Pergamon

Progress in Neurobiology Vol. 54, pp. 99 to 125, 1998

Printed in Great Britain
0301-0082/98/$19.00

PII: S0301-0082(97)00052-X

MICROGLIA AS EFFECTOR CELLS IN BRAIN DAMAGE AND REPAIR: FOCUS ON PROSTANOIDS AND NITRIC OXIDE

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(*Received* 16 *June* 1997)

Abstract—Microglial cells are believed to play an active role in brain inflammatory, immune and degenerative processes. Depending on the magnitude of microglial reaction, on the type of stimulus and on the concurrence of other local factors, microglia can contribute to host defence and repair, or to the establishment and maintenance of brain damage. Many of the effects of microglial cells can be ascribed to the numerous substances that these cells can synthesize and release in response to a variety of stimuli (cytokines, pro-inflammatory substances, neurotransmitters, toxins, etc.).

The present article deals with two classes of compounds that activated microglial cells can produce in large amounts: prostanoids (that derive from arachidonic acid through the cyclooxygenase pathway), and nitric oxide (that is synthesized from arginine by nitric oxide synthase).

Prostanoids and nitric oxide have a number of common targets, on which they may exert similar or opposite actions, and have a crucial role in the regulation of inflammation, immune responses and cell viability. Their synthesis can massively increase when the inducible isoforms of cyclooxygenase and nitric oxide synthase are expressed.

The metabolic pathways of prostanoids and nitric oxide are finely tuned by the respective end-products, by cyclic AMP and by a number of exogenous factors, such as cytokines, glucocorticoids, lipocortin-1 and others. Some of these factors (e.g. transforming growth factor-β1, interleukin-10, lipocortin-1) may be secreted by microglial cells themselves, and act in an autocrine-paracrine way.

In view of the neuroprotective role attributed to some prostaglandins and to the cytotoxicity of excessive levels of nitric oxide or its derivatives, the balance between prostanoid and nitric oxide levels may be crucial for orienting microglial reactions towards neuroprotection or neurotoxicity.

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ABBREVIATIONS

AA	arachidonic acid
AP-1	activator protein-1
ASA	acetyl salicylic acid
cAMP	cyclic adenosine monophosphate
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
COX	cyclooxygenase
CREB	cAMP responding element binding protein
EAE	experimental allergic encephalomyelitis
eNOS	endothelial nitric oxide synthase
EP	prostaglandin E receptor
FP	prostaglandin F receptor
GAP-43	growth-associated protein-43
IFN-γ	interferon-γ
IL	interleukin
INDO	indomethacin
iNOS	inducible nitric oxide synthase
LC-1	lipocortin 1
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide
MS	multiple sclerosis
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
PG	prostaglandin
PLA_2	phospholipase A_2
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso-N-acetyl-DL-penicillamine
TGF-β1	transforming growth factor-β1
TNF-α	tumour necrosis factor-α
TX	thromboxane

1. INTRODUCTION

Microglial cells, despite a long and protracted debate on their developmental origin (Ling and Wong, 1993), are generally considered the resident macrophages of the CNS. Evidence from immunocytochemical studies using macrophage-specific markers have shown that monocytes enter the developing CNS and give rise to microglia (Perry, 1994). In the immature brain, the cells show a rounded and simple morphology and are often referred to as ameboid microglia. These cells are thought to be involved in phagocytosis and removal of degenerating cells during CNS development. During the first postnatal weeks, the ameboid morphology of microglia undergoes a progressive change until the cells acquire long, fine, branched processes. These ''ramified'' or resting microglia, found in normal adult brain, show a down-regulated immunophenotype adapted to the specialized microenvironment of the CNS, but become rapidly activated in response to pathological events. Activated microglia express, in a graded fashion, antigenic and functional properties comparable to those of peripheral macrophages and tend to become again ameboid, phagocytic and motile (Perry, 1994; Kreutzberg, 1996; Moore and Thanos, 1996).

Although microglia represents a substantial fraction of all glial cells (10–20%, Perry, 1994), their role in the normal adult brain remains largely unknown. Undoubtedly, however, they are important effector cells in most pathological conditions. Due to their reactivity to a wide range of stimuli, they play a crucial role in host defence and facilitate neuroprotection and repair processes. On the other hand, the type or intensity of the noxious stimulus and/or the concurrence of other local factors may make them instrumental for the establishment or amplification of tissue damage (Banati and Graeber, 1994; Mallat and Chamak, 1994). It is therefore important to understand what are the conditions regulating the balance between neuroprotective and neurotoxic activities of these cells.

A common approach to study microglial functional properties is the isolation and culture of purified populations (>99%) of microglia from neonatal rodent brains (Giulian and Baker, 1986; Levi *et al*., 1993) or from fetal human brains (Lee *et al*., 1993b). The cells in these cultures show at least some of the morphological and antigenic features of ameboid cells, suggesting that they are partially activated. This may be due to their origin from the immature brain and to the manipulations required to obtain purified cultures. Although these models are very useful for analyzing several microglial properties, they may not be suitable for studying the functional features of resting microglia nor the initial events leading to cell activation. For this reason, methods for the bulk purification and culture of microglia from the adult rodent or human brain have been developed (Williams *et al*., 1992; Becher and Antel, 1996; Slepko and Levi, 1996). Bulk-isolated adult microglia exhibits a phenotype similar to that of resting, ramified microglia *in situ*. After a short period in culture, however, the cells progressively express a set of markers and functional features characteristic of activated microglia *in vivo* and of neonatal microglia *in vitro* or *in vivo*, even in the absence of added inducing stimuli.

Microglia has been studied also *in situ*, both in the normal brain and during disease in humans and experimental animals. The *in situ* studies have provided invaluable information concerning the progressive antigenic changes that these cells undergo in the various phases of different types of diseases. They also clarified some functional features related to microglia activation such as proliferation, phagocytosis and expression of inflammatory cytokines (Moore and Thanos, 1996 and references therein). The complexity of the *in vivo* system, however, is not suitable for approaching the analysis of several aspects of microglial functions at the cellular and molecular level.

A striking feature of microglial reactivity is the ability to synthesize and secrete a large number of substances, which, alone or in concert with factors derived from other brain or hematogenous cells, may have a crucial role in host defence or in the establishment or maintenance of brain damage. A list of secretory microglial products involved in in-

Table 1. Some products of activated microglia

	Species	References
Growth factors:		
Nerve growth factor	rodent	Mallat *et al*., 1989
Neurotrophin 3	rodent	Elkabes *et al*., 1996
Basic fibroblast growth factor	rodent, human	Presta *et al*., 1995; Shimojo *et al*., 1991
Transforming growth factor α	human	Walker *et al*., 1995
Transforming growth factor β	rodent, human	Walker *et al*., 1995
Cytokines:		
Interleukin-1α	human	Walker *et al*., 1995
Interleukin-1β	rodent, human	Hetier *et al*., 1991; Walker *et al*., 1995
Interleukin-3	human	Walker *et al*., 1995
Interleukin-5	rodent	Sawada *et al*., 1993a
Interleukin-6	rodent, human	Frei *et al*., 1989; Gottschall *et al*., 1995
Interleukin-10	rodent, human	Mizuno *et al*., 1994; Sheng *et al*., 1995
Interleukin-12	rodent	Aloisi *et al*., 1997; Lodge and Sriram, 1996
Tumor necrosis factor α	rodent, human	Chao *et al*., 1995b; Hetier *et al*., 1991
Coagulation factors:		
Urokinase type plasminogen activator	rodent	Nakajima *et al*., 1992a
Plasminogen	rodent	Nakajima *et al*., 1992b
Complement factors:		
C1, C3, C4	human	Walker *et al*., 1995
Lipid mediators:		
Arachidonic acid	rodent	Minghetti and Levi, 1995
Platelet activating factor	human	Jaranowska *et al*., 1995
Prostaglandin D_2, E_2, $F_{2\alpha}$	rodent, human	see Section 2.5
Thromboxane B_2	rodent	see Section 2.5
Leukotriene B_4	rodent	Matsuo *et al*., 1995
Free radicals:		
Superoxide anions	rodent, human	Colton *et al*., 1996; Chao *et al*., 1995a
Nitric oxide	rodent, human	see Section 3.5
Neurotoxins:		
Excitotoxin	rodent	Giulian *et al*., 1995
β-amyloid	rodent	Mönning *et al*., 1995
Quimolimic acid	human	Heyes *et al*., 1996
Enzymes:		
Elastase	rodent	Nakajima *et al*., 1993
Gelatinase	rodent	Gottschall *et al*., 1995
Extracellular matrix components:		
Thrombospondin	rodent	Chamak *et al*., 1994

flammatory and/or repair processes is reported in Table 1. The list is rapidly expanding and includes growth factors, cytokines, coagulation and complement factors, lipid mediators, extracellular matrix components, enzymes, free radicals and neurotoxins. Nonetheless, the circumstances under which each of them is produced and the factors regulating their synthesis and release are still largely unknown.

In the last few years, a number of comprehensive review articles (Banati and Graeber, 1994; Mallat and Chamak, 1994; Perry *et al*., 1994; McGeer and McGeer, 1995; Kreutzberg, 1996; Moore and Thanos, 1996) have discussed the role of these cells in brain inflammation, degeneration and ischemia. In the present article, we will restrict our discussion to two classes of compounds, prostanoids and nitric oxide (NO), which may be critical factors in determining the final outcome of microglial reaction to pathological stimuli.

Prostanoids, the arachidonic acid (AA) metabolites of the cyclooxygenase (COX) pathway, and NO, whose formation is catalyzed by nitric oxide synthase (NOS), are potent local mediators and play major roles in regulating inflammation, immune functions, vascular tone and neurotransmission (Appleton *et al*., 1996). While low levels of these molecules may participate to protective responses leading to enhanced disease resistance, their excessive production may be a cause of cytotoxicity.

Interactions between prostanoids and NO are of particular interest at inflammation sites, where their synthesis is often elicited in inflammatory cells by the same stimuli, and where they may have synergistic as well as antagonistic actions on common targets. To date, most of the information available on these compounds was obtained from *in vitro* or *in vivo* models of acute and chronic inflammation of peripheral organs. In spite of the close relationship between microglia and peripheral macrophages, however, it may not be appropriate to extend to the CNS the information acquired with peripheral cells. Indeed, inflammatory and immune responses in the CNS are peculiar (Perry and Gordon, 1991), probably due to the adaptation of the macrophagic phenotype of microglia to the unique cerebral environment.

In the next sections, we shall first provide a schematic description of prostanoid and NO metabolic pathways. Then we shall illustrate their regulation and reciprocal interactions in microglial cells, underlining the similarities and differences with peripheral macrophages.

2. PROSTANOIDS

2.1. Biosynthesis of Prostanoids and Other Arachidonic Acid Metabolites

The first event in the prostanoid cascade is the liberation of AA (5-8-11-14-eicosatetraenoic acid), a polyunsaturated 20-carbon fatty acid, from membrane glycerophospholipids by the enzyme phospholipase. Several forms of phospholipases, acting on different substrates, can induce AA release. However, in most inflammatory cells, where the bulk of AA resides in the sn-2 position of phospholipids, phospholipase A_2 (PLA_2) seems to have a prominent role. Two major subtypes of PLA_2 have been identified on the basis of their very different structural and biochemical properties (Glaser *et al.*, 1993). The secretory and low molecular mass (14–18 kDa) forms ($sPLA_2$) are mainly located in extracellular fluids, require millimolar concentrations of Ca^{2+} for optimal activity and do not show substrate specificity. The cellular and high molecular mass (31–110 kDa) forms ($cPLA_2$) were originally isolated from platelets and from the cytosol of the human monocytic cell line U937. They are activated bysubmicromolar concentrations of Ca^{2+} and have no homology with the known $sPLA_2$. At variance with $sPLA_2$, $cPLA_2$ has a marked preference for AA-containing phospholipids and appears to play a key role in the initiation of the AA cascade (Lin *et al.*, 1993; Piomelli, 1993). $cPLA_2$ is regulated by receptor-mediated events and is likely to function both in physiological and in pathological conditions. In fact, AA and its metabolites are implicated in normal CNS functions as well as in inflammation and degenerative processes. Pathological stimuli may determine an enhanced and prolonged activity of the enzyme and thus sustain the generation of high levels of AA metabolites and other potent lipid mediators, such as lysophospholipids and platelet activating factor. Interestingly, enhanced $cPLA_2$ immunoreactivity was reported in subpopulations of reactive astrocytes and microglia following global ischemia-reperfusion (Clemens *et al.*, 1996).

The enzyme COX rapidly converts the AA released to the biologically active prostaglandins (PGs) and thromboxanes (TXs), collectively termed prostanoids. Free AA can also take alternative routes: that of the 5-, 12- and 15-lipoxygenase pathways, which leads to the formation of leukotrienes, hydroxyeicosatetranoic acids and lipoxins, or that of the P450 epoxygenase pathway, which gives rise to dihydroxyicosatrienoic acids and epoxyeicosatrienoic acids (Fig. 1). The latter two metabolic pathways have been extensively covered elsewhere (Needleman *et al.*, 1986; Katsuki and Okuda, 1995) and will not be further discussed here. Furthermore, free AA can be re-incorporated into phospholipids by highly regulated mechanisms, which further contribute to the tight control of cellular levels of this important compound (reviewed by Chilton *et al.*, 1996).

2.2. Cyclooxygenase

COX (E.C. 1.14.99.1), also known as prostaglandin (PG) H synthase or prostaglandin endoperoxide

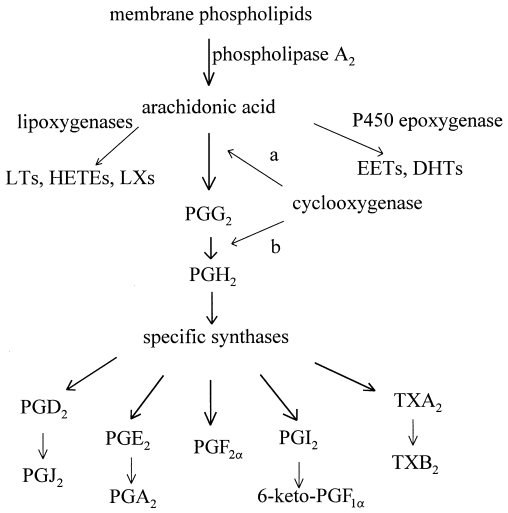

Fig. 1. A schematic diagram of arachidonic acid (AA) cascade. AA released from membrane phospholipids is rapidly converted into biologically active metabolites through several enzymatic pathways: 1) the 5-, 12-, and 15-lipoxygenase pathways give raise to leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins (LXs), respectively; 2) the P450 epoxygenase pathway leads to the production of epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHTs) and 3) the cyclooxygenase (COX) pathway gives rise to prostanoids. COX converts AA into an unstable endoperoxyde (PGG_2), by a cyclooxygenase reaction (a). PGG_2 is then reduced to PGH_2 by a peroxidase reaction (b). PGH_2 is converted into prostaglandin (PGs) and thromboxane (TX) by specific synthases. PGI_2 and TXA_2 rapidly degrade in biologically inactive compounds (6-keto-$PGF_{1\alpha}$ and TXB_2), while PGD_2 and PGE_2 can be converted non enzymatically into PGJ_2 and PGA_2, respectively (for further details, see Smith *et al.*, 1993).

synthase, exhibits both a bis-oxygenase activity (cyclooxygenase), which catalyzes PGG_2 formation from AA, and a peroxidase activity, which reduces PGG_2 to PGH_2, the final substrate for the specific synthases (Fig. 1). The two enzymatic activities occur at distinct, although interacting sites on the COX molecule and external factors can affect each of them independently. For example, aspirin (acetyl salicylic acid, ASA) and other widely used non-steroidal anti-inflammatory drugs (NSAIDs) abrogate prostanoid formation by inhibiting the COX activity, but do not influence the peroxidase activity of the enzyme (Smith *et al.*, 1993). The COX catalysis gives rise to an unstable protein intermediate, which has some probability of rearranging into inactive enzymatic species. This process, known as "suicide" inactivation, occurs on average once every 1300 turnovers (Marshall *et al.*, 1987). The "suicide" inactivation has been observed both *in vitro* and *in vivo*.

In addition to the availability of free AA, COX activity is an important limiting factor in prostanoid biosynthesis. So far little information is available on the mechanisms of activation of COX; in general, the enzymatic activity appears to be largely related to the COX protein concentration. The physiological "suicide" inactivation of the enzyme is consistent

with the fact that a continuous *de novo* synthesis of COX is required for the generation of prostanoids.

COX is an integral membrane glycoprotein, consisting of an homodimer of 72 kDa molecular mass subunits and having an associated heme group involved in both enzymatic activities (Smith *et al.*, 1993). Besides the constitutive isoform (COX-1), which is widely distributed in a variety of cell types and is thought to provide physiological levels of prostanoids, a second and inducible isoform, termed COX-2, has been recently identified. This isoform is rapidly expressed in several cell types upon activation by growth factors, cytokines and pro-inflammatory molecules. COX-2 is reputed to be responsible for the production of the high levels of prostanoids present in pathological conditions, such as acute or chronic inflammation, and is the major isoform expressed in inflammatory cells (Goppelt-Struebe, 1995; Appleton *et al.*, 1996; Herschman, 1996), including microglia (Minghetti and Levi, 1995).

The two isoforms are coded by two distinct single copy genes, which reside on different chromosomes. The COX-1 gene is on chromosome 2 and 9 in the murine and human system, respectively, and the COX-2 gene on chromosome 1 in both systems. The lengths of the two genes are quite different, being >22 kb for COX-1 and 8–9 kb for COX-2. However, the intron-exon structure and the hydropathy profile are very similar in both genes, suggesting that they may derive from a duplication of an ancestral gene. The most striking difference between the two genes is the presence of a large number of Shaw–Kamen sequences ATTTA in the untranslated region of the COX-2 sequence: these motifs occur in many immediate-early genes and confer enhanced mRNA instability. On the contrary, the sequence of the COX-1 gene is consistent with its classification as a "house-keeping" gene. The structure of the two genes and the regulation of their expression have been reviewed recently (Goppelt-Struebe, 1995; Herschman, 1996).

At the protein level, the two isoforms show over 60% homology in humans and rodents. While the functional sites are conserved, a few crucial substitutions cause some important conformational variations in the active site pocket of the two isoenzymes, which could account for the different sensitivities of COX-1 and COX-2 to specific inhibitors (Kurumbail *et al.*, 1996). One important difference between the two isoforms is the 18-amino acid insert near the COX-2 C-terminus, which is not present in COX-1, and has allowed the production of specific antibodies.

2.3. Cyclooxygenase-2 Induction

As mentioned earlier, a variety of agonists, including growth factors, cytokines, phorbol esters, bacterial endotoxin and platelet activating factor, can elicit COX-2 expression (Appleton *et al.*, 1996; Herschman, 1996). Many of these factors interact with serpentine receptors and tyrosine-receptors, which operate through a number of distinct signal transduction systems, including protein kinase C, protein kinase A or JAK/STAT kinases.

All these signalling pathways are likely to converge on the activation of transcription factors, which in turn, individually or in combination, can induce or enhance COX-2 gene expression. Indeed, the rather high instability of COX-2 mRNA, caused by the multiple AUUUA sequences in the 3′-untranslated region (Goppelt-Struebe, 1995), and the "suicide" inactivation of COX-2 during catalysis (Marshall *et al.*, 1987), render the control of the transcription of the COX-2 gene a crucial event in prostanoid biosynthesis. Several binding sites for transcription factors have been identified at the 5′-untranslated region, some of which are conserved among different species. Consensus sequences of the nuclear factor for interleukin 6 (NF-IL6) site and of cyclic AMP responding element (CRE) are present in the human (Kosaka *et al.*, 1994), and mouse (Fletcher *et al.*, 1992) COX-2 gene, while consensus sequences of the nuclear factor-kB (NF-kB) site are present in the human (Kosaka *et al.*, 1994) and rat (Bauer *et al.*, 1997) systems.

2.4. Cyclooxygenase-2 Expression in the CNS

The distribution of the two COX isoforms has been extensively studied in rat and human tissues (Herschman, 1996). In the majority of the tissues COX-1 appears to be the only isoform constitutively expressed, while in brain, testes and kidney macula densa cells both COX-1 and COX-2 are expressed under physiological conditions (Herschman, 1996). In rat and ovine brain, COX-1 and COX-2 immunoreactivities are present in discrete neuronal populations distributed in distinct areas of cerebral cortex and hippocampus. In other regions, such as midbrain, pons and medulla COX-1 immunoreactivity prevails (Breder *et al.*, 1995). The enriched expression of COX-2 in restricted subpopulations of excitatory neurons in cortex and hippocampus and its localization in dendritic processes are suggestive of a role of COX-2 in postsynaptic signalling of these neurons (Yamagata *et al.*, 1993; Breder *et al.*, 1995). Neuronal COX-2 expression appears to be regulated by normal synaptic activity in the developing and adult brain and can rapidly and transiently increase during seizures and (over)activation of *N*-methyl-D-aspartate (NMDA) receptors (Yamagata *et al.*, 1993; Chen *et al.*, 1995; Adams *et al.*, 1996; Marcheselli and Bazan, 1996). Moreover, the developmental profile of COX-2 expression in rat brain correlates with the critical period of activity-dependent synaptic remodelling (Kaufmann *et al.*, 1996). Nonetheless, the significance of the constitutive expression of COX-2 in brain is still unclear.

In addition to neurons, other cells of the CNS can express COX in physiological or pathological conditions. By using a monoclonal antibody that did not discriminate between COX-1 and COX-2 proteins, Tsubokura *et al.* (1991) found COX immunoreactivity in glial cells in normal monkey brain, although less intense than in neurones. More recently, Elmquist *et al.* (1997) observed the induction of COX-2 expression in perivascular microglia after intraperitoneal administration of lipopolysaccharide (LPS), while neuronal COX-2 remained unchanged. COX-2 expression and prostanoid pro-

duction can be certainly induced *in vitro* in both astrocytes and microglia, after challenging with appropriate stimuli (Minghetti and Levi, 1995; Fiebich *et al.*, 1996; O'Banion *et al.*, 1996; Bauer *et al.*, 1997). The glia-derived prostanoids could have specific functions in pathological conditions, as we shall describe in the following sections.

2.5. Microglia as a Cerebral Source of Prostanoids

Astrocytes have been considered for long time as the major source of prostanoids within the CNS. In primary cultures, these cells synthesize approximately 20 times more prostanoids than neurons, when stimulated with the calcium ionophore A23187 (Seregi *et al.*, 1984; Keller *et al.*, 1985). However, the establishment of highly purified glial cultures in the latest years has allowed to demonstrate that microglial cells synthesize even larger amounts of prostanoids upon activation.

In one of the first studies (Gebicke-Haerter *et al.*, 1989), the ability of microglial cells to release PGE_2 and PGD_2 upon stimulation with LPS and A23187 was found to be higher than that of astrocytes and lower than that of peripheral macrophages. In a later report Sawada *et al.* (1993b) reported that PGE_2 synthesis in microglial cell is preferentially elicited by LPS, while phorbol ester is a better stimulus in astrocytes. In agreement with these observations, we have shown that microglial cells are able to release higher levels of PGE_2, PGD_2 and TXB_2 than astrocytes (Fig. 2), after exposure to a 100-fold lower concentration of LPS (Minghetti and Levi, 1995). Moreover, Matsuo *et al.* (1995) detected AA metabolites in astrocyte supernatants after addition of A23187 only in the presence of contaminating microglial cells, suggesting that those AA metabolites originated mainly from microglia. This view was confirmed by a recent study (Giulian *et al.*, 1996), showing that activated microglia and not astrocytes can release TXB_2 and express TX-synthase protein. As observed in other cell types, phagocytic signals and LPS were potent stimulants

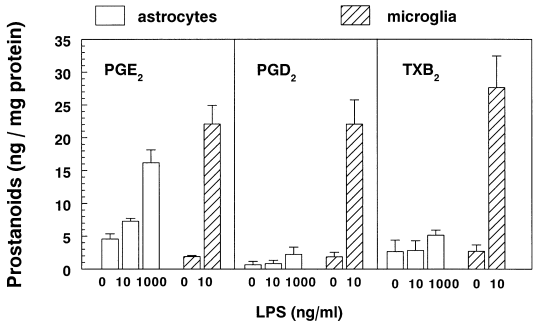

Fig. 2. Comparison between astroglial and microglial prostanoid production induced by LPS. Astrocytes and microglia derived from the same cell dissociation were subcultured for 24 h in 10% serum-containing medium, which was replaced with fresh medium before stimulation for further 24 h, in the presence of the indicated LPS concentrations. Cell-free supernatants were assayed for PGE_2, PGD_2 and TXB_2 content by using specific radioimmunoassays (Minghetti and Levi, 1995). Means $\pm$ S.E.M. of four independent experiments, run in duplicate.

of microglial TXB_2 release, while platelet activating factor and substance P were less effective. On the other hand, astrocytes are better responders than microglia to other types of stimuli. For example, a large production of PGE_2 can be elicited from astrocytes by interleukin-1β (IL-1β) (O'Banion *et al.*, 1996; Palma *et al.*, 1997), while this cytokine is scarcely if at all effective in microglia (Minghetti and Levi, 1995; Bauer *et al.*, 1997).

These observations suggest that both astrocytes and microglia can represent important sources of brain prostanoids and that the two cell populations respond to distinct and specific stimuli.

As to the molecular mechanisms involved in prostanoid synthesis in microglial cultures, we have shown that LPS induces prostanoid (PGE_2, PGD_2 and TXB_2) release by regulating both AA availability and COX-2 expression (Minghetti and Levi, 1995). Indeed, LPS significantly increased the release of ^{3}H-AA from pre-loaded microglial cells with a time lag of at least 1 h. Such a delay, together with the inhibitory effect of protein synthesis inhibitors, suggests that the effect of LPS on AA requires new protein synthesis. However, the signal transduction system evoked by LPS and the type of phospholipase involved remain to be identified.

As in the case of peripheral macrophages, COX-2 is the major isoform expressed by activated microglia. Indeed, the Western blot analysis of microglial proteins revealed a specific, dose-dependent induction of COX-2 by LPS, that was accompanied by an enhanced enzymatic activity (Fig. 3A). At the same time, the level of expression of COX-1 was very low or undetectable, both in control and LPS-stimulated cultures (Minghetti and Levi, 1995).

An increase in COX-2 mRNA levels in rat microglial cultures after exposure to adenosine, by a mechanism involving A_{2a} receptors and cyclic adenosine monophosphate (cAMP) formation, has been recently demonstrated (Fiebich *et al.*, 1996). As discussed in more detail later, cAMP appears to play a crucial role in COX-2 gene regulation.

Recently, Janabi *et al.* (1996) observed synthesis of $PGF_{2\alpha}$ and, to a lesser extent, of TXB_2 in human fetal microglia and astrocyte cultures after stimulation by a combination of the two pro-inflammatory cytokines IL-1β and interferon-γ (IFN-γ). As in the case of rodent cultures, the two cytokines were more effective in stimulating astrocytes than microglia. The kinetics and the protein synthesis-dependence of $PGF_{2\alpha}$ production suggest that the two cytokines acted by inducing *de novo* synthesis of COX-2. Since COX-2 mRNA or protein were not measured in this study, a further evaluation of these results is not possible.

The above mentioned observations agree in showing that COX-2 induction by appropriate stimuli results in an increased production of prostanoids by cultured neonatal microglia. We recently found that COX-2 is inducible also in adult microglial cells, where the level of COX-2 expression appears to have a major role in determining the ratio between the production of PGE_2 and that of TXA_2 (measured as TXB_2, a metabolically stable TXA_2 derivative) (Slepko and Levi, 1996; Slepko *et al.*, 1997). According to these studies (see Fig. 4), the

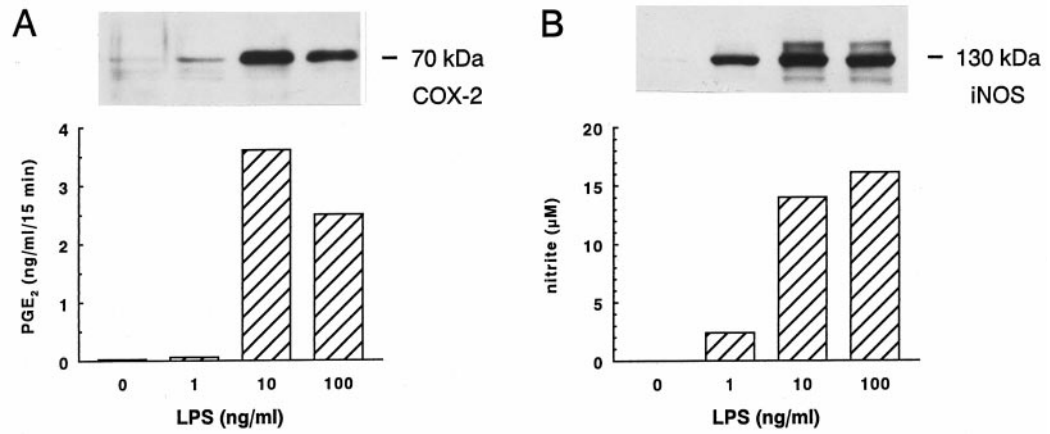

Fig. 3. Dose-dependence of COX-2 (A) and iNOS (B) induction by LPS in microglial cultures. Purified microglial cells were subcultured for 24 h in 10% serum-containing medium, which was replaced with fresh medium before stimulation. Cells were incubated for 24 h in the absence or in the presence of increasing concentrations of LPS. Microglial proteins were prepared and analyzed by Western blot, using anti-COX-2 and anti-iNOS specific antibodies. Protein bands were visualised by horseradish peroxidase-conjugated secondary antibodies and chemiluminescent reaction. The induced expression of the two enzymes correlated with their enzymatic activities (shown in the lower panels) which were measured as follows: (A) microglial cells were exposed to 10 μM AA for 15 min at the end of the stimulation period, to provide large availability of COX substrate, and PGE_2 production measured; (B) accumulation of nitrite, one of the end products of NO oxidation, into culture media was measured over the 24 h stimulation period by a modified procedure based on the Griess reaction (Minghetti *et al.*, 1996).

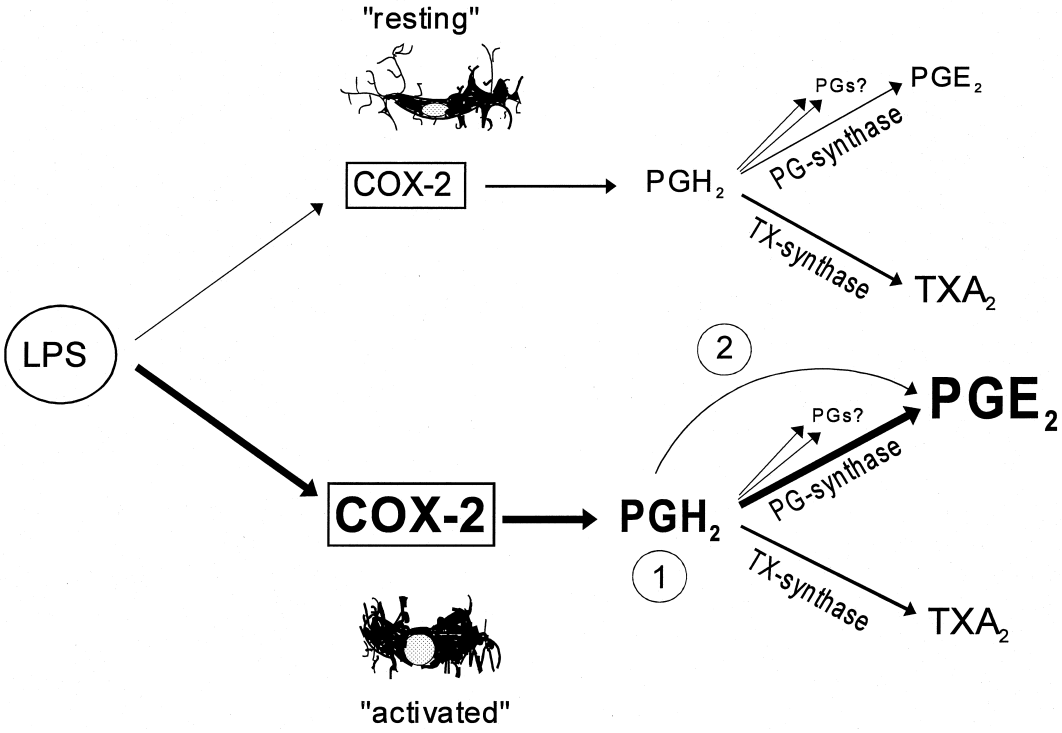

Fig. 4. Prostanoid production in adult microglial cultures at different stages of activation. COX-2 inducibility upon exposure to LPS appears to be related to the degree of activation occurring spontaneously with time in cultures. The greater LPS-induced expression of COX-2 is accompanied by an increased capacity to synthesize PGE_2, while the synthesis of another prostanoid, TXA_2, remains unchanged. The increased output of PGE_2 may be due to the higher availability of the substrate PGH_2 (1). PGH_2 may take preferentially the route of PGE synthase, which exhibits a higher Km for the substrate than TX synthase. A non enzymatic conversion of PGH_2 into PGE_2 is also possible (2).

Table 2. Prostanoid receptor classification

Receptor	Potency of prostanoids	Agonist*	Effector**	G protein
DP	$PGD_2 > > U\text{-}46619 > PGE_2 > PGI_2 > PGF_{2\alpha}$	BW245C	AC	Gs
EP1	$PGE_2 > PGI_2 > PGF_{2\alpha} > PGD_2$	17-phenyl-PGE_2	Ca^{2+} channel	unknown
EP2	$PGE_2 > > PGI_2 = PGF_{2\alpha} > PGD_2$	11-deoxy-16,16-dm-PGE_2	AC	Gs
EP3	$PGE_2 = PGE_1 > PGI_2 > PGF_{2\alpha} > PGD_2$	Sulprostone	AC	Gi
EP4	$PGE_2 = PGE_1 > > PGI_2 > PGF_{2\alpha} > PGD_2$	AH23848[a]	AC	Gs
FP	$PGF_{2\alpha} > PGF_1 > PGD_2 > U\text{-}46619 > PGE_2 > PGI_2$	Cloprostenol	PLC	Gq
IP	$PGI_2 > PGE_1 = PGD_2 > U\text{-}46619 > PGE_2 > PGF_{2\alpha}$	Cicaprost	AC, PLC	Gs, Gq
TP	$U\text{-}46619 > PGD_2 > PGF_{2\alpha} > PGE_2$	U-46619	PLC	Gq

*A single example of synthetic specific agonist is given; for a more extensive description see Coleman *et al.*, 1994.
**AC, adenylyl cyclase; PLC, phospholipase C.
[a]Antagonist.

level of COX-2 inducibility was related to the degree of microglial "activation" occurring spontaneously in culture. The greater LPS-induced expression of COX-2 observed in more "activated" cultures was accompanied by an increased capacity to synthesize PGE_2, but not TXA_2. The inability of TX-synthase to cope with the increased availability of the COX-2 product and prostanoid precursor PGH_2 may be at least partly responsible for the selective increase in PGE_2 production. In fact, TX-synthase activity was, if anything, decreased in the more activated cells (Slepko *et al.*, 1997; see also Nusing *et al.*, 1993 for observations on human monocytes). Moreover, in the presence of an excess of substrate, the enzymatic activity of PGE-synthase, which exhibits a higher Km when compared with TX-synthase, may be facilitated, with a consequent higher output of PGE_2. Whether the marked increase in LPS-stimulated PGE_2 production in more activated cells can be also related also to augmented PGE-synthase activity/expression (Matsumoto *et al.*, 1997) and to non enzymatic conversion of PGH_2 to PGE_2 (Smith *et al.*, 1993) remains to be established.

Altogether, the *in vitro* evidence indicates that the COX-2 pathway is elicited in microglia by pro-inflammatory stimuli in a similar fashion to what has been observed in peripheral macrophages, and suggests that microglia-derived prostanoids could play an important role in inflammatory and immune responses.

2.6. Prostanoid Receptors

Once synthesized, prostanoids are not stored within the cells but translocate to the extracellular space where they can exert their biological functions after interacting with specific membrane receptors. Prostanoids have been long thought to freely cross cell membranes by passive diffusion, without the need of any transport system. However, a prostaglandin transporter protein has been recently identified and characterized (Knai *et al.*, 1995), confirming the pioneering work of Bito and collaborators on the existence of a transport system for prostaglandins (Bito, 1975; Bito *et al.*, 1976).

Prostanoid membrane receptors can be classified into five basic types on the basis of sensitivity to the five naturally occurring prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. These receptors are termed P-receptors, with a preceding letter indicating the prostanoid to which the receptor is more sensitive (Kennedy *et al.*, 1982). In the case of PGE_2, four subtypes of prostaglandin E (EP) receptors (EP1, EP2, EP3 and EP4) have been characterized, on the basis of the response to distinct agonists and antagonists (Table 2).

Radioactive ligand binding studies revealed the existence of overlaps in receptor recognition, suggesting structural similarity of the receptors. Indeed, the amino acid sequences of P-receptors indicate that they all have seven hydrophobic putative transmembrane domains and that they all belong to the serpentine or rhodopsin-type receptor superfamily (Negishi *et al.*, 1995). P-receptors are coupled to different regulatory G-proteins, which can either activate or inhibit adenylyl cyclase activity, or stimulate phosphoinositide hydrolysis. The main characteristics of P-receptors are briefly summarized in Table 2.

Little is known about the regional and cellular distribution of P-receptors within the brain. Investigations over the past years, based on radioligand binding and autoradiographic techniques, have shown the occurrence of PGE_2, PGD_2 and $PGF_{2\alpha}$ binding sites in defined areas of the rat and monkey brain (Shimizu and Wolfe, 1990). More recent *in situ* hybridization studies revealed that EP3 mRNA is widely expressed in the mouse brain, where it appears to be mainly localized in neurons (Sugimoto *et al.*, 1994).

Most of the central effects of prostanoids have been attributed to their actions on neuronal receptors, but increasing evidence indicates that glial cells too are a potential target for these compounds. In fact, P-receptors have been detected in glial cultures. By using purified cultures of type-1 and type-2 astrocytes, Ito *et al.* (1992) have shown that type-1 astrocytes preferentially express PGF receptors (FP), whose activation leads to phosphoinositide hydrolysis and Ca^{2+} mobilization, whereas type-2 astrocytes express EP receptors, linked to cAMP generation. More recently Kitanaka *et al.* (1996) analyzed the presence of mRNAs for EP3, FP and thromboxane receptors in cultured rat astrocytes, oligodendrocytes and microglia. All three P-receptor mRNAs were detected in astrocyte and oligodendrocyte cultures, while microglial cells expressed EP3 and thromboxane receptors, but not FP mRNAs. Evidence for the presence of a putative EP2 receptor in microglial cells is provided by studies from our

group. We found that PGE_2 is able to induce an elevation of cAMP level in these cells (Patrizio *et al*., 1995; Patrizio *et al*., 1996), and that some biological effects of PGE_2 are mimicked by a selective EP2 receptor agonist (Minghetti *et al*., 1997a, Minghetti *et al*., 1997b). These observations suggest that different prostanoids can selectively regulate distinct cell types in the brain.

In addition to membrane receptors, nuclear receptors for the 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Forman *et al*., 1995; Kliewer *et al*., 1995) support the hypothesis that prostanoids could also function as physiological intracellular messengers, and directly regulate gene expression (see also Section 4.1.3. and Fig. 6).

2.7. Biological Functions of Prostanoids in the CNS

Although the presence of prostanoids in the CNS has been known for some time, the functions of these compounds remain largely unravelled. PGD_2, PGE_2 and $PGF_{2\alpha}$ have been identified in the brain of several mammalian species, including rodents and humans (Narumiya *et al*., 1982; Ogorochi *et al*., 1984). In normal conditions, cerebral prostanoid levels are very low, but they can dramatically increase in pathological conditions, including ischemia, seizures, injury and inflammation (Shimizu and Wolfe, 1990; Farooqui and Horrocks, 1991). Increased cerebral levels of PGE_2 have also been observed in multiple sclerosis (MS) (Fretland, 1992) and in human immunodeficiency virus-associated dementia (Froldi *et al*., 1992; Griffin *et al*., 1994).

The understanding of prostanoid functions is often complicated by the fact that, in some instances, individual prostanoids may exert opposite effects on common targets (Table 3). Prostanoids participate to the regulation of several neuronal functions, among which sleep–awake state (Hayaishi *et al*., 1993), body temperature (Rothwell, 1992), secretion of luteinizing hormone-releasing factor (Ojeda *et al*., 1982), activity of excitatory amino acids on Purkinje cell dendrites (Kimura *et al*., 1985), pain perception (Pitchford and Levine, 1991). These observations, together with the already reported neuronal expression of COX-2 mRNA and protein (see Section 2.4), suggest that neuron-derived prostanoids might be broadly involved in neuronal signalling.

On the other hand, prostanoids may also affect non-neuronal cell functions. In particular, PGE_2 has been shown to down-regulate important glial functions including cytokine secretion by astrocytes and microglia (Sawada *et al*., 1993b; Aloisi *et al*., 1997), expression of the co-stimulatory molecule B7-2 and NO production in activated microglial cells (Menèndez Iglesias *et al*., 1997; Minghetti *et al*., 1997a) and astrocyte proliferation (Sawada *et al*., 1993b). Many of these effects of PGE_2 seem to be mediated by increased cAMP levels, as discussed in Section 4.2.1.

2.8. Prostanoid Cascade, Neurodegeneration and Neuroprotection

The temporal correlation between increased levels of free AA and prostanoids and various neuropathological processes has led to the hypothesis that prostanoids contribute to neurodegeneration (Shimizu and Wolfe, 1990; Chen *et al*., 1995). The interpretation of such correlation, however, is made difficult by the fact that the activation of the prostanoid cascade is often accompanied by the generation of a broad range of other active molecules, including cytokines, platelet activating factor, AA itself and AA metabolites of other enzymatic pathways (see Section 2.1 and Fig. 1). For example, AA accumulation may contribute to cell damage by perturbing neuronal cell membranes, by affecting the activity of ion channels, by inhibiting glutamate uptake or by altering mitochondrial respiratory activities (see Katsuki and Okuda, 1995). In turn, leukotrienes could exacerbate tissue damage through their involvement in vasoconstriction and edema formation. Interestingly, lipoxygenase inhibitors and antioxidants, but not the COX inhibitor indomethacin, were able to attenuate AA toxicity towards primary neuronal cultures. Thus, the generation of oxygen free radicals through the lipoxygenase metabolism may be responsible for the AA-induced neuronal injury (Katsuki *et al*., 1995). On the other hand, the neurotoxicity of AA may well depend on its level, since low concentrations of AA had a neurotrophic effect and promoted neurite elongation (Okuda *et al*., 1994).

As in the case of leukotrienes, some prostanoids could cause neuronal damage by promoting vasoconstriction and platelet aggregation. However, the

Table 3. Prostanoid biological activities

Prostanoid[a]	Biological activity[b]
PGD_2	Growth inhibition, antiaggregation, smooth muscle constriction, hypothermia, sleep induction, narcolepsy, acetylcholine release, inhibition of prolactin, LH-RH and LH release, increase of serotonin turnover
PGE_2	Smooth muscle dilatation, Na^+ excretion, plasma exudation, inhibition of gastric secretion, hyperthermia, awake state, hyperalgia, stimulation of LH-RH release, catecholamine release, inhibition of neutrophyl O_2^- production, inhibition of T-cell mitogenesis and Th-1 cytokine secretion, cytoprotection
$PGF_{2\alpha}$	Bronchoconstriction, vasoconstriction, luteolysis, acetylcholine release
PGI_2	Vasodilation, antiaggregation, inhibition of gastric secretion, cytoprotection
TXA_2	Bronchoconstriction, vasoconstriction, aggregation

[a]The five major naturally occurring prostanoids are reported.

[b]An incomplete list of the main general biological activities is reported. Some of the activities can vary from species to species. (For more details see Needleman *et al*., 1986, Shimizu and Wolfe, 1990, Phipps *et al*., 1991).

final contribution of prostanoids to tissue damage or repair is likely to depend on the balance between different prostanoids affecting the vascular tone and platelet aggregation in opposite ways (see Table 3). A further indirect toxic effect could also be due to the production of oxygen free radicals by COX during prostanoid synthesis. In several, but not all cases, pharmacological manipulation of prostanoid synthesis either by COX or TX-synthase inhibitors led to an improvement of postischemic cerebral blood flow and to a reduction of neuronal death (Kochanek *et al*., 1988; Pettigrew *et al*., 1989; Nogawa *et al*., 1997). It has been postulated that the neuroprotective efficacy of prostanoid inhibition may depend on the severity of the ischemic episode (Patel *et al*., 1993).

On the other hand, there is an increasing evidence for a neuroprotective role of prostanoids, which would be consistent with the cytoprotective role of prostaglandins in various forms of stomach (Robert *et al*., 1979) and kidney injury (Paller and Manivel, 1992). PGE_2 and PGI_2 have been recently reported to protect cultured neurons from several kinds of noxious conditions, including hypoxia/reoxygenation, glutamate-induced injury (Cazevieille *et al*., 1993, Cazevieille *et al*., 1994; Akaike *et al*., 1994), and microglial toxic products (Thery *et al*., 1994). Interestingly, the neuroprotective effects of PGE_2 and PGI_2 are mediated by cAMP elevation. Moreover, prostaglandins, in particular PGE_2, could have an indirect protective role through the regulation of inflammatory and immune responses occurring in many brain pathologies. Indeed, PGE_2, in addition to its well-known pro-inflammatory activity, can limit the activation of macrophages and microglia, and regulate several functions of T and B cells (Weissmann, 1993; Phipps *et al*., 1991). The detection of PGE_2 during the remitting phase of experimental allergic encephalomyelitis (EAE), an animal model for MS (Khoury *et al*., 1992), and the suppression of clinical and histological EAE signs by a stable PGE analogue (Reder *et al*., 1994) are suggestive of an *in vivo* beneficial role of PGE_2 in this experimental disease. It is possible that PGE_2 exerts a beneficial role also in MS and other human brain diseases where increased levels of this prostaglandin have been detected (Fretland, 1992; Griffin *et al*., 1994; Froldi *et al*., 1992).

Concerning this aspect, an apparent paradox arises from the observations that some NSAIDs (whose main action is thought to result from the inhibition of COX activity and, thus, of prostanoid production) exert beneficial effects in EAE and MS (Fretland, 1992; Reder *et al*., 1994). Moreover, epidemiological observations indicate that aged people who underwent long-term treatment with NSAIDs had a lower probability to suffer from Alzheimer's disease (McGeer and McGeer, 1995). These studies would appear to favour the hypothesis that prostanoids promote the inflammatory processes involved in the pathogenesis of the above mentioned diseases.

A clear-cut explanation reconciling these conflicting results is still missing, but a suggestive hint comes from recent evidence for additional effects of NSAIDs. In particular, ASA and its derivative salicylate were shown to inhibit NF-kB activity, at doses comparable to those prescribed for anti-inflammatory treatments (Kopp and Ghosh, 1994; Frantz and O'Neill, 1995; Grilli *et al*., 1996). Such inhibition does not appear to be specific for NF-kB, and other transcription factors such as activator protein-1 (AP-1) and cyclic AMP responding element binding protein (CREB) may be affected (Beauparlant and Hiscott, 1996). In addition to ASA, other NSAIDs appear to affect NF-kB activation, at certain drug concentrations and in certain cell types (Aeberhard *et al*., 1995). The consequence of an inhibition of NF-kB activity is far reaching, since NF-kB controls the expression of a multitude of genes, that encode for cytokines and enzymes with a broad range of activities (Beauparlant and Hiscott, 1996).

3. NITRIC OXIDE

3.1. Nitric Oxide Synthesis

Nitric oxide is an inorganic free radical gas, generated from L-arginine through a complex enzymatic reaction catalyzed by NOS (E.C.1.14.13.39). The reaction requires O_2 and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates and tetrahydrobiopterin, flavin adenin nucleotide and flavin mononucleotide as cofactors.

As in the case of COX, constitutive and inducible isoforms of NOS have been identified. The constitutive NOS (cNOS or type I NOS) isoforms are typically expressed in neurons (neuronal NOS or nNOS) and endothelial cells (endothelial NOS or eNOS) (Nathan, 1992; Vincent, 1994). In the brain, a cNOS has been described also in astrocytes (Murphy *et al*., 1993). The inducible isoform (iNOS or type II NOS) is expressed in many cell types (including neutrophils, endothelial and muscle cells, macrophages and microglia) after stimulation with products of gram-positive and gram-negative bacteria and pro-inflammatory cytokines, such as IL-1, tumour necrosis-α (TNF-α) and IFN-γ (Nathan, 1992; Vincent, 1994).

Activation of cNOS occurs in response to agonist/receptor interactions, is Ca^{2+}/calmodulin-dependent and results in the release, for seconds or minutes, of small amounts of NO (picomolar concentrations). The NO produced by cNOS appears to regulate physiological activities, such as neurotransmission and vascular tone. Acetylcholine and glutamate are typical cNOS activators in blood vessels and brain, respectively (Lowenstein and Snyder, 1992). The interaction of acetylcholine and glutamate with their specific receptors causes an elevation of intracellular Ca^{2+}, which binds to calmodulin and activates cNOS.

At variance with cNOS, iNOS activity is Ca^{2+}-independent, since calmodulin permanently and tightly binds to the enzyme at the low basal level of intracellular Ca^{2+} (Cho *et al*., 1992). iNOS can generate a continuous flux of NO for long periods (hours or even days), until substrate is available, and can thus provide the high levels of NO responsible for defence against invading micro-organisms, pathological vasodilatation and tissue damage.

The isolation of the three major isoforms (nNOS, eNOS and iNOS) from several sources (for an extensive review see Nathan, 1992), their sequencing and cloning have provided important information on the structure and regulatory features of these enzymes.

The three isoforms, coded by three distinct genes, exhibit 50–60% identity at the nucleotide and amino acid levels and display significant homology with only one other mammalian enzyme, cytochrome P450 reductase. Such structural homology has suggested that, similar to cytochrome P450 reductase, NOS catalyzes the transfer of electrons from NADPH to the cofactors flavin adenin nucleotide, flavin mononucleotide and, eventually, to the heme moiety, to activate molecular oxygen. At the same time, L-arginine and tetrahydrobiopterin generate a reactive intermediate, which reacts with the activated oxygen to form NO, citrulline and water (Nathan, 1992; Vincent, 1994). In the absence of L-arginine and in the presence of NADPH, cerebellar NOS can donate electrons to reduce oxygen, cytochrome c, cytochrome P450 or tetrazolium salts (see Nathan, 1992 and references therein).

3.2. Regulation of Nitric Oxide Synthase Activity

The activity of the cNOS isoform is mainly regulated by Ca^{2+} levels. Nonetheless, the existence in the molecule of several consensus sites for cAMP-dependent phosphorylation suggests that phosphorylation represents an additional mechanism of cNOS regulation. Although several protein kinases can phosphorylate cNOS *in vitro*, the functional significance of these phosphorylations remains poorly understood (Vincent, 1994).

On the contrary, the regulation of iNOS activity occurs mainly at the transcription level. Several putative sequences for distinct transcription factors have been identified within the 5′-upstream region of exon 1 of the mouse (Lowenstein *et al.*, 1993; Xie *et al.*, 1994) and human (Chartrain *et al.*, 1994) iNOS gene, including sites for NF-kB, IFN-γ response element, TNF-α response element, NF-IL6 and AP-1. In addition, multiple AUUUA consensus sequences, which confer elevated mRNA instability, have been found within the 3′-untranslated region of iNOS mRNA. Thus, similar to COX-2 (see Section 2.2), the iNOS gene displays the typical characteristics of an immediate early gene (Gilbert and Herschman, 1993).

At present, only a limited number of observations indicate that also post-translational modifications can regulate iNOS activity. Despite the existence of several consensus sequences for phosphorylation by several kinases, there are no reports on iNOS phosphorylation *in vitro* or *in vivo*. Nonetheless, other factors can influence iNOS. For instance, the availability of substrates and cofactors may play an important role in limiting iNOS activity. In fibroblasts (Werner-Felmayer *et al.*, 1990), endothelial cells (Gross *et al.*, 1991) and smooth muscle cells (Gross and Levi, 1992), co-induction of iNOS and tetrahydrobiopterin is required for NO production, and inhibitors of tetrahydrobiopterin production reduce NO synthesis. Similarly, argininosuccinate synthetase and argininosuccinate lyase, the two enzymes responsible for L-arginine synthesis from citrulline, are co-induced with iNOS in stimulated macrophages (Nussler *et al.*, 1994) and smooth muscle cells (Hattori *et al.*, 1994). In addition, L-arginine uptake increases in macrophages during the generation of NO (Bogle *et al.*, 1992), suggesting that regulation of L-arginine availability may be critical for the control of NO synthesis.

3.3. Biological Functions of Nitric Oxide

NO is a pleiotropic molecule, involved not only in the regulation of a variety of important functions, such as blood pressure and neurotransmission, but also in the establishment of cytotoxicity and tissue damage.

The variety of NO effects is attained through direct interactions with targets as well as through the formation of reactive intermediates, which amplify the number of possible targets. In biological systems, NO can rapidly react with molecular oxygen (O_2), superoxide anion (O_2^-) and transition metals, and generate the reactive nitrogen oxygen species (NOx), peroxynitrite and metal adducts, respectively (Fig. 5). These reactive species can support nitrosative reactions at nucleophil centres, among which thiol groups represent the more reactive and prevalent species. Thus, metal- and thiol-containing proteins, including enzymes, ion channels, membrane receptors and transcription factors are all potential targets of NO activity (Table 4). In more stringent conditions, such as depletion of the thiol pool following oxidative stress, N-nitrosylation of DNA and covalent modifications of the tyrosine groups may also occur (Stamler, 1994; Gross and Wolin, 1995; Wink *et al.*, 1996; Lander, 1997).

NO concentration appears to be the main factor determining which type of effect (direct or indirect) will dominate in any specific circumstance (Wink *et*

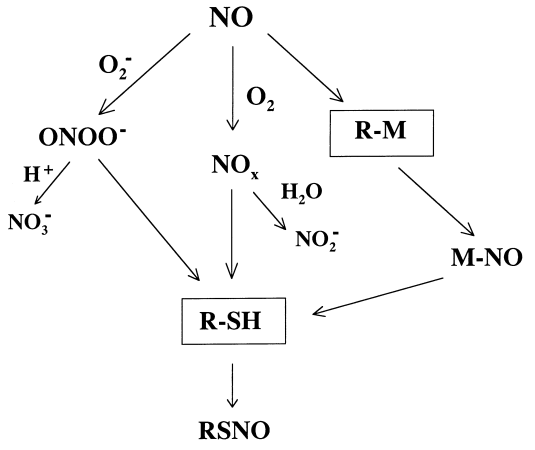

Fig. 5. A schematic diagram of NO biochemistry. In biological systems, NO rapidly reacts with superoxyde anion (O_2^-), molecular oxygen (O_2) and transition metals (M) to generate reactive nitrogen species (NOx), peroxynitrite ($ONOO^-$) and metal adducts (M-NO), respectively. These reactive species can support nitrosative reactions at nucleophil centres, among which thiol groups (-SH). Thus, metal- (R-M) and thiol- (R-SH) containing proteins are potential targets for NO-dependent modification.

Table 4. Biological targets of nitric oxide

Reaction site	Target*
Thiol	NMDA receptor
	Ca^{2+}-dependent K^+ channels
	G proteins/$p21^{ras}$
	Protein kinase C
	Adenylyl cyclase
	Glyceraldehyde-3-phosphate dehydrogenase
	SNAP-25
	GAP-43
	AP-1
	NF-kB
Metal	Guanylyl cyclase
	Hemoglobin
	Aconitase/IRE-BP
	Cyclooxygenases
	NO synthases
Unknown	CREB
	Ribonucleotide reductase

*For references see Stamler (1994) and Section 3.3.

al., 1996). The low levels of NO generated by cNOS are likely to determine direct effects, while the higher concentration of NO produced by iNOS can lead to both direct and indirect effects (Wink *et al.*, 1996; Vane *et al.*, 1994; Gross and Wolin, 1995).

The activation of the soluble isoform of guanylyl cyclase (and the consequent generation of cGMP) is considered the major physiological response to NO in the brain and represents a good example of a direct effect of NO (Vincent, 1994). The binding of NO to the heme iron of guanylyl cyclase apparently causes a dislocation of the heme, and consequently a conformational change, which activates the enzyme. NO is the most potent activator of soluble guanylyl cyclase, and may be uniquely capable, among small diffusible ligands, to induce such a conformational change (Traylor and Sharma, 1992). NO can regulate many other heme-containing proteins, but the result of NO-heme interactions may differ from that described above. For example, when the heme-iron is part of the catalytic site, as in the case of P450 enzyme, lipoxygenase, NOS itself and COX (but not of guanylyl cyclase), any modification of the heme iron by NO would abrogate enzymatic activity (Khatsenko *et al.*, 1993; Thomas *et al.*, 1994; Kanner *et al.*, 1992; Maccarone *et al.*, 1996). Thus, the activation of COX isoenzymes by NO observed in some systems is unlikely to be due to direct interaction of NO with the heme moiety (Salvemini *et al.*, 1993), and may be ascribed to modification of other functional groups by NO or peroxynitrite (Tsai, 1994; Hajjar *et al.*, 1995; Landino *et al.*, 1996).

Among the innumerable targets of NO indirect actions, we shall mention a few examples. The NMDA subtype of glutamate receptors (Lei *et al.*, 1992), protein kinase C (Gopalakrishna *et al.*, 1993) and type I adenylyl cyclase (Duhe *et al.*, 1994) all appear to be regulated by S-nitrosylation. In fact, S-nitrosylation prevents the Ca^{2+}-dependent activation of the above mentioned enzymes by down-regulating NMDA-gated Ca^{2+} currents. S-nitrosylation may control other proteins, such as neutrophil NADPH oxidase (Clancy *et al.*, 1992), the growth cone constituent growth-associated protein-43 (GAP-43), and S-nitroso-N-acetyl-DL-penicillamine (SNAP25), a synaptic protein involved in axon growth and synaptogenesis (Hess *et al.*, 1993). The requirement of critical thiol groups for the activity of the transcription factors AP-1, CREB and NF-kB may explain the responsiveness of these factors to NO (Peunova and Enikolopov, 1993; Lander *et al.*, 1993).

3.4. Cytotoxic and Cytoprotective Effects of Nitric Oxide in the CNS

Although the production of NO is an important component of antimicrobial and antineoplastic host defence, NO may also exert cytostatic and/or cytotoxic effects on normal cells by several mechanisms. It can cause DNA strand breaks, inactivation of iron containing enzymes (e.g. aconitase in the Krebs' cycle or coenzymes I and II in the mitochondrial electron transport chain) and depletion of ATP (through the ADP-ribosylation of regulatory proteins such as glyceraldehyde-3-phosphate dehydrogenase). The contribution of NO to lipid peroxidation, that can also cause cell damage, is still controversial. In fact, NO has been shown to potentiate superoxide-dependent oxidation (Hogg *et al.*, 1993a), and to decrease metal-mediated oxidation (Hogg *et al.*, 1993b; Rubbo *et al.*, 1994).

Macrophages, astrocytes, microglia and hepatocytes, that produce high levels of NO through the activity of iNOS, are particularly resistant to NO damage, being able to survive or to recover after exposure to high NO concentrations. Other cells, including NOS-negative neurons and oligodendrocytes are more susceptible to NO cytotoxicity. In the brain, both astrocytes and microglia may be a source of cytotoxic NO levels in pathological conditions. Cytokine-stimulated human astrocytes can damage neurons via a NO-mediated mechanism (Chao *et al.*, 1996). Microglia-produced NO and reactive nitrogen oxides may mediate neuronal degeneration occurring in ischemic and neurodegenerative disorders (Dawson *et al.*, 1991; Boje and Arora, 1992; Chao *et al.*, 1992, Chao *et al.*, 1993; Meda *et al.*, 1995), as well as oligodendrocyte damage in MS (Merrill *et al.*, 1993). Merrill and coworkers have shown that NO, generated by the synthetic donor SNAP, induced damage and necrotic cell death in oligodendrocyte cultures, but affected only minimally cell viability in astrocyte and microglial cultures (Mitrovic *et al.*, 1995). As NO-induced apoptosis does occur in a macrophage cell line (Meßmer and Brüne, 1996), microglia and peripheral macrophages might have different sensitivity to NO cytotoxicity.

The iNOS protein was detected in brain macrophage/microglia like cells, after viral infection and during EAE (Van Dam *et al.*, 1995) and elevated NOS mRNA was found in demyelinating regions in MS (Bö *et al.*, 1994; Bagasra *et al.*, 1995; DeGroot *et al.*, 1997). Although these and other observations support a role of NO in the pathogenesis of infectious and inflammatory brain diseases, pharmaco-

logical studies on the effects of NOS inhibitors on the development of EAE have provided conflicting results (Cross *et al*., 1994; Zielasek *et al*., 1995; Scott *et al*., 1996).

In certain circumstances NO may function as a neuroprotective agent. For example, NOS inhibitors aggravated cerebral cortex damage during ischemia-reperfusion. Moreover, NO itself prevented cell damage in both brain and heart ischemia (see Wink *et al*., 1993 and references therein). NO can react with a very high affinity with the superoxide anion (O_2^-) to generate peroxynitrite anion (see Section 3.3) which is considered a potent oxidant and toxic agent (Gross and Wolin, 1995). In spite of this, the administration of NO or peroxynitrite did not cause significant cell death in primary cultures of rat mesencephalic neurons (Wink *et al*., 1993). Moreover, when NO was generated in a limited and controlled fashion by synthetic donors known as NONOates, it protected neurons from the cytotoxic action of hydrogen peroxide and superoxide. A plausible explanation for the protective effect of NO is that NO can block the formation of the hydroxyl radicals ($OH^\cdot$) deriving from the metal-mediated reduction of hydrogen peroxide (Wink *et al*., 1993). Indeed, several studies indicate hydrogen peroxide-derived oxidants as the most effective reactive species in damaging neurons (Thery *et al*., 1991; Wink *et al*., 1993; Desagher *et al*., 1996).

Frequently, the same pathological stimuli can induce the production of NO and reactive oxygen species. The considerations made in the previous paragraph would help to explain why NO-producing cells (including NOS-positive neurons and microglia) are protected from reactive oxygen species damage (Lei *et al*., 1992).

3.5. Nitric Oxide Production in Macrophages and Microglia: Species Differences

The production of NO and its regulation in rodent macrophages are well established. In contrast, the demonstration of iNOS activity and NO production in human macrophagic cells remains controversial (for reviews, see Denis, 1994; Albina, 1995). Despite the fact that human monocytes/macrophages can express the iNOS gene (Weinberg *et al*., 1993; Reiling *et al*., 1994; Bukrinsky *et al*., 1995; Watkins *et al*., 1997), induction of NO release requires in any case a period of stimulation of several days and the amount of NO produced is substantially lower than in rodents' cells. Several factors may confound the study of NO synthesis in human macrophages: for example, the observation that iNOS induction in activated human monocytes/macrophages was not accompanied by a measurable accumulation of nitrite in cell supernatants suggested the existence of endogenous inhibitors or limiting factors (Albina, 1995). At any rate, it seems legitimate to conclude that the human macrophage iNOS is a low output and slow-acting system.

Similarly to their peripheral counterpart, mouse and rat microglia (Fig. 3B) as well as cell lines derived from them are potent producers of NO upon stimulation with LPS and/or pro-inflammatory cytokines (Zielasek *et al*., 1992; Chao *et al*., 1992; Betz-Corradin *et al*., 1993; Wood *et al*., 1994; Minghetti *et al*., 1996). In contrast, human microglial cells failed to produce detectable levels of NO under several circumstances (Lee *et al*., 1993a; Peterson *et al*., 1994, Janabi *et al*., 1996; Colton *et al*., 1996), with a few exceptions. Long-term subcultures of human ramified microglial cells, obtained from fetal brains (eight to 18 weeks of gestation), were reported to express iNOS protein and to produce large amounts of NO after stimulation with LPS and/or TNF-α. In this model, basic fibroblast growth factor and NO itself inhibited iNOS induction, by a mechanism involving prevention of NF-kB activation (Colasanti *et al*., 1995a, Colasanti *et al*., 1995b). In another study, human fetal microglial cells released only low levels of NO upon stimulation with IFN-γ plus LPS, which led the authors to propose that human macrophages have an inefficient IFN-γ-inducible iNOS and that in brain this deficiency could be advantageous for neighboring cells (Peterson *et al*., 1994). NO production by human glial cells has been documented in astrocyte cultures and in mixed glial cultures (Lee *et al*., 1993a; Koka *et al*., 1995), and according to some authors astrocytes rather than microglia represent the main intracerebral source of iNOS-derived NO (Lee *et al*., 1993b; Koka *et al*., 1995; Chao *et al*., 1996). More recently, a slow but substantial production of NO was detected in fetal human microglial cultures exposed to IL-1β plus IFN-α (Ding *et al*., 1997).

Species differences in generating NO have been demonstrated by comparing microglial cultures from several species. In these studies, hamster and porcine microglia were more similar to human than to mouse or rat cells in their capacity to synthesize NO and may thus provide experimental models that are closer to the human system. Interestingly, microglia from all species released similar amounts of superoxide anion and pro-inflammatory cytokines (Colton *et al*., 1996; Hu *et al*., 1996).

As a final comment, we would like to mention the possibility that the stimuli capable of inducing iNOS in human macrophages/microglia may not be identical to those effective in rodent cells. For example, it has been reported that human monocytes/macrophages can produce NO after ingestion of live microbes or exposure to tumour cells (Denis, 1991; Zembala *et al*., 1994). Specific deficiencies along the signal transduction cascade required for the induction of mouse iNOS gene seem to be responsible for the hypo-responsiveness of human iNOS gene to LPS/IFN-γ (Zhang *et al*., 1996). Other stimuli, which utilize different signal transduction systems, may effectively induce iNOS in human macrophagic cells. The possible existence of species differences in the sensitivity of target cells to NO should be also taken into consideration, in order to better evaluate the functional significance of the macrophages/microglia NO system in humans versus rodents. Until these aspects are better elucidated, any conclusion on the role of microglial NO in humans seems premature.

4. REGULATION OF PROSTANOID AND NITRIC OXIDE BIOSYNTHESIS IN MICROGLIAL CELLS

4.1. Prostanoid and Nitric Oxide Reciprocal Interactions

Prostanoids and NO have several common targets in both physiological and pathological conditions. Their normally low concentrations can increase during inflammation, trauma, ischemia, viral infection, etc., largely due to the expression of two inducible rate-limiting enzymes, COX-2 and iNOS. In several, but not all cases, the stimuli capable of inducing the expression of these enzymes are the same. In the brain, two populations of effector cells, astrocytes and microglia, appear to express promptly COX-2 and iNOS in response to pathological stimuli. As previously mentioned, microglial cells are particularly effective in this respect, at least in the rodent brain. It seems therefore important to understand the factors (endogenous or exogenous) regulating these two metabolic pathways and to assess whether the two pathways are mutually linked, as it has been observed in other systems (see Di Rosa *et al*., 1996 and references therein). In the following section we shall summarize our studies and those of others in this topic, focusing on observations made on microglial cells.

4.1.1. *Regulation of Prostanoid and Nitric Oxide Production by Nitric Oxide*

Using purified neonatal rat microglial cultures, we found that NO depresses the LPS-induced production of prostanoids and that this occurs essentially through a down-regulation of COX-2 expression, as shown by the following experimental evidence. First, the abrogation of endogenous NO synthesis, obtained by the omission of L-arginine from culture media or by the addition of micromolar concentrations of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), resulted into a remarkable enhancement of LPS-induced COX-2 expression and prostanoid synthesis. Second, the addition to the incubation medium of NO donors, such as 3-morpholinosydnonimine (SIN-1), SNAP (Minghetti *et al*., 1996; Guastadisegni *et al*., 1997) and NONOate compounds (unpublished observations), depressed the LPS-induced expression of COX-2 and its enzymatic activity. Therefore, both exogenous and endogenously synthesized NO had a similar depressing effect on COX-2 expression in LPS-activated rat microglial cultures.

As previously mentioned, modulation of the COX pathway by NO has been studied in several systems, both *in vitro* and *in vivo*, with non uniform results. Inhibitory activity of NO was reported in vascular endothelial cells (Doni *et al*., 1988), rat Kupffer cells (Stadler *et al*., 1993), the J774.2 macrophage cell line (Swierkosz *et al*., 1995), rat peritoneal macrophages (Habib *et al*., 1997) and rabbit chondrocytes (Stadler *et al*., 1991). Stimulatory activity has been documented, among others, in the mouse macrophage cell line RAW 264.7 (Salvemini *et al*., 1993), in rat aorta smooth muscle cells (Inoue *et al*., 1993), in rat islets of Langerhans (Corbett *et al*., 1993), in mesangial cells (Tetsuka *et al*., 1994), in 3T3 fibroblasts (Kelner and Uglik, 1994) and in rat astrocytes (Molina-Holgado *et al*., 1995). In other cases, NO did not affect the COX pathway (Curtis *et al*., 1996; Jarvinen *et al*., 1996).

Distinct mechanisms may account for the reported opposite effects of NO on prostanoid synthesis. In most studies, the stimulatory effect of NO was not dependent on cGMP formation and was suggested to involve a direct interaction of NO with the heme of COX, which in turn would facilitate COX enzymatic activity (see Section 2.2.3). On the other hand, the main target of NO inhibitory action seems to be the expression rather then the activity of COX-2 (Minghetti *et al*., 1996; Swierkosz *et al*., 1995). Thus, NO is likely to interfere with the COX pathway at distinct levels, possibly by using different signal transduction systems. The final outcome on prostanoid synthesis in a specific cell type may depend on several factors, including cell redox state and the NO concentration, which can both determine the type of NO reactivity. In a comparative study on microglia and the macrophage cell line RAW 267.4 we have observed that the two cell types have different susceptibility to reactive nitrogen species (Guastadisegni *et al*., submitted). In microglia, both SNAP (a donor of NO) and SIN-1 (a donor of NO and superoxide, which combine to form peroxynitrite), inhibited PGE_2 production by preventing COX-2 expression. In RAW 267.4 cells, SIN-1 enhanced basal and LPS-stimulated PGE_2 synthesis by up-regulating COX-2, while SNAP stimulated basal production and slightly inhibited LPS-induced production. As SNAP enhanced AA release and depressed COX-2 expression in RAW 267.4 cells, its effect on PGE_2 must be the net result of these opposite actions. In a different macrophage system, the J774.2 cells, low concentrations of NO stimulated prostanoid formation, whereas higher concentrations inhibited it (Swierkosz *et al*., 1995). More relevant to the aim of this review, in cytokine-stimulated human fetal microglia the production of $PGF_{2\alpha}$ was moderately decreased in the presence of millimolar concentrations of the NOS inhibitor L-NMMA, despite the fact that NO was not detectable in culture supernatants, even in the absence of L-NMMA (Janabi *et al*., 1996). These observations led the authors to hypothesize that very low levels of endogenous NO (below detection limits) produced by a putative cNOS, could stimulate COX activity. The question of whether NO stimulated microglial COX-2 expression remains open, since COX-2 expression was not analyzed in this study. The fact that high concentrations of the NOS inhibitors L-NAME and L-NMMA can affect the activity of other enzymes, including COX (Peterson *et al*., 1992; Southan and Szabó, 1996), adds further uncertainty to the proposal that NO can stimulate prostanoid synthesis, in the absence of more direct evidence (Appleton *et al*., 1996).

Positive as well as inhibitory interactions between NO and COX pathways have been documented *in vivo*, in animal models for acute or chronic inflammation (Di Rosa *et al*., 1996: Appleton *et al*., 1996). However, it is hard to say whether the interactions described are direct or mediated by indirect effects.

As an example, during acute inflammation, NO alters vascular permeability and causes plasma extravasation, which can promote prostanoid formation (Appleton *et al*., 1996). Consequently, abrogation of NO production will cause prostanoid inhibition indirectly. One should also consider the possibility that changes in cytokine profile observed in inflammation (Vane *et al*., 1994) might favour the iNOS or the COX-2 pathway over the other (see Section 4.2.2.).

Besides modulating prostanoid synthesis, NO can regulate its own production. In alveolar macrophages, exogenous NO inhibited iNOS activity (Griscavage *et al*., 1993). In astrocytes, endothelial cells, the mouse macrophage cell line ANA-1 and human ramified microglia, NO down-regulated the expression of both iNOS mRNA and protein, by preventing NF-kB activation (Park *et al*., 1994: Peng *et al*., 1995: Sheffler *et al*., 1995; Colasanti *et al*., 1995b). In rat microglial cultures, the expression of iNOS protein was moderately decreased in the presence of the NO donor SIN-1 (100 μM), but was not significantly affected by the abrogation of endogenous NO synthesis by micromolar concentrations of L-NMMA (Minghetti *et al*., unpublished observation).

4.1.2. *Regulation of Nitric Oxide and Prostanoid Production by Exogenous Prostaglandin E_2*

As in the case of prostanoid regulation by NO, both positive and negative modulations of NOS activity by PGE_2 have been documented in distinct macrophage models, such as bone marrow-derived macrophages or established cell lines (Marotta *et al*., 1992; Mauël *et al*., 1995; Milano *et al*., 1995).

In rat microglial cultures stimulated for 24 h with LPS, the presence of increasing amounts of PGE_2 caused a dose-dependent reduction of nitrite release and iNOS protein expression (Minghetti *et al*., 1997a). The effect of PGE_2 was no longer present if the prostaglandin was administered 2 h after LPS, when iNOS protein was already detectable. The observed time course is suggestive of a reduction of the transcription rate rather than of the stability of iNOS mRNA (Minghetti *et al*., 1997a).

Interestingly, in the same culture conditions, exogenous PGE_2 showed an opposite regulation of COX-2 expression (Minghetti *et al*., 1997b). In fact, nanomolar concentrations of PGE_2 potentiated the LPS-induced COX-2 expression and activity. As described in Section 4.2.1., the effect of PGE_2 on both pathways appeared to involve an elevation of cAMP levels (Minghetti *et al*., 1997a, Minghetti *et al*., 1997b).

4.1.3. *Regulation of Nitric Oxide and Prostanoid Production by COX Inhibitors*

Unexpectedly, the inhibitory effect of exogenous PGE_2 on NO production increased in the presence of indomethacin (INDO), a strong inhibitor of COX activity. This observation suggests that endogenous prostanoids partially counteracted the inhibitory effect of exogenous PGE_2 on the NO pathway. The finding that INDO, as well as two other COX inhibitors, ASA and 6-methoxynaphthalene acetic acid, caused by themselves a reproducible decrease of LPS-induced nitrite accumulation and iNOS expression, is consistent with this hypothesis (Minghetti *et al*., 1997a). One could argue that these drugs might exert a direct inhibitory effect on iNOS synthesis, as they do in other cells when used at high concentrations (see also Section 2.7). For example, in the macrophage cell line RAW 264.7 and in rat cardiac fibroblasts, millimolar concentrations of ASA or of its derivative salicylate decreased the expression of iNOS (Amin *et al*., 1995; Farivar *et al*., 1996; Kepka-Lenhart *et al*., 1996). In alveolar macrophages, in addition to ASA and salicylate also INDO (>1mM) inhibited iNOS expression (Aeberhard *et al*., 1995). In our experiments, however, INDO, ASA, and 6-methoxynaphthalene acetic acid showed a comparable ability to down-regulate iNOS at micromolar concentrations. Thus, the action of these compounds was presumably due to the block of prostanoid production, rather than to a direct effect on iNOS expression.

The observation that INDO and 6-methoxynaphthalene acetic acid caused an up-regulation of LPS-induced COX-2 expression was also unexpected and in apparent contrast with the above reported up-regulation of LPS-induced COX-2 expression by exogenous PGE_2 (Minghetti *et al*., 1997b). As discussed elsewhere (Minghetti *et al*., 1997b), a direct stimulatory effect of the drugs on COX-2 seems unlikely.

Thus, anti-inflammatory drugs appear to regulate in opposite ways iNOS and COX-2 expression in microglial cells and, paradoxically, their effect on iNOS and COX-2 expression is qualitatively similar to that of exogenous PGE_2. The paradox could be explained by assuming that endogenous prostanoids other than PGE_2 affect LPS-induced iNOS and COX-2 expression. Alternatively, endogenous PGE_2 might act intracellularly, through a distinct signalling pathway. The recent identification of nuclear prostanoid receptors for the PGJ_2 series (Forman *et al*., 1995; Kliewer *et al*., 1995) and the localization of COX-2 at the inner side of the nuclear membrane (Morita *et al*., 1995) suggest that endogenously produced prostanoids may indeed act at the nuclear level (Fig. 6). Moreover, we can not exclude that blockade of the COX pathway may limit AA utilization or shift it towards the lipoxygenase pathway. In this case, lipoxygenase metabolites or arachidonic acid itself may down-regulate or up-regulate iNOS and COX-2 expression, respectively.

4.2. Factors Regulating Prostanoid and Nitric Oxide Production in Microglial Cells

4.2.1. *Effects of Cyclic AMP Elevating Agents on Nitric Oxide and Prostanoid Production*

Cyclic AMP is one of the major second messengers, and regulates a number of glial functions, including the secretion of cytokines and growth factors and the expression of major histocompatibility complex antigens (Sasaki *et al*., 1990, Grimaldi *et al*., 1994, Hetier *et al*., 1991, Frohman *et al*., 1988).

To study the possible role of cAMP in the regulation of microglial prostanoid and NO synthesis,

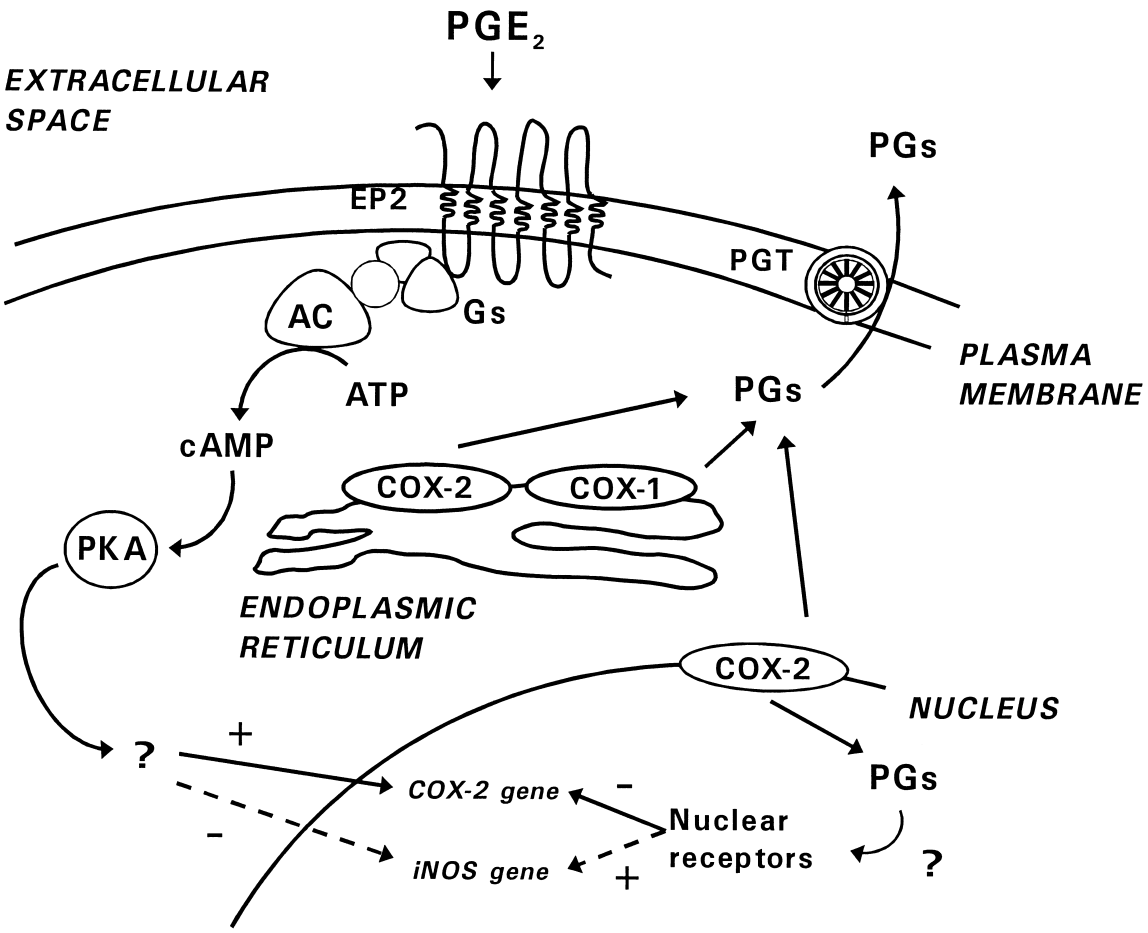

Fig. 6. Proposed mechanisms for intracellular and extracellular prostaglandin biological actions. AC adenylyl cyclase; PGs, prostaglandins; PKA, protein kinase A; PGT, prostaglandin transporter. For further detail see Sections 2.8; 4.1.2. and 4.1.3.

we utilized the β-adrenergic agonist isoproterenol and PGE_2 itself, which elevate cAMP levels in these cells (Patrizio *et al*., 1995, Patrizio *et al*., 1996). In rat microglial cultures activated by LPS, both agents enhanced the production of prostanoids and inhibited that of NO. Both compounds acted mainly on the expression of the two rate limiting enzymes COX-2 and iNOS, the former being up-regulated and the latter down-regulated (Minghetti and Levi, 1995; Minghetti *et al*., 1997a; Minghetti *et al*., 1997b).

Several observations support the involvement of cAMP in the regulation of COX and iNOS pathways. First, the effects of isoproterenol and PGE_2 were mimicked by the adenylyl cyclase activator forskolin and by two cAMP active analogues (dibutyryl-cAMP and the thiolated cAMP-Sp isomer). Second, the effect of PGE_2 was prevented by a specific inhibitor of adenylyl cyclase (SQ 22 1,536) or by the inactive thiolated cAMP-Rp isomer, a competitive inhibitor of cAMP at the level of protein kinase A. Third, the effect of PGE_2 was mimicked by an agonist of EP2 receptors whose activation leads to an accumulation of cAMP (11-deoxy-16,16-dm-PGE_2), but not by an agonist of EP3 and EP1 receptors (sulprostone), that are associated with an inhibition of adenylyl cyclase activity and with the accumulation of inositol trisphosphate and Ca^{2+}. Thus, the action of exogenous PGE_2 on prostanoid and NO synthesis in LPS-activated rat microglia involves an elevation of cAMP, possibly achieved through the activation of EP2 receptors (Minghetti *et al*., 1997b). Similarly to isoproterenol and exogenous PGE_2, adenosine, which evokes cAMP accumulation by interacting with its A_{2a} receptor, can induce COX-2 expression in rat microglial cultures. This effect was observed even in the absence of other microglial activators (Fiebich *et al*., 1996). Interestingly, also isoproterenol, but not PGE_2, slightly enhanced COX-2 expression in the absence of LPS. Such enhancement was not accompanied by increased prostanoid synthesis, probably due to the insufficient availability of AA in these conditions (Minghetti and Levi, 1995). The specific features of cAMP generation by β-adrenergic as compared to EP2 receptor activation, such as kinetics and level of accumulation, may account for the different behavior of isoproterenol and PGE_2 with regard to COX-2 expression in LPS-free cultures.

To summarize, our data indicate that PGE_2, β-adrenergic agonists, and possibly other cAMP ele-

vating agents can shift the balance between prostanoids and NO in favour of prostanoids, by acting at the level of the two key enzymes regulating their production. The consequences of this unbalance may be far reaching. Microglia-derived NO and prostanoids could influence the local evolution of inflammatory, immune and degenerative processes in the CNS by fostering brain damage, on one side, and neuroprotection and repair on the other (see Sections 2.8 and 3.4).

4.2.2. *Cytokines*

The pro-inflammatory cytokine IFN-γ is one of the most typical inducers of iNOS in macrophages and microglia (Murphy *et al*., 1993). IFN-γ is produced mainly by Th1 lymphocytes and NK cells. Its cerebral level can increase when these cells invade the nervous system in the course of inflammatory and other types of brain diseases. Besides stimulating NO synthesis, IFN-γ has numerous other actions, by itself or in combination with other cytokines, and may play a prominent role in the pathogenesis of lesions in inflammatory diseases, such as MS (Navikas and Link, 1996, Agresti *et al*., 1996). In view of the central role of this cytokine, we assessed its effect on the production of prostanoids by activated microglial cells. In neonatal rat microglial cultures, the cytokine by itself did not affect prostanoid synthesis, but depressed the LPS-induced production of the three prostanoids examined (PGE_2, PGD_2 and TXB_2). The decrease in prostanoid production was concomitant with the enhancement of the LPS-induced NO synthesis. In spite of this, the bulk of the inhibitory effect of IFN-γ on prostanoid production was not related to the augmented release of NO, since it occurred also in conditions in which the synthesis of NO was virtually abrogated. IFN-γ did not limit the LPS-evoked release of AA from microglial membranes, but substantially prevented the up-regulation of COX-2 expression caused by LPS. Thus, although NO and IFN-γ had COX-2 as a common target, each of these two agents appeared to down-regulate the expression of the enzyme by independent mechanisms (Minghetti *et al*., 1996). What seems particularly relevant with regard to IFN-γ-related neuropathogenesis is the fact that the cytokine can cause a substantial shift in the balance between prostanoid and NO concentrations in favour of the latter, through its concerted actions at the level COX-2 and iNOS expression.

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine havig, among others, immunoregulatory and macrophage deactivating functions. A rapid and abundant expression of TGF-β1 is observed in several experimental (Lindholm *et al*., 1992; Kiefer *et al*., 1993; McNeill *et al*., 1994; Khoury *et al*., 1992) and human diseases (Cupp *et al*., 1993; Wahl *et al*., 1991; Finch *et al*., 1993; Navikas and Link, 1996) associated with microglial and astrocytic activation. The observations that, in most of the lesions studied, activated microglia and macrophages are the predominant cell types expressing both TGF-β1 mRNA and protein (Kiefer *et al*., 1993; Morgan *et al*., 1993), and that microglial cell *in vitro* synthesize TGF-β1 (Constam *et al*., 1992; da Cunha *et al*., 1993), suggest that these cells, once activated, represent one of the major intrathecal sources of the cytokine. Abundant experimental evidence stands for a beneficial role of TGF-β1 in neurological diseases, which may be at least partly related to a depression of microglial activation. Interestingly, in neonatal rat microglial cultures the regulation of prostanoid and NO synthesis by TGF-β1 was opposite to that of the pro-inflammatory cytokine IFN-γ (Minghetti *et al*., submitted). Indeed, TGF-β1 inhibited NO synthesis by depressing the LPS-induced iNOS expression, as previously reported for adult mouse microglial cultures (Lodge and Sriram, 1996), and increased PGE_2 production, by enhancing COX-2 expression. As in the case of IFN-γ (Minghetti *et al*., 1996) experiments performed in the presence of specific inhibitors of iNOS and COX-2 activities suggested that the stimulatory and inhibitory effects of TGF-β1 on COX-2 and iNOS, respectively, did not depend on the availability of end products of the two enzymes (Minghetti *et al*., 1997c).

Interleukin 10 (IL-10) is another pleiotropic cytokine, produced by a variety of cell types including Th2 lymphocytes, B cells, mast cells and macrophages. IL-10 is a potent suppressor of the effector functions of macrophages, T cells and NK cells, but also exerts an immunostimulatory effect on B cells (Moore *et al*., 1993). IL-10 protein has been detected in cerebrospinal fluids of individuals with infectious and inflammatory meningeal diseases (Gallo *et al*., 1994) and within MS lesions (Cannella and Raine, 1995), and IL-10 mRNA is expressed in EAE (Kennedy *et al*., 1992). It has therefore been suggested that this cytokine is implicated in some CNS diseases and that its intrathecal synthesis may be relevant for the regulation of inflammatory and immune responses. As in the case of TGF-β1, IL-10 can be secreted by activated microglial cultures (Sheng *et al*., 1995; Lodge and Sriram, 1996). Since microglia also express IL-10 receptors (Mizuno *et al*., 1994), these cells can be both a source and a target of this cytokine.

Although both TGF-β1 and IL-10 have immunosuppressive and anti-inflammatory actions, their effects on the synthesis of prostanoids and NO in LPS-activated rat microglial cultures did not completely overlap. In fact, IL-10 inhibited the expression of both iNOS and COX-2, and consequently reduced not only NO (as did TGF-β), but also PGE_2 production (that was stimulated by TGF-β).

The fact that pro- and anti-inflammatory cytokines can specifically regulate the synthesis of potent local effectors, such as PGE_2 and NO, may provide a novel clue for understanding the specific role of these cytokines in several neurological diseases, in which their cerebral level is elevated. To name only few of the possible functional consequences of these regulations, the enhancement of PGE_2 synthesis by TGF-β1 could be one of the mechanisms by which the cytokine exerts its immunoregulatory and macrophage-deactivating functions. Furthermore, the depression of PGE_2 production by IL-10 could be part of an autocrine feedback regulation by

which IL-10 limits its own synthesis. In fact, PGE_2 has been reported to increase IL-10 synthesis in monocytes and macrophages (van der Pouw Kraan *et al*., 1995; Strassmann *et al*., 1994). Such autocrine regulation may prevent excessive depression of microglial reactivity by IL-10 in response to pathological conditions. On the other hand, the increased synthesis of NO induced by IFN-γ and its inhibition by TGF-β1 and IL-10 could promote or limit, respectively, the establishment of neuronal death or oligodendrocyte damage (Boje and Arora, 1992; Chao *et al*., 1992, Chao *et al*., 1993; Merrill *et al*., 1993; Mitrovic *et al*., 1994).

4.2.3. *Glucocorticoids and Lipocortin-1*

Glucocorticoids are complex anti-inflammatory drugs and are frequently the next line of therapy, when NSAIDs are ineffective in suppressing inflammation. Their potent activity is due to multiple actions on distinct molecular targets, including COX-2 and iNOS. The synthetic glucocorticoid dexamethasone inhibits COX-2 and iNOS expression *in vivo* (Masferrer *et al*., 1992; Mitchell *et al*., 1994; Salvemini *et al*., 1995) and in several types of cultured cells, including monocytes/macrophages (Fu *et al*., 1990; O'Banion *et al*., 1992; Lee *et al*., 1992; Di Rosa *et al*., 1990) and microglia (Bauer *et al*., 1997; Minghetti *et al*., unpublished observations). Since the promoter region of COX-2 does not contain a negative glucocorticoid response element (Goppelt-Struebe, 1995), other mechanisms of regulation must intervene. Recent experiments have demonstrated that glucocorticoids can interfere with essential transcription factors (Beauparlant and Hiscott, 1996). In particular, they inhibit the activation of NF-kB, which is an important regulatory step in microglial COX-2 and iNOS expression (Bauer *et al*., 1997; Colasanti *et al*., 1995b).

In addition to prostanoid and NO synthesis, glucocorticoids depress other microglial functions, such as proliferation, cytokine production and major histocompatibility complex antigen expression (Kiefer and Kreutzberg, 1991; Chao *et al*., 1992; Ganter *et al*., 1992). However, in spite of this high responsiveness to glucocortocoids *in vitro*, microglia did not readily respond to manipulation of glucocorticoid levels *in vivo*, during Wallerian degeneration of mouse optic nerve (Castaño *et al*., 1996). The lack of response could be due to the fact that *in vivo* microglial glucocorticoid receptors are down-regulated. Indeed, brain glucocorticoid receptors have been mainly localized on neurons and astrocytes (Aronsson *et al*., 1988; Chou *et al*., 1990; Cintra *et al*., 1994).

Several of the anti-inflammatory effects of glucocorticoids are mediated by an endogenous protein, lipocortin-1 (LC-1), whose expression can be induced or enhanced by glucocorticoids themselves, both *in vivo* and *in vitro* (see Wu *et al*., 1995, and review by Flower and Rothwell, 1994). LC-1 belongs to a family of calcium and phospholipid-binding proteins, called either lipocortins or annexins. Lipocortins are characterized by the presence of homologous repeats, which form the core of the proteins, while they differ in their N-terminus region (Flower and Rothwell, 1994). Intensive studies on LC-1 have revealed that this protein possesses diverse and unexpected properties, some of which are independent of glucocorticoids. Among others, LC-1 has antipyretic activity and protects neurons from ischemic and NMDA-mediated death (Relton *et al*., 1991). In the brain, LC-1 immunoreactivity is localized predominantly in astrocytes and microglia (McKanna, 1993: Go *et al*., 1994). After mechanical injury, LC-1 immunostaining increases several folds, primarily in microglia surrounding degenerating neurons, which led to the proposal that LC-1 may limit the inflammatory response and neuronal damage (McKanna, 1993). Increased levels of LC-1 have been also found in MS and EAE (Elderfield *et al*., 1992).

LC-1 might prevent tissue damage by limiting or controlling microglial activation. To test this hypothesis, we studied the biological effects of a peptide derived from human LC-1 N-terminus (Ac 2–26), which retains most of the functions of the parent protein (Flower and Rothwell, 1994). We found that Ac 2–26 inhibited, in a dose-dependent way, both PGE_2 and NO production, mainly by down-regulating the expression of COX-2 and iNOS. The pre-incubation with specific anti-LC-1 antibodies partially prevented the inhibitory effect of Ac 2–26 on both biosynthetic pathways. Compared to LC-1, dexamethasone was much more effective, and anti-LC-1 antibodies partially counteracted its inhibitory effect on NO, but not on PGE_2 production (Minghetti *et al*., in preparation). At variance with our results, LC-1 (fragment 1–188) did not depress LPS-induced COX-2 expression (but did inhibit iNOS expression) in the macrophage cell line J774.2 (Wu *et al*., 1995). It is difficult to say whether this apparent discrepancy is due to the different cell type, to the different LC-1 fragment used or to other factors. Interestingly, however, anti-LC-1 antibodies antagonized the inhibitory effect of dexamethasone on NO, but not on PGE_2 synthesis in J774.2 cells, just the same as in microglial cells. In conclusion, our data support the hypothesis that LC-1 contributes to the down-regulation of microglia by limiting the expression of the two inducible enzymes COX-2 and iNOS. Further data are required to support the possibility that LC-1 mediates the effects of dexamethasone on both metabolic pathways.

4.2.4. *Other Factors*

In addition to LPS and IFN-γ, a few other substances can stimulate NO production in microglial cells. Among these, chromogranin A and β-amyloid are worth mentioning for their potential role in triggering and/or sustaining neurodegeneration.

Chromogranin A is a ubiquitous secretory protein, stored and released from many neuroendocrine cells and neurons. In human brain, chromogranin A is commonly found in regions undergoing degenerative processes, such as Lewy bodies in Parkinson's disease and senile plaques in Alzheimer's disease. Recent evidence indicates that chromogranin A can activate microglial cultures by inducing a dramatic change in cell morphology, accompanied by a reor-

Table 5. Regulation of COX-2 and iNOS expression in activated rat microglial cells

Regulatory agents	Second messengers	Transcription factors	COX-2	iNOS
PGE_2	cAMP	CREB?	↑	↓
β-adenergic agonist	cAMP	CREB?	↑	↓
Adenylyl cyclase activators	cAMP	CREB?	↑	↓
TGF-β		CREB?	↑	↓
NSAIDs*			↑	↓
Nitric oxide		NF-kB**	↓	=/↓**
IFN-γ			↓	↑
IL-10			↓	↓
Lipocortin-1 peptide 2–26			↓	↓
Dexamethasone			↓	↓

*Effect mediated by non-identified endogenous AA metabolites.
**Findings in human ramified microglia (Colasanti *et al*., 1995b).

ganization of the actin network, and by stimulating NO production (Taupenot *et al*., 1996).

β-amyloid protein, the main constituent of extracellular deposits present in the typical lesions (senile plaques) of Alzheimer's disease, was shown to stimulate NO release from microglial cultures when the cells were also exposed to IFN-γ (Meda *et al*., 1995; Goodwin *et al*., 1995).

These observations suggest that both chromogranin A and β-amyloid protein could participate to the pathogenic processes leading to neurodegeneration through the induction of NO release from microglia. To date, it is not known whether these two products can also affect the production of prostanoids.

5. CONCLUSIONS

Microglial cells have been generally considered as aggressive cells, capable of inducing neuronal and oligodendroglial damage through their secretory products (nitrogen and oxygen radicals, inflammatory cytokines, glutamate, other excitotoxins, etc.) and of promoting autoimmune processes through their ability to present antigens and stimulate T-cell responses. It has recently become clear, however, that this is just one of the facets of microglial pathophysiology. As mentioned at the beginning of this article and in several recent reviews, microglial reactivity should be primarily viewed as addressed to host defence and neuroprotection. Only when the cells are induced to over-react and other concomitant mechanisms of defence fail, microglia would be involved in the establishment and/or maintenance of brain damage.

It is obviously difficult to determine what is the threshold beyond which microglial reactivity results in brain damage. In this respect, the view of the pathologist may be biased by the fact that his observations refer to terminal stages of diseases.

To date very few studies have dealt with the roles of prostanoids and nitric oxide as mediators and/or modulators of microglial protective or toxic functions. A recent investigation on this matter (Caggiano and Kraig, 1996) indicates that pharmacological manipulation of the two metabolic pathways *in vivo* strongly affects microglial activation.

The present article addresses one aspect of the above problems. We have shown that microglia can produce large amounts of prostanoids and NO, whose beneficial or neurotoxic role may depend on the concentration achieved in the tissue. We have also shown that the two metabolic pathways mutually interact through the activity of their end products, that can also regulate their own output by feedback mechanisms. Finally, we have provided evidence that a number of factors present in the pathological brain (cytokines, lipocortins) and anti-inflammatory drugs can influence in a complex way the microglial production of both prostanoids and NO (Table 5). In several cases (IFN-γ, TGF-β1, NSAIDs) such regulation has an opposite sign for the two classes of compounds, and can thus alter the balance between them in a substantial way. In our discussion we favoured the hypothesis that a high prostanoid/NO production ratio is more likely to promote neuroprotection than a high NO/prostanoid ratio, which is more likely to result into neurodegeneration. Such an hypothesis is supported by an increasing literature, that recognises a protective role to prostaglandins (Bazan *et al*., 1995) and a cytotoxic role to NO and its derivatives (Chao *et al*., 1992; Merrill *et al*., 1993; Adamson *et al*., 1996), both within the CNS and peripherally.

Although attractive, this hypothesis, like most biological hypotheses, must be taken with some caution and requires confirmation in animal models of brain diseases. In particular, the hypothesis may be valid within a certain range of concentrations of the compounds considered. For example, we have already mentioned that NO may be protective or cytotoxic, depending on its concentration.

Another note of caution derives from the doubts on the role of human microglia/macrophages in the production of NO (see Section 3.5). In this respect, however, it is worth recalling that in the human brain NO can be produced by other cell types. In turn, microglia can generate other cytotoxic free radicals (like oxygen radicals), when activated by stimuli that induce NO production in rodent cells.

In the case of prostaglandins (in particular of PGE_2), indirect evidence for their protective role *in vivo* derives from the detection of increased cerebral levels of PGE_2 during the remitting phase of EAE (Khoury *et al*., 1992) and from the beneficial role of other cAMP elevating agents, such as type IV phos-

phodiesterase inhibitors, β-adrenergic agonists and PGE_2 itself, in ischemia or EAE (Kato *et al*., 1995; Genain *et al*., 1995; Sommer *et al*., 1995; Wiegmann *et al*., 1995). In this respect, it would be interesting to evaluate whether the well-known neuroprotective effect of adenosine (von Lubitz *et al*., 1995) is related to the enhanced production of prostanoids induced by the activation of A_{2a} receptors (Fiebich *et al*., 1996). It may be pertinent to recall that such receptors are linked to cAMP generation. It is also intriguing that the cyclopentanone prostaglandins (PGJ_2 and PGA_1), which derive non-enzymatically from PGD_2 and PGE_2, respectively, possess a potent antiviral activity against a wide variety of viruses, including HIV-1 (Rozera *et al*., 1996). The increased level of PGE_2 and PGD_2 found in the brain (CSF) of AIDS patients (Griffin *et al*., 1994; Froldi *et al*., 1992) might thus have a protective role, not only towards neuronal damage, but also towards viral spreading in the CNS, if local concentrations of cyclopentanone prostaglandins could achieve sufficiently high levels.

A further important aspect to be considered is the controversial protective effect of NSAIDs, which would support the notion that prostanoids exacerbate brain damage. Indeed, the action of this widely used class of drugs should be reconsidered in the light of their ability (see also Section 2.8) to interfere with the activation of transcription factors that regulate the expression (among others) of iNOS and pro- or anti-inflammatory cytokines (Beauparlant and Hiscott, 1996). Thus NSAIDs may have important indirect effects not mediated by the blockade of COX activity.

The hypotheses advanced in this article were largely based on *in vitro* observations. An *in vivo* validation of such hypotheses may disclose new avenues for the pharmacological treatment or prevention of brain damage in at least some neurological diseases.

Acknowledgements—The original work presented in this article was supported by the following grants: Project on AIDS of the Italian Ministry of Health; Project on Multiple Sclerosis of the Istituto Superiore di Sanità; Project on Ageing, subproject on Gerontobiology of the Italian National Research Council. The secretarial help of Ms. Estella Sansonetti is gratefully acknowledged.

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