Differential effects of interleukin-1 alpha and beta on interleukin-6 and chemokine synthesis in neurones

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Interleukin (IL)-1 is a key mediator of neuroinflammation via actions of two agonists IL-1 α and β that bind to the IL-1 type I receptor (IL-1RI), and are thought to trigger identical responses. However, evidence suggests that IL-1 α and β may have differential actions in the central nervous system (CNS). The objective of this study was to test the hypothesis that IL-1 α and β differentially regulate the expression of IL-6 and chemokines KC, IP-10 and MCP-1 in primary neurones. Here we demonstrate that, whilst IL-1 β induced significant synthesis of IL-6 in neurones, IL-1 α had no effect. In contrast, IL-1 α and β induced strong synthesis and constitutive release of chemokines KC, IP-10 and MCP-1 from neurones, and these responses were IL-1RI-dependent. Whilst IL-1 β -induced IL-6 synthesis was dependent on the nSMase/Src kinase signalling cascade, specific inhibitors of nSMase (3-OMS) and Src kinase (PP2) failed to inhibit IL-1 α - and IL-1 β -induced chemokines synthesis, suggesting the existence of alternative signalling pathway(s) in neurones.

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Introduction

Interleukin (IL)-1 is a prototypic proinflammatory cytokine involved in the immune response to infection and injury, and is a primary mediator of inflammatory responses to acute and chronic central nervous system (CNS) disorders (Allan et al., 2005; Rothwell, 2003). IL-1 also contributes to the regulation of physiological functions such as sleep, memory and long-term potentiation (LTP), as well as host defence responses such as fever and sickness behaviour (Bilbo et al., 2005; Dantzer et al., 1998; Ross et al., 2003; Schneider et al., 1998).

IL-1 α and β are the two established functional agonists of the IL-1 family that bind to a specific, membrane-associated IL-1 type I receptor (IL-1RI) (Sims et al., 1988), which associates with the IL-1 receptor accessory protein (IL-1RAcP) (Wesche et al., 1997) to activate downstream signalling cascades. In the CNS, IL-1 α and β trigger in glial cells the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF κ B), leading to the

production of key inflammatory mediators such as IL-6, prostaglandins E2 and chemokines (Kim et al., 2004; Parker et al., 2002), and all these actions are blocked by the naturally occurring IL-1 receptor antagonist (IL-1RA). We have recently reported that IL-1 β also induces in neurones the synthesis of IL-6 but via distinct pathways including neutral sphingomyelinase (nSMase)/ceramide and Src kinase (Tsakiri et al., 2008a), but whether this signalling pathway is activated by IL-1 α in neuronal cells to produce IL-6 is unknown.

IL-1 α and β are believed to induce identical cellular responses, but there have been reports of differences in the activity of these two ligands both peripherally and centrally in rodents. For instance, T cell-dependent antibody production is mediated by IL-1 β , but not IL-1 α (Nakae et al., 2001) and the ligands have differential effects on some aspects of cutaneous immune function (Cumberbatch et al., 2002) and tumour development (Song et al., 2003). In the rat CNS, IL-1 β is much more effective at inducing fever than IL-1 α when injected intracerebroventrically (icv) (Anforth et al., 1998). Furthermore IL-1 β , but not IL-1 α , induces nerve growth factor (NGF) release from astrocytes (Juric and Carman-Krzan, 2001) and, despite inducing similar levels of activation of MAPKs/NF κ B in glia, IL-1 β is more potent than IL-1 α at inducing IL-6 release from these cells (Andre et al., 2005). The effect of IL-1 α on neuronal IL-6 production is not known and the aim of this study was to compare the effects of IL-1 α and IL-1 β on expression of IL-6 and other inflammatory mediators identified by cDNA microarray analysis, such as chemokines KC (CXCL1), IP-10 (CXCL10) and MCP-1 (CCL2) in primary neurones. We show here that, unlike IL-1 β , IL-1 α fails to induce IL-6 synthesis in neurones, and demonstrate for the first time that both ligands are potent inducers of chemokines synthesis and release, via an nSMase/Src kinase-independent signalling mechanism.

Results

IL-1 β , but not IL-1 α , induces IL-6 mRNA and protein synthesis in primary neurones

IL-1 β (0.003 to 3 U/ml) dose-dependently induced significant IL-6 gene expression after 4 h in primary neurones, with maximum induced expression (57-fold) obtained with the dose of 0.3 U/ml

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(Fig. 1A). In contrast, IL-1 α failed to induce IL-6 mRNA expression and all concentrations tested were found to be less potent in inducing IL-6 gene expression than the equivalent concentrations of IL-1 β . A statistically significant difference was observed between IL-1 α and IL-1 β -induced IL-6 gene expression at a dose of 0.3 U/ml. IL-1 β (0.03 to 30 U/ml) also induced significant synthesis of IL-6 protein in the cells, with maximum synthesis detected in response to the concentration of 0.3 U/ml, but IL-1 α again had no effect on IL-6 protein expression in the cell supernatant or lysates of treated cells (Fig. 1B). IL-1 α induced only a slight, but non-significant increase in IL-6 protein levels in the cell lysates at all concentrations tested, and no significant release of the protein in the culture medium. IL-1 β produced significantly higher levels of IL-6 compared to IL-1 α at the concentration of 0.03–3 U/ml.

IL-1 α and IL-1 β induce synthesis of the chemokines KC, IP-10 and MCP-1 in primary neurones

To identify the profile of changes in gene expression induced by IL-1 in neurones, we used cDNA microarray-based gene expression

Fig. 1. IL-6 mRNA and protein expression in IL-1 α - and IL-1 β -treated neuronal cell cultures. Primary cortical neuronal cell cultures were treated with vehicle (C), IL-1 α or IL-1 β (0.003 to 30 U/ml) for 4 or 7 h. (A) cDNA from the cultures was prepared and used for SYBR-green real-time PCR using specific primers for IL-6 and β -actin. Two melting curves for primer binding were constructed to ensure that cDNA amplification was due to a single PCR product. Expression of the two genes was determined by the standard curves, and IL-6 expression was normalised to β -actin expression levels. Data are presented as the mean $\pm$ SD of three independent experiments * p < 0.05, ** p < 0.001, C vs. IL-1 β , # p < 0.05, IL-1 α vs. IL-1 β , one-way ANOVA with Bonferroni's multiple comparison post-hoc test. (B) Cell lysates and supernatants were collected and assayed with a mouse-specific IL-6 ELISA. Data are presented as the mean $\pm$ SD of seven independent experiments * p < 0.05, ** p < 0.01, *** p < 0.001, C vs. IL-1 β , # p < 0.05, ### p < 0.001, IL-1 α vs. IL-1 β , one-way ANOVA with Bonferroni's multiple comparison post-hoc test.

profiling in neuronal cultures treated with IL-1 β . The genes that showed significant changes in their expression were consistently upregulated by IL-1 β across all three independent experiments, and were all identified as inflammatory mediators (Fig. 2); In addition to IL-6, marked induction of gene expression was seen for chemokines e.g. the murine homologue of GRO α (KC, also known as CXCL1) (62.8-fold), interferon- γ inducible protein 10 kDa (IP-10, also known as CXCL10) (13.2-fold), the murine homologue of MCP-1 (MCP-1, also known as CCL2) (14.4-fold), as well as macrophage colony stimulating factor (M-CSF) (4.0-fold) and the intercellular adhesion molecule 1 (ICAM-1) (1.9-fold). All these effects were reversed by co-incubation with IL-1RA.

The expression profile of KC, IP-10 and MCP-1 proteins was studied in cortical neurones treated with the same biologically active dose of IL-1 α or IL-1 β (0.3 U/ml) for 1 to 24 h. Significant up-regulation of KC, IP-10 and MCP-1 protein levels were detected in response to IL-1 α or IL-1 β compared to vehicle (C), as early as 2 h for KC and MCP-1, and 3 h for IP-10 (Fig. 3), and the effects were similar for both forms of IL-1. The dramatic increase in the levels of all three chemokines KC, IP-10 and MCP-1 was observed in cell lysates and in culture supernatants, suggesting that, unlike IL-6, the proteins were released constitutively in response to IL-1. In addition to increases in the levels of the chemokines detected in the culture supernatants, a significant increase in MCP-1 levels was detected in the cell lysates (Fig. 3C). IL-1 α and IL-1 β produced similar up-regulation in the protein levels of the chemokines, and a similar time course of release. Up-regulation of KC, IP-10 and MCP-1 by IL-1 α or IL-1 β was reversed by IL-1RA, whilst IL-1RA alone had no significant effect on the regulation of chemokines protein levels (not shown).

IL-1 α - and IL-1 β -induced release of KC, IP-10 and MCP-1 is independent of nSMase and Src kinase signalling pathway in primary neurones

We have demonstrated previously that IL-1 β -induced IL-6 synthesis in neurones is dependent on the activation of the neuronal-specific nSMase/Src kinase signalling pathway (Tsakiri et al., 2008a). We therefore determined whether the same pathway is involved in the synthesis of chemokines induced by IL-1. Co-incubation of cultures with IL-1 α or IL-1 β and the Src kinase inhibitor PP2 (10 μ M) or nSMase inhibitor 3-OMS (25 μ M), induced a slight, but non-significant reduction of KC and IP-10 in culture supernatants, whereas PP2 or 3-OMS had no effect on the intracellular levels of KC and IP-10 (Fig. 4A/B). PP2 or 3-OMS had no effect on IL-1-induced MCP-1 synthesis (Fig. 4C).

Discussion

In this study we demonstrate that unlike having a differentially effect on IL-6 synthesis, IL-1 α and β are potent inducers of chemokines synthesis and release, via an nSMase/Src kinase-independent signalling mechanism, suggesting the existence an alternative signalling pathway in neurones.

Proinflammatory actions of IL-1 α and β are mediated in the CNS primarily by glial cells that produce various inflammatory mediators including IL-6 and chemokines (Kim et al., 2004; Parker et al., 2002). IL-1 β also induces IL-6 synthesis in neurones (Tsakiri et al., 2008a,b), but the effect of IL-1 α was unknown. In the present study, we confirm that IL-1 β induces significant synthesis of IL-6 in neurones, and demonstrate that the same biologically active doses of

Fig. 2. Analysis of GeneChip® microarray-based gene expression profiling from IL-1 β -treated neurones. Genes whose expression changed significantly (over 2.5 arbitrary units (AU) between the different treatments were selected and identified as key inflammatory mediators (KC, MCP-1 and IP-10, the macrophage colony stimulating factor (M-CSF), and intercellular adhesion molecule 1 (ICAM-1)). These genes were upregulated by IL-1 β , and this effect was reversed by co-incubation with IL-1RA. The arithmetic values of the table represent the mean expression (AU) of each gene from the three experiments, and the coloured boxed underneath represents the expression intensity of each gene in each individual experiment.

IL-1 α fail to induce IL-6 synthesis, providing further evidence of differential actions of these two ligands in the CNS. Indeed we have demonstrated previously that IL-1 β is more potent than IL-1 α at inducing fever and IL-6 production from glia (Anforth et al., 1998; Andre et al., 2005). Furthermore we have investigated the effect of these two ligands on the expression of other inflammatory mediators in neuronal cells, and we demonstrate for the first time that IL-1 α and IL-1 β produce a strong and rapid synthesis of three major chemokines KC, IP-10 and MCP-1 in primary neurones (Figs. 2 and 3). The similar effect of both IL-1 ligands on chemokine synthesis demonstrates that the preparations of recombinant IL-1 used in our study are equally bioactive. KC, IP-10 and MCP-1 are known to be highly expressed after CNS injury, and are key mediators of inflammation, via actions on glia and neurones, which express the functional receptors CXCR2 (for KC), CCR2 (for MCP-1), and CXCR3 (for IP-10) (Barbero et al., 2002; Cho and Miller, 2002). Our data are the first to demonstrate that IL-1 can directly activate neuronal cells to produce chemokines, which could be an important mechanism for the recruitment of microglia to the site of neuronal injury.

Importantly, both IL-1 ligands appeared to be equally potent in the regulation of expression of these chemokines, in the time-course

and magnitude of expression, indicating that the marked difference in their effects on IL-6 could not be ascribed to differences in biological activity. In addition, these chemokines appeared to be released from the cells into the culture supernatants, in contrast to IL-6, which was stored intracellularly and needs a secondary stimulus for its release (Tsakiri et al., 2008b). Here again, both IL-1 ligands had similar potency on the release of these chemokines. The fact that IL-1 α and IL-1 β have identical effects on KC, IP-10 and MCP-1 expression, but differentially induce IL-6, cannot be attributed to signalling through another receptor since the effects of both IL-1 ligands were abolished by IL-1RA, which blocks actions through IL-1R1. Moreover both IL-1 α and IL-1 β have similar binding affinities for IL-1R1 (Dower et al., 1986). The differential effects of IL-1 α and IL-1 β could be explained by the recruitment of additional/alternative receptor accessory protein or adaptor proteins to the receptor complex, which could trigger differential binding affinity for one of the ligands, activation of other intracellular signalling pathways and/or more prolonged activation of the existing pathway.

IL-1 β is known to induce in neurones the activation of Src kinase through nSMase, leading to an increase in intracellular Ca²⁺ concentration from NMDA receptors that controls IL-6 synthesis (Tsakiri et

Fig. 3. Effect of IL-1α and IL-1β on chemokine expression in primary cortical neurones. Cortical neurones were treated with vehicle (C) for 7 h, IL-1α or IL-1β (0.3 U/ml) for 1 to 24 h. Cultures were also treated with IL-1α or IL-1β in the presence or absence of IL-1RA (50 ng/ml) for 1 or 7 h. Cell lysates and supernatants were assayed for KC (A), IP-10 (B) and MCP-1 (C) protein by ELISA. Data are presented as mean ± SD of three independent experiments carried out on separate cultures. * $p < 0.05$, ** $p < 0.01$, C vs. IL-1α or IL-1β; # $p < 0.05$, ### $p < 0.01$, IL-1α vs. similar concentration of IL-1β, using one-way ANOVA and Dunnett's multiple comparison post-hoc test.

al., 2008a). In order to understand whether the nSMase/Src kinase also governs the regulation of KC, IP-10 and MCP-1 in neurones, we tested the effect of specific inhibitors of nSMase (3-OMS) and Src kinase (PP2) on IL-1-induced chemokine synthesis. We show that 3-OMS and PP2 did not induce significant reduction in the protein levels of the chemokines, whilst IL-6 levels in the same samples were significantly reduced (Tsakiri et al., 2008a). These results suggest that the regulation of chemokine synthesis by IL-1 in neurones occurs independently of nSMase/Src kinase signalling pathway activation.

In conclusion, we show that, in contrast to their differential effects on IL-6 synthesis, both IL-1 ligands induce strong synthesis and

release of chemokines KC, IP-10 and MCP-1, via an IL-1R1-dependent and nSMase/Src kinase-independent mechanism, suggesting the existence of additional neuronal IL-1 signalling pathway(s).

Experimental methods

Animals

All studies were performed using cell cultures prepared from wild type mice (C57/BL6x129sv, Charles River, UK). Animal care and experimental procedures were conducted in accordance with the guidelines set by the

Fig. 4. Effect of Src kinase and nSMase inhibitors on IL-1 α - or IL-1 β -induced chemokines expression. Cortical neurones were treated with vehicle (C), IL-1 α or IL-1 β (0.3 U/ml) for 7 h in the absence or presence of the Src kinase inhibitor PP2 (10 μ M) or the nSMase inhibitor 3-OMS (25 μ M). As a control, neurones were also treated with 3-OMS or PP2 alone for 7 h. Cell lysates and supernatants were assayed for KC (A), IP-10 (B) and MCP-1 (C) proteins by ELISA. Data are presented as mean $\pm$ SD of three independent experiments carried out on separate cultures. * p < 0.05, ** p < 0.01, *** p < 0.001, significant differences between control and treated groups, non-significant (ns) differences between IL-1 and IL-1 + PP2/3-OMS treatments, using one-way ANOVA with Bonferroni's multiple comparison post-hoc tests.

European Council directives (86/609/EEC) and the UK Home Office, Animals (Scientific Procedures) Act 1986.

Reagents and drugs

General laboratory reagents were obtained from Sigma (UK) or BDH Ltd (UK). Tissue culture reagents were supplied by Invitrogen (UK). Rat recombinant IL-1 α , IL-1 β and human recombinant IL-1 receptor antagonist

(IL-1RA) were provided by Dr. Steve Poole from the National Institute of Biological Standards and Controls (NIBSC, UK).

Primary cortical neuronal cell culture and treatments

Primary, neuronal cell cultures were prepared from embryonic (day 16) mice as described previously (Moore et al., 2002). The cultures were used on days 12–14. Cell-specific immunostaining revealed that all cultures

were highly enriched of neurones with no glial contamination (data not shown).

For IL-6 mRNA expression, cultures were treated with rat recombinant IL-1 α or IL-1 β (0.003, 0.015, 0.03, 0.3, 3 or 30 U/ml, diluted in vehicle (0.1% low endotoxin bovine serum albumin (BSA) in sterile 0.9% NaCl) for 4 h (optimal time point of IL-6 mRNA expression) or 7 h (optimal time point for protein expression) (Tsakiri et al., 2008b). For cDNA microarray experiments, cultures were treated with vehicle or IL-1 β (0.3 U/ml), in the absence or presence of IL-1RA (50 ng/ml) for 4 h. To assess the effect of IL-1 α and IL-1 β on the synthesis of KC (CXCL1), IP-10 (CXCL2) and MCP-1 (CCL2) proteins in neurones, primary cultures were treated with vehicle, IL-1 α or IL-1 β (0.3 U/ml) for 1 to 24 h. Cultures were also treated with IL-1 α or IL-1 β (0.3 U/ml) for 1 or 7 h in the presence of IL-1RA (50 ng/ml). To investigate the involvement of Src kinase and nSMase on IL-1-induced chemokine expression, cultures were treated with vehicle, IL-1 α or IL-1 β (0.3 U/ml) in the absence or presence of the Src kinase inhibitor PP2 (10 μ M, Calbiochem, UK), or the nSMase inhibitor 3-O-methyl-sphingomyelin (3-OMS, 25 μ M, Biomol, USA) for 7 h.

Real-time PCR

Real-time PCR for IL-6 was performed as previously described (Tsakiri et al., 2008b) using specific oligonucleotide primers for IL-6 and β -actin (sequences available on request). For all experiments, control reactions using total RNA were performed to ensure that amplification was not a result of contamination with genomic DNA.

cDNA microarray analysis

Microarray analysis was performed with the GeneChip® Mouse Genome 430 2.0 Array (oligonucleotide arrays), which contains over 45,000 probe sets, representing well established mouse genes and unknown EST sequences. The sequences used for the probe design had been selected from the GeneBank®, dbEST and RefSeq. The oligonucleotide probes had been synthesised *in situ* on the arrays, and eleven probes were used to measure the level of transcription of each sequence on the array. The arrays contained a set of mouse housekeeping genes that allowed the normalisation of the data. Biotin labelled cRNA samples were hybridised on the array platforms and stained with streptavidin-phycoerythrin. The arrays were scanned in GeneChipScanner® and the results were analysed with the GeneChip Operating Software®. Data were normalised by global scaling, and data present in at least two of the three triplicate arrays were used for further analysis. The results were analysed with a parametric test that assumes equal variances, Benjamin and Hochberg False Discovery Rate multiple testing correction and Tukey's post-hoc test with a cut-off point of $p < 0.05$, which produced a list of 152 genes.

ELISA

Immunoreactive IL-6, KC, IP-10 and MCP-1 in neuronal cell culture supernatants and cell lysates were assayed using a validated mouse-specific ELISAs (DuoSet) purchased from R&D Systems (UK). Standards were assayed in triplicate and samples (100 μ l) in duplicate. The absorbance was then measured using a plate reader (MRX, Dynatech, UK), and results were calculated from the standard curve. The detection limits of the assays were 3 pg/ml.

Data analysis

Data were analysed with GraphPad Prism 4.0 software, and expressed as the mean $\pm$ standard deviation (SD) of at least three independent experiments carried out on separate cultures. Differences between treated and control groups were analysed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post-hoc test. Differences between IL-1 alone and IL-1 in the presence of inhibitors were analysed by one-way ANOVA with Bonferroni's post-hoc test. For all analyses, a value of $p < 0.05$ was considered statistically significant.

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