Bacterial lipopolysaccharide induces type 2 deiodinase in cultured rat astrocytes

Audrey Lamirand1,2,3, Martine Ramaugé1,2,3, Michel Pierre1,2,3 and Françoise Courtin1,2,3

1INSERM UMR 788, Stéroïdes, neuroprotection et neurogénèse, 94275 Le Kremlin-Bicêtre cedex, France
2INSERM UMR 854, Récepteurs, signallisations et physiopathologie thyroïdiennes et de la reproduction, 94275 Le Kremlin-Bicêtre cedex, France
3University Paris-Sud 11, 91405 Orsay cedex, France

Correspondence should be addressed to F Courtin who is now at CNRS UMR 8200 Institut Gustave Roussy 39, Rue Camille Desmoulins, 94805 Villejuif, France; Email: francoise.courtin@igr.fr

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 mechanism, support the possible implication of brain D2 in adaptive responses to an infectious stress.

Journal of Endocrinology (2011) 208, 183–192

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 (Crandt 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–throid 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, Margalla 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-kB than the rat Dio2 promoter (150- vs 3-fold increase) in HC11 cells. Recent findings have underlined the existence of an NF-kB-binding site with transactivation potency of the p65 subunit of NF-kB 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-kB sulfasalazine. Finally, we tested the ability of the transcription factors NF-kB 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. Exgen5000 was obtained from Euromedex (Souffelweyersheim, France). [¹²⁵]T₃ (3 mCi/μg) and [¹²⁵]T₄ (1.5 mCi/μg) were purchased from Amersham International. Sprague–Dawley rats were purchased from Ifa-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 via a passage through a nylon mesh (82 μm pore size) into 40 ml DMEM supplemented with 6 g/l glucose, 2-4 g/l sodium bicarbonate, antibiotics (100 U/ml penicillin, 100 μg/l streptomycin, and 0.25 μg/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 mN sodium selenite, 10 μg/ml insulin, and 10 μg/ml transferrin followed by 1 additional day in DMEM/F12 supplemented with 30 mN sodium selenite, 10 μg/ml transferrin, and eventually 1 μM 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 rdio2 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-kB subcloned in the pcDNA3.1 expression vector and C/EBPα, C/EBPβ, and C/EBPδ subcloned in the expression vector pREP4 were obtained from M Raymondjean (Antonio et al. 2002). Liver activating protein (LAP) or C/EBPβ, 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 Exgen5000 (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 $-80^\circ$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 $[{^{125}}\text{I}]$T4, and 1 nM $[{^{125}}\text{I}]$T4 for 20–60 min at 37 $^\circ$C. Addition of 50 nM T3 prevented 5-deiodination of both $[{^{125}}\text{I}]$T4 and D2-generated $[{^{125}}\text{I}]$T3 into $[{^{125}}\text{I}]$3,3',5'-triiodothyronine (reverse T3) and $[{^{125}}\text{I}]$3',3'-diiodothyronine (3,3'-T2) respectively, according to the previous in vitro studies on type 3 iodothyronine deiodinase (D3) activity in cultured astrocytes (Esfandiar et al. 1992). Reactions were stopped by adding 10 µl of 10 M NH$_4$OH containing 10 µM iodide, 10 µM T3, and 10 µM T4. The $[{^{125}}\text{I}]$T3 produced was separated from $[{^{125}}\text{I}]$T4 by descending paper chromatography (Courtin et al. 1986). Then, the radioactive products were counted for determination of D2 activity, expressed as femtomoles of T3/min per mg protein. Kinetic analysis was performed in sonicates of LPS-treated cells using 0.05–2 nM $[{^{125}}\text{I}]$T4 as substrate and 20 mM DTT as cofactor. For D3 activity assays, homogenates were incubated at 37 $^\circ$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 $[{^{125}}\text{I}]$T3. Reactions were stopped by adding 10 µl of 10 M NH$_4$OH containing 10 µM T3 and 10 µM T4. The $[{^{125}}\text{I}]$3,3'-T2 produced was separated from $[{^{125}}\text{I}]$T3 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'-T2/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 µg/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 µg/ml (significantly different using a $t$-test, $P<0.001$) and was maximal for 1 µg/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 T4 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

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**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 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).

![Figure 1](image1.png) **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, \( *P<0.05; **P<0.01; ***P<0.001 \)).

![Figure 2](image2.png) **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 results obtained from three dishes (two-way ANOVA analysis; \( ***P<0.001; **P<0.01; *P<0.05 \)). (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 the absence of dexamethasone; \( ***P<0.001; **P<0.01; *P<0.05 \); LPS-treated conditions with or without RU486 in the presence of dexamethasone versus the absence of dexamethasone, \( ***P<0.001 \)).
H2O2, which increases D3 and inhibits D2 (Lamirand et al. 2007), has been previously shown to diminish in astrocytes the effects of LPS. NAC, a powerful antioxidant, has been shown to 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-κB signaling pathway is able to stimulate D2 expression in rat astrocytes cultured in the presence or absence of cortisol.

Since NF-κB 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 examined the implication of NF-κB 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-κB inhibitor sulfasalazine (Fig. 4A and B). This NF-κB 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-κB signaling pathway is able to stimulate D2 expression in rat astrocytes cultured in the presence or absence of cortisol.

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. 2004, Rada & Leto 2008), 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, NO synthase (NOS), did not affect LPS-induced D2 activity, showing that NO was not involved in this regulation.

Figure 3 Implication of p38-MAPK and ERK pathways in D2 induction by LPS. Astrocytes were cultured in the presence of 1 μM cortisol for the last 24 h. Cells were untreated (white bars) or treated for 12 h with 1 μg/ml LPS (black bars) before harvesting. (A and B) Implication of p38-MAPK and ERK pathways; 0.1% DMSO (vehicle), 5 μM SB203580 (SB), or 5 μM U0126 were added 20 min before LPS. (C) Absence of implication of H2O2 or NO. L-NAME or 20 mM N-acetyl cysteine (NAC) 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 ± 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 NF-κB in D2 induction by LPS and its effect on the rat dio2 promoter in rat astrocytes

To examine the implication of NF-κB 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-κB inhibitor sulfasalazine (Fig. 4A and B). This NF-κB 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-κB signaling pathway is able to stimulate D2 expression in rat astrocytes cultured in the presence or absence of cortisol.

Since NF-κB 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 examined the implication of NF-κB signaling pathway in D2 induction by LPS. Activation of the rat dio2 promoter in astrocytes by p65 and p50 NF-κB. (A and B) Astrocytes were cultured in the presence of 1 μM cortisol for the last 24 h. Cells were untreated (white bars) or treated for 12 h with 1 μg/ml LPS (black bars) before harvesting; 0.1% DMSO (vehicle), sulfasalazine (SSZ) at 0.2 mM 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 ± 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).

Figure 4 Implication of NF-κB pathway in D2 induction by LPS. Activation of the rat dio2 promoter in astrocytes by p65 and p50 NF-κB. (A and B) Astrocytes were cultured in the presence of 1 μM cortisol for the last 24 h. Cells were untreated (white bars) or treated for 12 h with 1 μg/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 ± 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).
empty pREP4 vector for each construct. Data are the means ± S.D. of the results obtained from three dishes (two-way ANOVA analysis; compared with control by Student’s t-test).}

**Figure 5** C/EBPβ 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 ± 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 ± 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-κB 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-κB, 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 transcriptional 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/EBPβ) 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/EBPα or C/EBPβ 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 induction. D2 was not induced by LPS in cultured rat astrocytes. Previously, LPS was shown to induce D2 activity in primary cultures of neonatal rat astrocytes (Courtin et al. 1989). LPS-induced D2 increase in astrocytes was inhibited by the NF-κB inhibitor sulfasalazine, indicating the implication of the NF-κB pathway in the presence or absence of glucocorticoids. Consequently, glucocorticoids do not inhibit NF-κB 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-κB 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

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is also strongly increased by glucocorticoids in astrocytes (Courtin et al. 1989). However, TPA-induced D2 activity was not inhibited by NF-kB 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 \(H_2O_2\) (Tournier et al. 1997, Lennon et al. 2002) and LPS plus interferon \(\gamma\) 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 \(H_2O_2\) production and suggests another activating mechanism than \(H_2O_2\) 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 UGTA16 (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 tanyocytes 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-kB inhibitor sulfasalazine inhibited LPS-induced D2 increase in astrocytes, we also checked that NF-kB, a transcription factor classically known to be involved in gene expression activation by LPS, was able to stimulate \(dio2\) promoter in astrocytes. NF-kB 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-kB 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-kB-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-kB 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\(\beta\), 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\(\alpha\), C/EBP\(\beta\), and C/EBP\(\delta\)) appeared able to transactivate the D2 rat promoter. Interestingly, knocking out C/EBP\(\alpha\) 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\(\beta\) and C/EBP\(\delta\) by the cAMP pathway in astrocytes (Cardinaux & Magistretti 1996) more generally brings into question their role in the control of D2. Interestingly, C/EBP\(\beta\) 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 tanyocytes of the rat mediobasal hypothalamus increases T\(3\) 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 T\(_4\)-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 T\(_4\) after LPS, whereas the increased D2 activity in mediobasal hypothalamus is independent of the associated fall in circulating T\(_4\).

Thyroid hormone has been shown to be critical for development and functioning of central nervous system (Bernal 2002). Paracrine signaling by glial-cell-derived T\(_3\) 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 T\(_3\)-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, T\(_4\), and not T\(_3\), partly protects the brain against ischemic
stroke (D’Aley 1997) and T3 protects the neurones in culture from apoptosis (Müller 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 (Margail 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 (Margail 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.

Funding

This work was supported by the Institut National de la Sante et de la Recherche Médicale, INSERM.

Acknowledgements

We are very grateful to P R Larsen (Boston, MA) for his kind gift of D2 promoter constructs. We thank M Raymondjean (Paris, France) for providing p50 NF-kB, p65 NF-kB, and C/EBPs expression vectors and M Pomerance (Châtenay-Malabry, France) for providing LAP, LIP, and CHOP expression vectors. We also thank K Rajkowski (Le Kremlin-Bicêtre, France) for his helpful advice and the Maison des Langues (Paris Descartes University, France) for English corrections.

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Journal of Endocrinology (2011) 208, 183–192

www.endocrinology-journals.org


Received in final form 7 October 2010
Accepted 10 November 2010
Made available online as an Accepted Preprint 10 November 2010