence of cortisol. D2 activity was fourfold increased by cortisol in cells in basal

conditions as well as in cells treated with LPS (Fig. 2A). D2 mRNA was also threefold increased by cortisol in cells treated with LPS (Fig. 2B). In basal conditions, D2 mRNA was twofold increased by cortisol that appeared significantly different ($P < 0.01$) by *t*-test analysis (not shown). We checked that 10 nM dexamethasone, a synthetic unmetabolizable glucocorticoid, which binds the glucocorticoid receptor and mineralocorticoid receptor, also enhanced D2 activity and mRNA expression in LPS-treated or untreated astrocytes (Fig. 2C and D). We specified the mechanism of action of glucocorticoids by studying the effect of 1 µM RU486, a glucocorticoid receptor antagonist, on the enhancement of D2 by dexamethasone. We found that RU486 alone had no effect on D2 activity or mRNA and did not increase the action of LPS. Furthermore, RU486 suppressed the effect of dexamethasone on basal D2 activity and mRNA accumulation and also on LPS-induced D2 activity or

Figure 2 D2 induction by LPS is enhanced by glucocorticoids. (A and B) Astrocytes were cultured in the absence or presence of 1 µM cortisol for the last 24 h. Astrocytes were untreated (white bars) or treated for 12 h with 1 µg/ml LPS (black bars) before harvesting. D2 activity (A) and D2 mRNA accumulation (B) were measured as described in section 'Materials and Methods'. Data are the means ± s.d. of the results obtained from three dishes (two-way ANOVA analysis; *** $P < 0.001$; ** $P < 0.01$). (C and D) Astrocytes were cultured in the presence of vehicle (0.1% ethanol) or in the presence of 10 nM dexamethasone (Dex), 1 µM RU486, or both agents for the last 24 h. Astrocytes were treated for 12 h with 1 µg/ml LPS before harvesting. D2 activity (C) and D2 mRNA accumulation (D) were measured as described in section 'Materials and Methods'. Data are the means ± s.d. of results obtained from three dishes (two-way ANOVA analysis; basal conditions with or without RU486 in the presence of dexamethasone versus in the absence of dexamethasone; §§ $P < 0.01$; § $P < 0.05$; LPS-treated conditions with or without RU486 in the presence of dexamethasone versus in the absence of dexamethasone, *** $P < 0.001$).

Figure 3 Implication of p38-MAPK and ERK pathways in D2 induction by LPS. Astrocytes were cultured in the presence of 1 μ M cortisol for the last 24 h. Cells were untreated (white bars) or treated for 12 h with 1 μ g/ml LPS (black bars) before harvesting. (A and B) Implication of p38-MAPK and ERK pathways; 0.1% DMSO (vehicle), 5 μ M SB203580 (SB), or 5 μ M U0126 were added 20 min before LPS. (C) Absence of implication of H₂O₂ or NO. L-NAME (0.5 mM) or 20 mM N-acetyl cysteine (NAC) were added 20 min before LPS. D2 activity (A and C) and D2 mRNA accumulation (B) were measured as described in section 'Materials and Methods'. Data are the means $\pm$ s.d. of the results obtained from three dishes (two-way ANOVA analysis; different signaling pathway inhibitors in the presence or absence of LPS versus vehicle in the presence or absence of LPS, *** P <0.001; ** P <0.01).

mRNA accumulation. Thus, these effects of glucocorticoids implicate their binding to the glucocorticoid receptor.

Implication of MAP kinases in D2 induction by LPS

Since the activation of MAP kinases is involved in LPS signaling in astrocytes (Bhat *et al.* 1998, Lee *et al.* 2003, Shin *et al.* 2007), LPS effects on D2 activity and mRNA accumulation were studied in the presence of U0126 and SB203580, inhibitors respectively of ERK and p38-MAPK pathways (Fig. 3A and B). The effects of LPS (12 h treatment) on D2 mRNA accumulation and activity were inhibited by U0126 and SB203580. Thus, activation of ERK and p38-MAPK is certainly at stake in the effects of LPS.

Since production of reactive oxygen species (ROS) and nitric oxide (NO) are classical responses to LPS treatment, including in astrocytes (Bhat *et al.* 1998, 2002, Pawate *et al.* 2004, Rada & Leto 2008), we assessed their hypothetical role in D2 activation by LPS. NAC, a powerful antioxidant, has been previously shown to diminish in astrocytes the effects of H₂O₂, which increases D3 and inhibits D2 (Lamirand *et al.* 2008). NAC (20 mM) had no effect on the activation of D2 activity by LPS (Fig. 3C). This eliminated a role of H₂O₂ in the effect of LPS on D2. L-NAME, an inhibitor of NO synthase (NOS), did not affect LPS-induced D2 activity, showing that NO was not involved in this regulation.

Implication of NF- κ B in D2 induction by LPS and its effect on the rat *dio2* promoter in rat astrocytes

To examine the implication of NF- κ B signaling pathway in LPS-induced D2 in rat astrocytes, effects of LPS on D2 activity and mRNA accumulation were studied in the presence of NF- κ B inhibitor sulfasalazine (Fig. 4A and B). This NF- κ B inhibitor has been previously shown to partially block LPS-induced D2 activity in mesothelioma cells (Zeold *et al.* 2006). LPS-induced increase in D2 activity was blocked by sulfasalazine in the presence of cortisol (Fig. 4A). We also showed the inhibition by sulfasalazine of LPS-induced D2 mRNA accumulation (Fig. 4B). Sulfasalazine also caused a 65 and 80% reduction in LPS-induced D2 activity and mRNA accumulation in the absence of cortisol. These data suggest that the LPS/NF- κ B signaling pathway is able to stimulate D2 expression in rat astrocytes cultured in the presence or absence of cortisol.

Since NF- κ B has been shown to be able to activate transcription of *dio2* promoter in different cell lines including glioblastoma (Fekete *et al.* 2004, Zeold *et al.* 2006), we also

Figure 4 Implication of NF- κ B pathway in D2 induction by LPS. Activation of the rat *dio2* promoter in astrocytes by p65 and p50 NF- κ B. (A and B) Astrocytes were cultured in the presence of 1 μ M cortisol for the last 24 h. Cells were untreated (white bars) or treated for 12 h with 1 μ g/ml LPS (black bars) before harvesting; 0.1% DMSO (vehicle), sulfasalazine (SSZ) at 0.2 or 0.5 mM were added 20 min before LPS. D2 activity (A) and D2 mRNA accumulation (B) were measured as described in section 'Materials and Methods'. Data are the means $\pm$ s.d. of the results obtained from three dishes (two-way ANOVA analysis; SSZ at different concentrations in the presence or absence of LPS versus vehicle in the presence or absence of LPS, *** P <0.001; ** P <0.01). (C) The rdio2#1, rdio2#3, rdio2#4, and rdio2#5 constructs (containing respectively 3.8 kb, 658, 83 and 37 bp rat *dio2* 5'-FR) were cotransfected with empty pcDNA3.1 vector, pcDNA3.1-p65, or pcDNA3.1-p50 for 48 h. Normalized CAT activity was measured as described in section 'Materials and Methods' and expressed as fold induction of the empty pcDNA3.1 vector for each construct. Data are the means $\pm$ s.d. of the results obtained from three dishes (two-way ANOVA analysis; cotransfection with pcDNA3.1 vector containing p65 or p50 versus the respective controls cotransfected with the empty pcDNA3.1 vector, ** P <0.01; *** P <0.001).

Figure 5 C/EBP β activates the rat *dio2* promoter in astrocytes. (A) The rdio2#1, rdio2#3, rdio2#4, and rdio2#5 constructs (containing respectively 3.8 kb, 658, 83, and 37 bp rat *dio2* 5'-FR) were cotransfected with pREP4-LAP plasmid or empty pREP4 vector for 48 h. Normalized CAT activity was measured as described in section 'Materials and Methods' and expressed as fold induction of empty pREP4 vector for each construct. Data are the means $\pm$ s.d. of the results obtained from three dishes (two-way ANOVA analysis; cotransfection with pREP4 vector containing LAP versus the respective controls cotransfected with the empty pREP4 vector, *** $P < 0.001$). (B and C) The rdio2#4 construct was cotransfected with 100 ng pREP4-LAP plasmid associated with 500 ng empty pREP4 vector or pREP-LIP (B), or associated with 100 ng empty pREP4 vector or pREP-CHOP (C). Normalized CAT activity was measured as described in section 'Materials and Methods' and expressed as % of transactivation when empty pREP4 vector was added with pREP4-LAP. Data are the means $\pm$ s.d. of the results obtained from three dishes (* $P < 0.05$; *** $P < 0.001$ when compared with control by Student's *t*-test).

checked the effect of this transcription factor on rat *dio2* promoter in our cultured rat astrocytes. In astrocytes, co-expression of p65 NF- κ B with the 3.8 kb form of the rat *dio2* promoter (rdio2#1) led to a sevenfold increase in the transcriptional activity (Fig. 4C). The other component of NF- κ B, p50, led to a threefold induction of the rdio2#1 construct transcriptional activity. The truncated promoters (rdio2#3, rdio2#4, and rdio2#5) remained unresponsive. LPS did not increase the transcription activity of the transfected rat *dio2* promoter in cultured astrocytes (not shown). LPS was also

unable to increase the transcriptional activity of transfected IL6 promoter in cultured astrocytes (not shown). Finally, LPS did not increase endogenous D2 activity in the transfected astrocytes (not shown), suggesting that these transfection conditions interfere with LPS signaling in astrocytes.

Effect of C/EBPs on the rat *dio2* promoter in rat astrocytes

We also examined the action of other transcription factors, C/EBPs that are classically known to be involved in gene expression activation by LPS. The co-expression of LAP (C/EBP β) very strongly induced the D2 promoter (long and truncated forms containing CCAAT, i.e. rdio2#1, rdio2#3 and rdio2#4) (Fig. 5A). Co-expression of LAP did not affect the 37 bp minimal promoter (rdio2#5), which did not contain CCAAT, a putative C/EBP-responsive element. Overexpression of C/EBP α or C/EBP δ also strongly activated rdio2#1, rdio2#3, and rdio2#4 (not shown). We also checked that LIP and CHOP, which are competitive inhibitors of LAP by binding to the regulatory CCAAT sequences, effectively suppressed these effects (Fig. 5B and C).

Discussion

In the present work, we show for the first time that the bacterial LPS induces type 2 deiodinase in primary cultures of rat astrocytes. D2 activity induction by LPS observed in cultured astrocytes after a lag of 4–8 h involves D2 mRNA increase. D3 was not induced by LPS in cultured rat astrocytes. Previously, LPS was shown to induce D2 activity in MSTO-211H cells (Zeold *et al.* 2006) but not in GH3 cells (Baur *et al.* 2000). Very recently, LPS was reported to induce D2 in H4 glioma cells (Freitas *et al.* 2010).

Interestingly, the D2 induction by LPS was increased by cortisol or dexamethasone, acting through the glucocorticoid receptor because dexamethasone effect was blocked by the glucocorticoid receptor antagonist RU486 (Jung-Testas & Baulieu 1984). Basal D2 activity and mRNA were also increased by glucocorticoids as previously reported in astrocytes for D2 activity (Courtin *et al.* 1989). LPS-induced D2 increase in astrocytes was inhibited by the NF- κ B inhibitor sulfasalazine, indicating the implication of the NF- κ B pathway in the presence or absence of glucocorticoids. Consequently, glucocorticoids do not inhibit NF- κ B pathway implicated in D2 induction by LPS. However, glucocorticoids block IL6 expression by LPS in astrocytes (Grimaldi *et al.* 1998) as we also checked in our culture conditions (not shown), allowing one to discard a role for IL6 in the LPS effects on D2 in cultured astrocytes. Repression of NF- κ B activity by glucocorticoids has indeed been reported to depend on promoter and cell type (De Bosscher *et al.* 2003). Moreover, emerging literature suggests that glucocorticoid pro-inflammatory effects can co-exist with their anti-inflammatory properties, particularly in the central nervous system (Sorrells *et al.* 2009). D2 induction by TPA

is also strongly increased by glucocorticoids in astrocytes (Courtin *et al.* 1989). However, TPA-induced D2 activity was not inhibited by NF- κ B inhibitor sulfasalazine (not shown). D2 induction by LPS is shown in this study to require the ERK and p38-MAPK pathways, which have been demonstrated to be activated by LPS in astrocytes (Bhat *et al.* 1998, Lee *et al.* 2003, Shin *et al.* 2007). Interestingly, MAPK pathways can be activated by H₂O₂ (Tournier *et al.* 1997, Lennon *et al.* 2002) and LPS plus interferon γ can increase NOS through ROS production and MAPK pathways (Pawate *et al.* 2004). However, D2 induction by LPS was not affected by NAC, which is a powerful antioxidant. This indicates that D2 induction by LPS is independent of H₂O₂ production and suggests another activating mechanism than H₂O₂ for ERK and p38-MAPK pathways. Moreover, LPS-induced D2 activity was not affected by L-NAME, a NOS inhibitor, whereas NO and NOS were shown, in astrocytes, to be implicated in regulation by LPS of some genes, including UGT1A16 (Heurtaux *et al.* 2006). D2 induction by LPS is therefore independent of NO production, which was shown to be blocked by glucocorticoids (Kozuka *et al.* 2007).

The increase in LPS-induced D2 by glucocorticoids in astrocytes might be physiologically significant because glucocorticoids are increased *in vivo* by various type of stress, including infectious stress (Sternberg 2001). LPS can enter the brain by crossing the blood–brain barrier and bind some receptors that are notably localized in astrocytes (Bowman *et al.* 2003). In mediobasal hypothalamus including the median eminence, a structure particularly sensitive to LPS as it is devoid of blood–brain barrier (Zlokovic 1995), the LPS-induced D2 mRNA is not mediated by the associated increase in glucocorticoids (Sanchez *et al.* 2008). However, this does not exclude a glucocorticoid contribution to the effect of LPS as observed in cultured astrocytes. Moreover, D2 is predominantly expressed in tanycytes and not in astrocytes in the mediobasal hypothalamus (Guadano-Ferraz *et al.* 1997, Tu *et al.* 1997, Fekete *et al.* 2004, Sanchez *et al.* 2008), bringing a potential explanation of the difference observed in glucocorticoid effects.

Since NF- κ B inhibitor sulfasalazine inhibited LPS-induced D2 increase in astrocytes, we also checked that NF- κ B, a transcription factor classically known to be involved in gene expression activation by LPS, was able to stimulate *dio2* promoter in astrocytes. NF- κ B induction of human and rat *dio2* promoter has been previously observed in human cell lines HC11 and HEK-293 (Fekete *et al.* 2004, Zeold *et al.* 2006). We observed a sevenfold transactivation of rat *dio2* promoter by p65 NF- κ B in astrocytes, a transactivation of the same order as that previously described (threefold) for the rat *dio2* promoter in HC11 cells (Fekete *et al.* 2004). Among all forms of promoter we tested, only the rdio2#1, which is the longest construct (3.8 kb), responded to p65 and p50, excluding the putative NF- κ B-responsive element at –194 bp, which presents a high homology with the transactivation site recently identified as functional in the human *dio2* promoter (Zeold *et al.* 2006). Thus, the

responsive sequence of NF- κ B in rat *dio2* promoter is probably located in a different position from the one characterized in human *dio2* promoter.

We also looked for another transcription factor candidate for D2 induction by LPS, C/EBP β , which is upregulated by LPS in glial cells (Ejarque-Ortiz *et al.* 2007). Furthermore, C/EBPs are crucial for regulating transcription of genes involved in some LPS responses in astrocytes (Cardinaux *et al.* 2000, Kelicen & Tindberg 2004, Perez-Capote *et al.* 2006). Potential consensus sequences for C/EBPs appeared in mouse, rat, and human *dio2* promoter near the TSS (Song *et al.* 2000, Gereben *et al.* 2001). We found that all forms of the *dio2* promoter containing the consensus sequences were potently transactivated by C/EBPs. Moreover, different C/EBPs (C/EBP α , C/EBP β , and C/EBP δ) appeared able to transactivate the D2 rat promoter. Interestingly, knocking out C/EBP α leads to a decrease in D2 expression in brown adipose tissue (Carmona *et al.* 2002). Further directed mutagenesis experiments might allow us to confirm the implication of the C/EBP-binding site in the rat *dio2* promoter. Note that C/EBPs are targets for MAPK pathways (Bhat *et al.* 2002) that we have found implicated in D2 induction by LPS. The induction of C/EBP β and C/EBP δ by the cAMP pathway in astrocytes (Cardinaux & Magistretti 1996) more generally brings into question their role in the control of D2. Interestingly, C/EBP β mRNA and D2 mRNA are reported to be increased *in vivo* in the mediobasal hypothalamus of Japanese quail after photoperiod signal (Nakao *et al.* 2008).

The *in vivo* significance of the D2 induction by LPS in cultured astrocytes remains to be explored. The D2 induction by LPS in the tanycytes of the rat mediobasal hypothalamus increases T₃ generation, providing local negative feedback in the hypothalamus and accounting for the impaired response of the hypothalamo–pituitary–thyroid axis observed in nonthyroidal illness syndrome (Fekete *et al.* 2004). I.p. injection of LPS in rats also increased D2 activity in the cerebral cortex (Fekete *et al.* 2004). In T₄-replaced thyroidectomized rats, LPS administration also increased D2 activity in the mediobasal hypothalamus but not in the cerebral cortex (Fekete *et al.* 2005). Hence, this suggests that the increased D2 activity in the cerebral cortex is due to falling T₄ after LPS, whereas the increased D2 activity in mediobasal hypothalamus is independent of the associated fall in circulating T₄.

Thyroid hormone has been shown to be critical for development and functioning of central nervous system (Bernal 2002). Paracrine signaling by glial-cell-derived T₃ activates neuronal gene expression in a co-cultured system of D2-expressing H4 glioma cells and D3-expressing neuroblastoma cells (Freitas *et al.* 2010). LPS increased D2 activity and neuronal T₃-dependent gene expression in this co-cultured system (Freitas *et al.* 2010). The induction of D2 by LPS in primary cultures of astrocytes might represent a brain protective mechanism against inflammation. In the same way, T₄, and not T₃, partly protects the brain against ischemic

stroke (D'Alecy 1997) and T₃ protects the neurones in culture from apoptosis (Muller *et al.* 1995). Induction of D2 activity and mRNA was also reported in the ipsilateral cortex and striatum within 24 h after ischemia, suggesting a neuroprotective role of D2 (Margaill *et al.* 2005). Ischemia increased also D3 immunoreactivity in the neurones in the cortex and hippocampus after 1 h (Freitas *et al.* 2010). However, no increase in D3 activity was observed in striatum and cortex at 6 and 24 h after ischemia (Margaill *et al.* 2005), suggesting that the initial increase in neuronal D3 immunoreactivity might be a transient regulation before D2 induction as observed in proximal nerve segment after peripheral nerve injury (Li *et al.* 2001, Courtin *et al.* 2005).

In conclusion, we have shown the D2 induction by LPS in primary cultures of astrocytes and specified some characteristics of D2 induction mechanism. Our observations support the possible implication of brain D2 in adaptive responses to an infectious stress.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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