Differential modulation of interleukin-6 expression by interleukin-1β in neuronal and glial cultures

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ABSTRACT. We analysed the specific effects of IL-1β immunoneutralization on the expression of IL-6 in different pure cultures of neurones and glia after both experimental subliminal hypoxia and recovery. Whereas the IL-1β-deprivation signal induced a decrease in IL-6 expression and release of normoxic neurones, it provoked an increase in IL-6 protein in hypoxic neurones. Moreover, the direct correlation between IL-1β and IL-6, observed in normal and recovering neuronal cultures, was reversed in hypoxic conditions. These reversals were not observed in glial cells, in which IL-1β immunosuppression led to a decrease in IL-6 under all conditions considered. In conclusion, the IL-1β modulates IL-6 in different ways according to the ambient physiological or pathological conditions, and also acts *via* different mechanisms, depending on the cellular phenotype.

Keywords: IL-1β, IL-6, neurones, glia, hypoxia

INTRODUCTION

Conflicting roles in neuroinjury have been ascribed to some cytokines, such as interleukin (IL)-1β, tumour necrosis factor-alpha (TNF-α), and IL-6. Although it has been proposed that IL-1β acts principally as a mediator in the pathogenesis of ischemic/hypoxic damage, and many results confirm this opinion [1-3], data from other researchers and our own research [4-7] suggest a modulatory and neuroprotective role for IL-1β. Furthermore, IL-1β induces, in a variety of cells, the production of cytokines and factors such as IL-1β itself, TNF-α, and IL-6 [8]. IL-6 is a multifunctional and pleiotropic cytokine which plays a key role in neuroimmune interactions, and potentially exerts a neuroprotective effect against ischemic/hypoxic damage and excitotoxic neuronal loss [9-12] *via* direct and indirect mechanisms. All data from *in vivo* and *in vitro* experimental systems have reported an increase in the expression and release of both cytokines (IL-1β and IL-6) in response to neuronal insult [6-7, 13-14], and consequently, particular interest is focussed on the interaction between, and the opposing actions of, IL-1β and IL-6. The present work examines the specific effects of IL-1β immunoneutralization on the expression of IL-6, at the transcription and translation levels, in pure cultures of hippocampal neurones and of glial cells under physiological conditions, and after both experimental subliminal hypoxia and recovery. Our previous research demonstrated the constitutive release of IL-1β from neurones of the hippocampus and an increase in this protein during exposure to hypoxia [6]. Furthermore, IL-1β is not defined exclusively as a disease-related factor, and the IL-1β signal influences the production of factors and cytokines, such as NGF and TNF-α, depending upon physiological or pathological conditions [7]. In this study, we continue investigation of the interactions among proinflammatory cytokines. In addition the different mechanisms by which different types of neural cells react to injury and their different contributions to the process.

MATERIALS AND METHODS

Neuronal cultures

Hippocampal cultures were generated from pregnant Sprague-Dawley rats at 17-18 days gestation. Brains were removed from foetuses and hippocampi were dissected under a stereomicroscope. The tissue was collected under sterile conditions and dissociated both chemically (papain, 20 U/ml) and mechanically (10 – 12 pipette aspirations). Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% horse serum (HS), and plated into 3.5-mm poly-D-lysine-precoated dishes at 2×10^5 cells/ml. After 3-4 hours, the medium was replaced with N_2-supplemented serum-free medium, in order to inhibit non-neuronal cell proliferation and to avoid serum interference in the experimental

procedures that followed. Cultures were placed in a humidified CO_2 incubator, and after 3 days in culture the division of non-neuronal cells was halted by the addition of 10 μM cytosine arabinoside. The culture medium was never changed, and the cells were not reefed until experimental hypoxia. The neuronal cultures were used for experiments after 8 – 10 days *in vitro*.

Astrocyte cultures

Primary cultures of rat cerebral cortical astrocytes were prepared from cortices of new-born (P2-P3) Sprague-Dawley rats [15]. Briefly, cortices were collected and dissociated as described above. Cell suspensions were plated in untreated 75-ml flasks in 10 ml of DMEM/F12 medium with 10% foetal calf serum (FCS). The medium was changed every 3 days, and after about 10 days the cultures were shaken at 400 rpm for 10 min, and the medium renewed to remove most of the contaminating ameboid microglia and oligodendroglia [16]. To purify cultures further, the culture flasks were shaken overnight at 250 rpm at 37°C, and the following day the medium was changed for medium containing AraC (10^{-4} M) for 1-3 days. Cells were detached from the flask surfaces by brief exposure to 0.1% trypsin, resuspended in DMEM + N_2 supplement and were allowed to adhere to plastic culture dishes. Astrocytes were used at confluence after 7-10 days.

Immunocytochemistry

Immunocytochemistry was performed after 8 days *in vitro* to assess the characterisation of neuronal and glial cultures. Cultures were double stained with antibodies for neuronal and glial cells. Specifically, monoclonal antibody IgG_1 to neurofilament (NF) (BioGenex, San Ramon, CA, USA MU073-UC), followed by the avidine-biotin immunoperoxidase method (Vectastain Elite ABC Kit, Vector, Burligame, CA, USA) or anti-mouse IgG TRITC conjugate (T-5393, Sigma) were used to mark the cytoskeletal protein of neurones, whereas polyclonal antibody to glial fibrillary acidic protein (GFAP) (BioGenex, HK099-5K) revealed by anti-rabbit IgG-fluorescein, $F(ab')_2$ fragment, was used to mark astrocytes. The cultures were then washed in PBS, labelled with the nuclear fluorochrome bisbenzimide (Hoechst 33342; Sigma), mounted in aqueous mounting medium (BioGenex, HK099-5K), observed and photographed using a Diaplan Leitz fluorescence microscope.

Immunoneutralization

For passive immunoneutralization, an IgG1 anti-mouse-IL-1β monoclonal antibody (10 μg/ml; 1997-01, Genzyme Corp., Cambridge, MA, USA) was added to the culture media immediately before exposure to hypoxia and maintained until the end of recovery. This antibody recognises the IL-1β precursor and mature, secreted forms of IL-1β, but not IL-1α. As reported in the manufacturer's protocol, no detectable cross-reactivity was observed with TNF-α or IFN-γ. As a sham control, parallel cultures were treated with an irrelevant antibody, IgG1 MOPC 300, at the same concentration [7]. The cultures used in all experiment were always sister cultures from a single dissection.

In vitro *hypoxia model*

Cultures were exposed reversibly to hypoxia by placing the plates in an airtight sealed Plexiglas chamber. The air pressure in the chamber was reduced to 20 mmHg by aspiration and replaced by flushing for 15 minutes with a gas mixture of 95% N_2 and 5% CO_2. The resultant atmosphere contained no oxygen, as confirmed by an oxymeter (BodyGuard 4 Gas Detector, Bacharach, Pittsburgh, PA 15238, USA). The relative humidity was maintained close to 95% by filling the bottom of the chamber with deionized, sterile water. To maintain the temperature at 37°C, the chamber was placed inside an incubator [6-7, 17] and hypoxia was maintained for 6 hours. The chamber was opened and plates reoxygenated in the incubator for a further 3 hours.

Viability cell assays

To evaluate the viability of neuronal and glial cultures, an assay was performed by adding 1 volume of CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corporation, San Luis Obispo, CA, USA) to 5 volumes of culture medium in each well. After 1 hour at 37°C in a humidified 5% CO_2 atmosphere, the absorbance of the cultures at 490 nm was recorded using an ELISA plate reader.

RT-PCR analysis

Total RNA was extracted from collected cells (TRIzol Reagent, Invitrogen, Life Technologies, Scotland, UK.) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using RETROscript™ (Ambion, Cambridgeshire, UK) with random decamers used as first-strand primers. The cDNA (2 μl) was used as the template for amplification in a 20-μl PCR reaction. Multiplex RT-PCR was performed using the Gene-Specific Relative RT-PCR kit (Ambion) to amplify IL-1β and IL-6 mRNAs. The kit provides 18S rRNA as an internal control, which is used in combination with 18S competimers and a gene-specific PCR primer pair. Competimer technology is used to modulate the amplification efficiency of a PCR template, and the correct ratio between the 18S rRNA primers and 18S competimers was determined empirically. We used a 2:8 ratio of 18S primer/competimer for both IL-1β and IL-6 primers. The 18S rRNA primer set produced a product of 495 bp, the IL-6-specific primer pair produced an amplification product of 414 bp, and the product of the IL-1β primers was 240 bp. The number of cycles was determined empirically by sampling IL-6 and IL-1β amplicons between 22 and 40 cycles and selecting the approximate midpoint of the linear amplifications (35 cycles). PCR thermal cycling was performed under the following conditions: initial denaturation for 2 minutes at 94°C; followed by the appropriate number of cycles of 30 seconds at 94°C, 40 seconds at 61°C, 30 seconds at 72°C; and a final extension of 5 minutes at 72°C. All PCRs were performed in duplicate. PCR products were separated on a 2% agarose gel stained with ethidium bromide, and the relative densities of the PCR fragments were determined and normalised using a semiquantitative densitometric analysis (Total Lab, Phoretix, NEI 3JA UK). Values are given as relative units (RU).

ELISAs

Protein levels were evaluated in the culture media of different neuronal and astrocyte cultures. The medium of each culture was collected in a sterile cryotube and stored at – 80°C until assay. The culture media were thawed, centrifuged briefly, and processed with a rat IL-6 ELISA kit (ER2-IL-6; Endogen Inc., Woburn, MA, USA) and a rat IL-1β kit (ER2-IL-1β; Endogen Inc., Woburn, MA, USA) according to the manufacturer's protocol. The sensitivity of this assay was 16 pg/ml and each sample was analysed in duplicate.

Statistics

Data are presented as means ± standard errors (SEM). One-way ANOVA was performed to determine the significant differences in expression under different conditions. Scheffè's *post hoc* test was used for the statistical analysis of group differences. Wilcoxon's test was used for paired samples. All data were analysed with SPSS software. Statistical significance was assumed at $p < 0.05$.

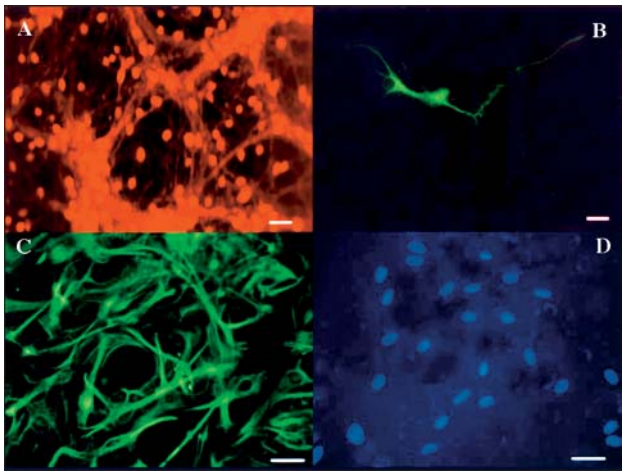

Figure 1
Immunostaining of neuronal and glial cultures. Neuronal cultures double-marked with NF and GFAP antibodies. A and B show the same microscopic field with different UV filters. Scale bar: 100 µm. C, immunofluorescence detection of GFAP in glial cultures. D, the same microscopic field of C, stained with Hoechst nuclear fluorochrome. Scale bar: 50 µm.

RESULTS

Culture characterisation and cell mortality evaluation.

The purity of neuronal and glial cultures was assessed by immunocytochemistry using specific anti-neurofilament (NF) and anti-glial fibrillary acidic protein (GFAP) antibodies. In pure hippocampal cultures, more than 95% of cells were immunopositive for NF, the remaining small percentage was marked with GFAP [6-7] (Figure 1A, B). Close to 98% of cells in the glial cultures were polygonal GFAP-positive (Figure 1C): in our hands no cells were NF immunopositive but we do not exclude that the remaining percentage (about 2%) could have been microglia. As previously demonstrated, hypoxic stress was applied long enough to elicit the biochemical response but was below the mortality threshold. In fact, the percentage of dead neuronal cells under subliminal hypoxia did not differ significantly from the percentage of dead cells under normoxic conditions [7]. No mortality in astroglial cultures was observed after six hours of hypoxia.

Protein expression and release

The expression and release of IL-1β protein were measured in 10 normal, hypoxic, or reoxygenated, cultured neuronal and astroglial cell cultures. IL-1β mRNA was significantly increased in pure neurones and astrocytes after mild hypoxia (Figure 2A). This increase was followed by a consequent release in protein, as demonstrated by ELISA (Figure 2B). In contrast, the level of IL-6 displayed a different and characteristic time course in neuronal cultures (N = 12). In fact, in untreated cultures, both the expression and release of IL-6 decreased significantly after mild hypoxia and did not completely recover after three hours of reoxygenation, as shown in Figures 3A and 3B. Interestingly, this behaviour was reversed by pre-treatment with anti-IL-1β antibody. The effect of IL-1β neutralisation had already been observed in normoxic neuronal cells, as IL-6 decreased significantly in treated cultures (Figure 3A, B). Conversely, the immunoneutralization of IL-1β induced a significant increase in IL-6 of at least two-fold in hypoxic-pre-treated cultures as compared with hypoxic-untreated cultures. This activatory effect persisted in the recovery state when compared with untreated cultures, even though data from gene expression, indicated a return towards the level for treated normoxic cells (Figures 3A, 3B).

Under physiological conditions, the activation of IL-6 by the IL-1β signal is evident from the direct correlation between the levels of transcripts of these cytokines

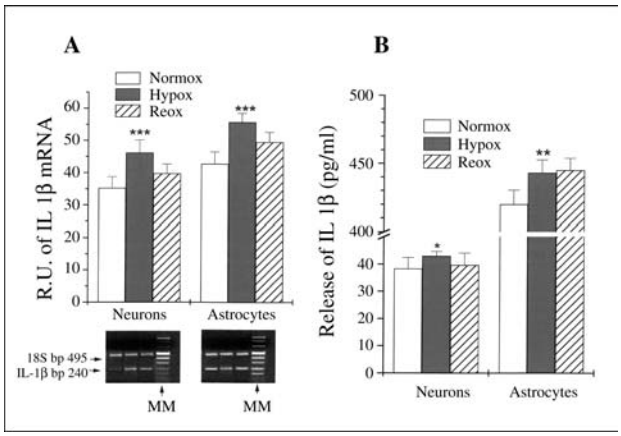

Figure 2 A
Upper side: representative histogram of IL-1β mRNA densitometric analysis in neuronal and astrocyte cultures measured under each set of conditions; N = 10 for both kinds of cell cultures. *** means $p < 0.005$ of hypoxic cultures *versus* normoxic and reoxygenation conditions (Wilcoxon's test for paired data); A, lower side: an example of multiplex RT-PCR for IL-1β mRNA and the 18S rRNA internal standard respectively, for neuronal and glial cultures. Molecular weight markers are indicated by "MM". B, IL-1β release measured by ELISA in culture medium under the same conditions. * $p = 0.05$ *versus* normoxic and $p = 0.02$ *versus* reoxygenated neuronal cultures. ** means $p < 0.05$ *versus* normoxic cultures of astrocytes (Wilcoxon's test).

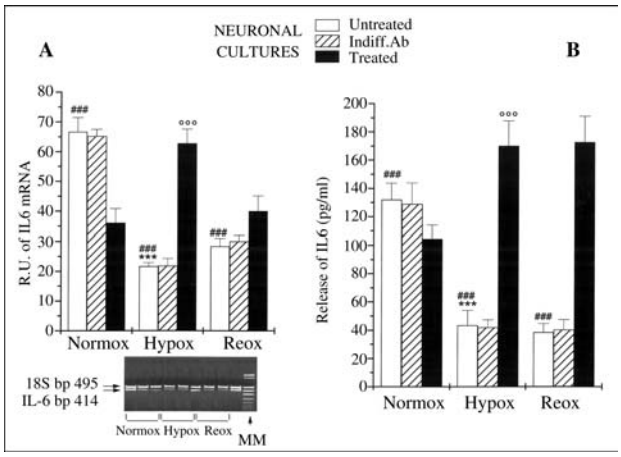

Figure 3
Neuronal cultures.
A, upper panel: graphical representation of data from densitometric analysis of IL-6 RT – PCR amplifications. N = 12. *** and °°° under hypoxic state means $p < 0.005$ *versus* normoxic and reoxygenated levels as determined by one-way ANOVA followed by *post-hoc,* multiple comparison analysis with Scheffe's test. For each condition, data from treated cultures were significantly different from untreated ($p < 0.005$, Wilcoxon's test, not shown). Lower panel: results of multiplex RT-PCR amplification showing one representative experiment for each conditions. "MM" means molecular weight marker, B: Protein release, measured in the media of the same hippocampal neuronal cultures, parallels transcript expression. Significant differences (*** and °°° = $p < 0.005$) were evident after exposure to mild hypoxia (ANOVA followed by Sheffe's test), and between treated and untreated neuronal cultures for each condition (### = $p < 0.005$, Wilcoxon's test).

(Figure 4A). The significant correlation also persists at the protein level, as demonstrated by ELISA (Figure 4D). In contrast, a negative correlation between the levels of IL-1β and IL-6 mRNAs was demonstrated when neuronal cultures were exposed to hypoxia (Figure 4B), and also between the release of IL-1β and IL-6 proteins subsequent to gene expression (Figure 4E). After three hours of reoxygenation, the direct correlation had been restored and was significant for both transcript expression and release (Figure 4C, F).

The influence of IL-1β neutralisation on the expression of IL-6 and its release from astrocytes was also studied. An increasing trend in IL-6 mRNA expression, from normoxic to recovery conditions, was detected in both pre-treated and untreated glial cultures. Significant decreases in IL-6 mRNA were measured in treated cells relative to untreated cells under normoxic and hypoxic conditions (Figure 5A). Protein release exhibited the same behaviour, except during reoxygenation where the effects of immunoneutralization were not evident as was also seen for protein mRNA (Figure 5A, 5B). Interestingly, as shown in Figure 5B, the IL-1β-deprivation signal inhibited IL-6 expression in normal and stressed glial cells, but activated IL-6 expression during recovery. Linear regression analysis demonstrated that the levels of IL-6 and IL-1β in untreated glial cultures, even if there was a positive linear trend, did not significantly correlate with each other, under either normoxic, hypoxic or reoxygenation conditions (data not shown). Application of the control antibody to both neurones or glia did not significantly affect either the expression of IL-6 mRNA or the release of IL-6 protein.

DISCUSSION

In this study, we have demonstrated, for the first time, a phenotype-related response to insult, insofar as the mechanisms by which neurones and glia react to injury are quite different, at least in terms of IL-1β and IL-6 expression. Our results can be summarised in three main statements. Firstly, neurones promptly react to mild injury; one symptom of this phenomenon is a change in the regulation of cytokine expression. Secondly, IL-6 expression is inhibited in neurones by IL-1β immunoneutralization under physiological conditions. In contrast, the same treatment provokes a significant increase in IL-6 when a pathological stimulus is applied. Thirdly, the anti-IL-1β neutralising monoclonal antibodies inhibit IL-6 expression by both normal and stressed astrocytes. Subliminal hypoxic stress was used as it mimics both a state of "ischemic penumbra", found in neural cells far from the "core" and involved in delayed neurodegeneration, and a phenomenon known as "ischemic tolerance", that induces neuronal resistance to otherwise lethal ischemia. This device allowed us to evaluate the potential mechanism involved in the cells' response to insult, and to investigate the harmful or protective processes initiated by mild injury. Hippocampal neurones, mainly in CA1 zone, are most susceptible to injury following ischemia/hypoxia, and a reduction of protein synthesis that correlates with selective vulnerability has been demonstrated [18]. On the other hand, the ischemic tolerance of CA1-selectively vulnerable neurones, is associated with early recovery of protein synthesis [19]. In a previous work [7], we reported a decrease in TNF-α and NGF in neurones; here, we demonstrate the decrease in IL-6 under the same conditions, but contemporarily we observed an increase in IL-1β. The supposition of a role for IL-1β in fine-tuning the regulation of IL-6 is supported by the fact that the correlation between the expression of these two proteins changes when conditions change. In fact, as we have shown here and previously, the relationship exists not only in the presence of injury but also under physiological conditions. This regulating signal might influence the expression of IL-6 directly at the transcriptional and translational levels, because protein release was observed immediately after gene expression. IL-1β fulfils opposing functions according to the prevailing biological conditions, particularly in neurones. The reaction of neuronal cells to subliminal stress is immediate and quite different, insofar as IL-1β mRNA increases with a consequent release of protein and IL-6 mRNA decreases with a parallel decrease in protein. These results are surprising, especially the amplitude and speed of the responses (Figures 2, 3, 5), suggesting that, of the resident brain cells, neurones are the first to react to insult. Obviously, this feature is a double-edged sword because fast reactivity can make neurones more vulnerable. The complementary roles of glia and neurones are also highlighted by the data presented here. We demonstrate, for the first time, an alternative way to respond to injury. Interleukin-1β exhibits a different influence in astrocytes, because immunoneutralization of IL-1β in glial cultures always induced a decrease in IL-6, under both physiological and pathological conditions. This is an intriguing phenomenon, in view of our understanding of the protective and harmful mechanisms initiated by mild

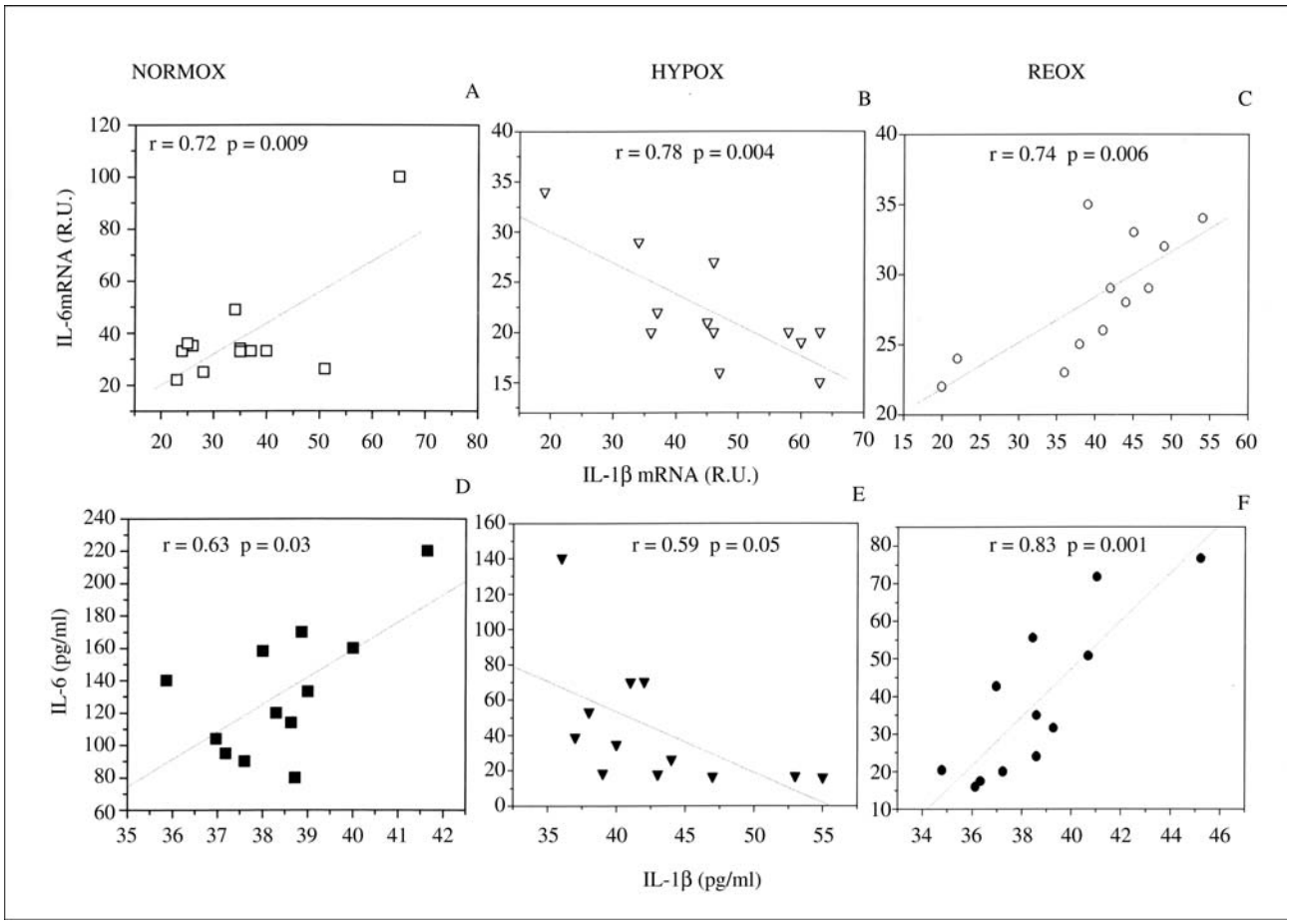

Figure 4

IL-6 expression as a function of IL-1β expression in untreated hippocampal neurones. A, B and C: linear regression analysis of IL-6 and IL-1β mRNAs under normoxic conditions, after mild hypoxia and after three hours of reoxygenation respectively. D, E, and F: linear regression analysis of IL-6 and IL-1β release measured in the culture media of the same cultures, parallels transcript expression. Each point represents a single experiment. The lines are computer-generated least-squares linear regression plots (Pearson's correlation).

injury. The influence of IL-1β on IL-6 can be compared with a similar regulatory role previously reported for NGF [7]. The synergistic action of IL-6 and NGF as growth factors [20], the increased vulnerability of normoxic neurones deprived of the IL-1β signal, and the inversion of this effect during hypoxic stress may suggest a pivotal role for IL-1β in the CNS [7]. From our data, the increase of IL-1β in both neuronal and glial cultures was significant, in spite the fact that it was rather small. Moreover, even if we can not exclude that α form of IL-1 could have influenced the observed responses, linear regression analysis, particularly from neuronal cultures, demonstrated a tight relationship between IL-1β and IL-6.

The neuronal source of cytokines such as IL-1β [21-23] and IL-6 [24], which are mainly expressed by microglia and astrocytes, indicates that neurones are capable of participating in the CNS cytokine network more directly than previously thought, and that they may play an important role in the response to injury. IL-1β itself does not seem to be directly involved in toxic activity in normal neurones [4,6], but can enhance hypoxic brain injury through its interaction with other molecules released or induced by damage. We infer that the context of the mediators, at any given time after brain injury, may well determine whether the effects of IL-1β are protective or toxic. Our finding that mild hypoxic injury without subsequent neuronal cell death is sufficient to alter the expression of inflammatory cytokines in the brain has fundamental implications for our understanding of CNS immunoregulation. The results produced in an *in vitro* system can contribute to our understanding of the basic mechanisms involved in the preservation of homeostasis or in damage, although they inevitably lack a complete biological *in vivo* context. Nevertheless, both neuronal and glial cells in culture retain all their physiological characteristics and perform biochemical reactions. Therefore, by separately testing the main kinds of nervous system cells, we hoped to achieve a better understanding of their specific capacities, with particular regard to cytokine regulation. To this end, a simplified model should be very valuable in analysing the basic relationships and interactions among these factors in specific neural phenotypes. An enhanced understanding of cytokine interactions and signalling pathways may define a potentially therapeutic role for combinatorial protective factors in the treatment of neurotoxicity and oxidative stress.

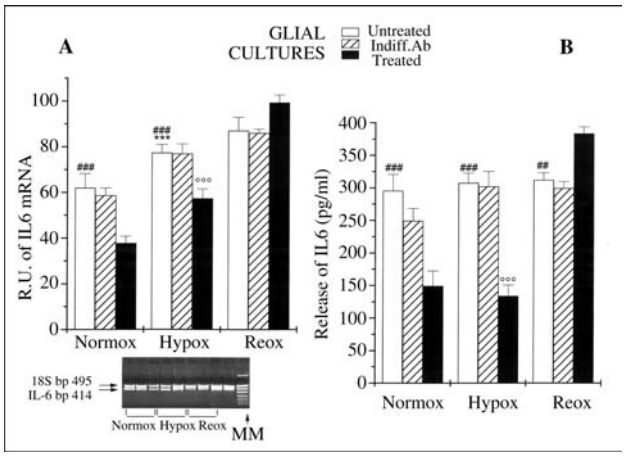

Figure 5

Comparative histogram showing the effects of IL-1β immunoneutralization on IL-6 levels in cultured astrocytes. A, upper side: the densitometric analysis of mRNA amplifications showed a significant difference between IL-1β-immunoneutralised and untreated cultures of astrocytes during normal condition and after mild hypoxia (### means $p < 0.005$, Wilcoxon's test). After recovery, no significant differences were observed between treated and untreated cultures. *** means $p < 0.005$ under hypoxic-untreated cultures *versus* normoxic- and reoxygenated-untreated cultures (ANOVA followed by Sheffe's test). The hypoxic immunoneutralised cultures of astrocytes showed significant differences when compared with normoxic and reoxygenated cultures (°°°$p < 0.005$, ANOVA followed by Sheffe's test) N = 12 for each condition. A, lower side: RT – PCR products amplification. One representative experiment showing each condition. "MM" indicates molecular weight marker. B: shows protein histogram of IL-6 protein measured by ELISA. In all conditions, significant differences were observed between treated and untreated cultures (## means $p < 0.05$, ### means $p < 0.005$, Paired data were analysed by Wilcoxon's rank test. °°° $p < 0.005$ by ANOVA, followed by Sheffe's test.

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