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CHAPTER 35

Nitric oxide-mediated mitochondrial impairment in neural cells: a role for glucose metabolism in neuroprotection

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Introduction

Nitric oxide ($^{\bullet}$NO) is a highly diffusible, short-lived physiological messenger present in the central nervous system (CNS) (Garthwaite et al., 1988) that is synthesised by a family of nitric oxide synthases (NOSs) which catalyze the conversion of arginine to citrulline and $^{\bullet}$NO (Bredt and Snyder, 1990; Knowles and Moncada, 1994). All CNS cells synthesise $^{\bullet}$NO (Murphy et al., 1993). Neurones produce $^{\bullet}$NO by calcium-dependent activation of neuronal, constitutive NOS (nNOS or NOS1), whereas glial cells synthesise $^{\bullet}$NO in a calcium-independent way that requires previous transcriptional induction of NOS (inducible NOS, iNOS or NOS2) (Galea et al., 1992; Simmons and Murphy, 1992). Astrocytes also synthesise $^{\bullet}$NO through NNOS activity (Murphy et al., 1990, 1991; Agulló and García, 1992a,b). A third isoform of NOS (endothelial NOS, eNOS or NOS3) is associated with brain vasculature.

In general, $^{\bullet}$NO participates in the transduction pathway leading to elevations in intracellular cyclic GMP levels (Bredt and Snyder, 1989; Knowles et al., 1989) and therefore participates in cyclic GMP functions (Wang and Robinson, 1997). However, an increasing body of evidence is now arising to suggest that $^{\bullet}$NO and its most active metabolite, the peroxynitrite anion ($ONOO^-$), may be involved in the regulation of brain energy metabolism. This chapter will specifically focus on the mechanisms involving $^{\bullet}$NO and $ONOO^-$-mediated interference with brain mitochondrial energy production and the modulating role of glutathione in cell energy metabolism. Finally, we discuss recent evidence that strongly suggests the importance of cell glucose utilisation in maintaining glutathione homeostasis and hence in preventing nitric oxide-mediated mitochondrial impairment.

Astrocytic INOS induction and mitochondrial function

In astrocytes, the induction of INOS by lipopolysaccharide (LPS, 1 μg/ml) plus interferon-γ (IFN-γ, 100 U/ml) for 18 h is accompanied by a selective and irreversible inhibition of complex IV (cytochrome *c* oxidase) activity of the mitochondrial respiratory chain (Fig. 1) (Bolaños et al., 1994). This effect is prevented by the inhibition of NOS activity with *N*-monomethyl-L-arginine (NMMA, 1 mM; Fig. 1), suggesting that the biosynthesis of $^{\bullet}$NO is a necessity for the effect on complex IV. More-

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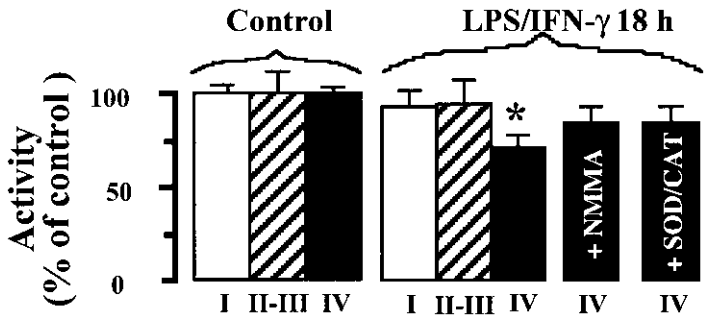

Fig. 1. iNOS induction is accompanied by the inhibition of mitochondrial complex IV activity in astrocytes. Incubation of astrocytes with LPS/IFN-γ (1 μg/ml plus 100 U/ml, respectively) for 18 h increased NOS activity by about 100-fold (not shown) and this was accompanied by the inhibition of complex IV (C IV) activity, without affecting those of complexes I (C I) and II–III (C II–III). The presence of NMMA (1 mM), or SOD/CAT (250 U/ml each) during the 18-h incubation period fully prevented the inhibition of complex IV. Control values were as follows: complex I, 65.5 ± 3.5 nmol/min/mg; complex II–III, 11.31 ± 1.41 nmol/min/mg; complex IV, 2.12 ± 0.08 k/min/mg, where k is the first rate order constant. *Statistically different ($P < 0.05$) from the respective control value. Data obtained with permission from Bolaños et al. (1994).

over, incubation with LPS/IFN-γ supplemented with superoxide dismutase (SOD) plus catalase (CAT) (250 U/ml each) prevents such complex IV damage (Fig. 1), suggesting the involvement of the endogenous formation of superoxide anion ($O_2^{\bullet -}$). In fact, INOS activity synthesises $O_2^{\bullet -}$ as well as $^{\bullet}NO$ (Xia et al., 1998) and therefore peroxynitrite anion ($ONOO^-$) can be formed endogenously by the direct reaction of $^{\bullet}NO$ with $O_2^{\bullet -}$ (Beckman et al., 1990). Consequently, It may be inferred that the irreversible damage to complex IV after INOS induction in astrocytes would be due to $ONOO^-$ rather than to $^{\bullet}NO$ itself.

Despite irreversible complex IV damage by the endogenous formation of $ONOO^-$ in astrocytes, simultaneous measurement of $^{\bullet}NO$ and oxygen concentrations in activated astrocytes has uncovered a reversible effect of $^{\bullet}NO$ on oxygen consumption (Brown et al., 1995). This reversible inhibition of oxygen consumption is possibly due to the competition of endogenously formed $^{\bullet}NO$ with O_2 for complex IV, as previously reported in synaptosomes using authentic $^{\bullet}NO$ (Brown and Cooper, 1994). In addition, an irreversible inhibition of oxygen consumption has also been observed, because the presence of NMMA only restores the rate of O_2 consumption in LPS/IFN-γ-treated astrocytes to 73% as compared with controls (Brown and Cooper, 1994). Subsequently, Sharpe and Cooper (1998), using purified complex IV, demonstrated that its activity is directly and irreversibly inhibited by $ONOO^-$, confirming the former results by Bolaños et al. (1994) in intact. It now appears clear that either in situ $^{\bullet}NO$ formation, or exogenous $^{\bullet}NO$ addition, reversibly causes complex IV inhibition, possibly through the interaction of $^{\bullet}NO$ with an intermediate turnover with a partially reduced cytochrome a_3-Cu_B binuclear centre (Giuffrè et al., 1996). However, under appropriate conditions, when $ONOO^-$ is also formed instead of $^{\bullet}NO$, an additional irreversible complex IV inhibition is directly brought about by $ONOO^-$. This seems to be due to a reduction in the V_{max} and an increase in the K_m of the enzyme for oxygen, consistent with irreversible damage to the Cu_A centre (Sharpe and Cooper, 1998). Moreover, whilst the former phenomenon seems to be a physiological mechanism focused on tonically regulating mitochondrial function (Brown, 1995), the latter would be a pathophysiological mechanism responsible for the mitochondrial dysfunction observed under certain neuropathological situations (Bolaños et al., 1997).

Of interest is that, as an attempt to model the in vivo situation, we have previously used LPS/IFN-γ-activated astrocytes that were cocultured with neurones, only separated by an appropriate membrane that allows later analysis of neurones and astrocytes independently (Bolaños et al., 1996). Using this system, it was observed that neurones that were exposed for 24 h to these astrocytes suffered an ATP deficiency (Bolaños et al., 1996) and specific damage to complex IV activity (Stewart

et al., 1998). Also, using mixed astrocyte–neuronal cocultures, others have described the neurotoxicity elicited by astrocyte-released •NO (Dawson et al., 1994; Chao et al., 1996).

Relevance for INOS induction in neurodegenerative diseases

Provided we can extrapolate the above results to the in vivo situation, it can be advanced that glial activation may have an important role in neurodegeneration. In fact, irreversible complex IV damage has been observed in a number of neurodegenerative diseases (Beal et al., 1993), such as Alzheimer's disease (Kish et al., 1992; Mutisya et al., 1994; Chagnon et al., 1995) or Parkinson's disease (Bindoff et al., 1991; Benecke et al., 1993). Taking into account the relevance of cytokine formation within the brain in these diseases (Boka et al., 1994; Mrak et al., 1995), our results pointing to selective and irreversible complex IV damage after endogenous $ONOO^-$ synthesis suggest that such damage may be a contributing factor in the development of those neurological disorders. Furthermore, in brain samples from several neurodegenerative diseases, the presence of nitrotyrosinated proteins has been demonstrated, consistent with endogenous $ONOO^-$ formation in these disorders (Beal, 1997). In addition, an increasing body of evidence now suggest INOS induction in the brain of patients that died from multiple sclerosis (Bö et al., 1994) and in the cerebrospinal fluid of multiple sclerosis patients (Johnson et al., 1995). Interestingly, in vitro, interferon-β, which is currently used in the treatment of this disease, impairs INOS induction in astrocytes (Stewart et al., 1997), underscoring the role of INOS in the development of multiple sclerosis and pointing to the possible value of therapeutic strategies aimed at preventing excessive •NO formation in certain neurological disorders.

Other mitochondrial targets for nitric oxide/peroxynitrite

Other mitochondrial targets may also be responsible for nitric oxide- and/or peroxynitrite-mediated energy deficiency. Thus, at least in macrophages and hepatocytes, Hibbs and colleagues (Drapier and Hibbs, 1988; Hibbs et al., 1988) have shown that INOS induction is accompanied by a decreased O_2 consumption compatible with the inhibition of aconitase activity. More recently, Hausladen and Fridovich (1994) and Castro et al. (1994) have shown that $ONOO^-$ and $O_2^{\bullet -}$, but not •NO, are responsible for such inhibition, possibly due to an irreversible interaction of $O_2^{\bullet -}$ and $ONOO^-$ with the iron–sulphur prosthetic group of the purified enzyme. On the other hand, treatment of microglial cells with a combination of azide plus LPS causes inhibition of α-ketoglutarate dehydrogenase activity (Park et al., 1999), an effect that appears to be due to endogenously produced $ONOO^-$. Whether $O_2^{\bullet -}$ and/or $ONOO^-$mediated blockade of these Krebs cycle enzymes results in a disruption of Krebs cycle activity followed by neurotoxicity remains to be established. If so, such a mechanism might subsequently deplete $NADH(H^+)$ and $FADH_2$, hence contributing to the mitochondrial energy impairment caused by $ONOO^-$.

Glutamate-mediated activation of NNOS and neurotoxicity

Excitotoxicity, i.e. neurotoxicity due to the exposure of neurones to glutamate is, at least partially, •NO-mediated (Dawson et al., 1991; Schulz et al., 1995). Thus, brief exposure of neuronal cultures to glutamate receptor agonists, such as *N*-methyl-D-aspartate (NMDA) transiently elevates intracellular calcium concentrations, which return to basal levels within seconds. However, this short-term calcium elevation may initiate processes that contribute to delayed neurotoxicity. Despite this, it should be mentioned that some laboratories have not been able to reproduce the •NO-mediated neurotoxicity of glutamate-receptor activation (Pauwels and Leysen, 1992; Garthwaite and Garthwaite, 1994; Maiese et al., 1995), and have therefore questioned the absolute role of •NO in excitotoxicity. Nevertheless, this kind of excitotoxicity requires $O_2^{\bullet -}$ (Patel et al., 1996) or •NO plus $O_2^{\bullet -}$ formation (Lafon-Cazal et al., 1993; Gunasekar et al., 1995), suggesting that in situ $ONOO^-$ formation is required for excitotoxicity (Lipton et al., 1993).

Possible mechanisms have been proposed to explain ATP deficiency in excitotoxicity. Thus, nitric oxide- and/or peroxynitrite-mediated activation

of poly(ADP-ribose) synthetase, an ATP-consuming nuclear enzyme that synthesises poly(ADP-ribose) from NAD^+, has been shown to lower cellular energy levels (Zhang et al., 1994). Nitrosylation and possible inhibition of the activity of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Molina y Vedia et al., 1992; Zhang and Snyder, 1992) have also been proposed. However, this hypothesis has been questioned on the grounds that only under severe anaerobic conditions will such inhibition of glycolysis be relevant. Furthermore, as shown below, induction of INOS in astrocytes is accompanied by the activation of glycolysis (Bolaños et al., 1994), despite possible modifications of glyceraldehyde-3-phosphate dehydrogenase.

Among the hypotheses advanced to account for the mechanism for excitotoxicity, the mitochondrial depolarisation that occurs shortly after treatment seems to be the most plausible explanation (Wang et al., 1994; Ankarcrona et al., 1995; Isaev, 1996; Khodorov et al., 1996; Schindler, 1996; White and Reynolds, 1996) (reviewed in Nicholls and Ward, 2000). Moreover, this phenomenon has recently been shown to be an •NO-mediated process (Almeida et al., 1999; Keelan et al., 1999). In fact, short-term exposure of neurones to glutamate irreversibly disrupts the mitochondrial respiratory chain, specifically at complex II–III (Almeida et al., 1998), possibly contributing to mitochondrial depolarisation. However, the primary event leading to membrane potential collapse appears to be related to the mitochondrial Ca^{2+} overload that is brought about shortly after glutamate treatment (White and Reynolds, 1996). This massive Ca^{2+} accumulation within this organelle may account for mitochondrial permeability pore opening that also occurs in these circumstances (reviewed in Crompton, 1999; Nicholls and Ward, 2000). Apparently, glutamate-mediated mitochondrial pore opening may be related to neuronal apoptosis observed under certain conditions (Ankarcrona et al., 1995; Bonfoco et al., 1995). Nevertheless, the exact mechanism(s) leading/rescuing to/from necrosis or apoptosis remains to be fully elucidated. In this context, it should be mentioned that glutamate receptor stimulation is accompanied by nuclear factor κB activation (Kaltschmidt et al., 1995), strongly suggesting that activation of this factor during excitotoxicity may be involved in a compensative neuroprotective mechanism that still needs to be explained in full (reviewed in Mattson et al., 2000).

Different cell susceptibility to nitric oxide-mediated mitochondrial damage and cell death

Mitrovic et al. (1994) showed that oligodendrocytes are the most susceptible glial cell type to •NO-mediated succinate dehydrogenase inhibition and cell death. Bearing in mind the relevance of INOS induction and oligodendrocyte degeneration in multiple sclerosis, the results of Mitrovic et al. (1994) suggest one possible mechanism that could be invoked to explain the demyelinating process accompanying this neurological disease. Furthermore, this study also reported that different cell types have different susceptibilities to •NO-mediated damage. Thus, a subsequent study by Bolaños et al. (1995) reported a highly dose-dependent (0.01–0.5 mM) susceptibility of neurones towards complex II–III and IV inhibition (Fig. 2). In contrast, astrocytes were completely resistant to peroxynitrite-mediated mitochondrial damage, at least up to 2 mM (Fig. 2).

Peroxynitrite-mediated inhibition of mitochondrial complex activities precedes neuronal cell death (Fig. 3) (Bolaños et al., 1995), suggesting that the impairment of mitochondrial ATP biosynthesis might be a mechanism involved in this neurotoxicity. The exact mechanism leading to mitochondrial complex II–III and IV impairment after exogenously added $ONOO^-$ is difficult to ascertain, but a direct effect of $ONOO^-$ on mitochondrial complexes may be advanced. In this sense, as mentioned above, Sharpe and Cooper (1998) reported the direct inhibition of purified complex IV brought about by $ONOO^-$. However, the possibility that, in the intact cell, exogenous $ONOO^-$ might react with other cellular components before it reaches the mitochondrial complex proteins cannot be ruled out. This issue is examined in the following section.

Glutathione contents govern the susceptibility to nitric oxide/peroxynitrite-mediated mitochondrial damage in neural cells

It is interesting to note that $ONOO^-$ avidly reacts with thiol-containing groups (Radi et al., 1991), such

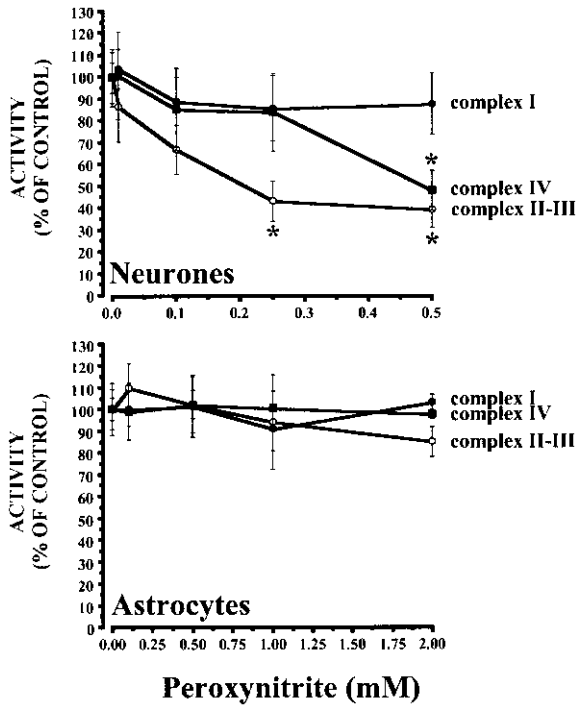

Fig. 2. Peroxynitrite inhibits the mitochondrial respiratory chain in neurones, but not in astrocytes. Neurones or astrocytes were incubated with peroxynitrite at the indicated concentrations for 5 min, but cells were further incubated for 24 h in DMEM. This treatment caused a dose-dependent inhibition of complexes II–III and IV, without affecting the activity of complex I in neurones (top panel). However, astrocytes were unaffected after incubation with peroxynitrite up to 2 mM (bottom panel). Control values were as follows. For neurones: complex I, 10.0 ± 0.7 nmol/min/mg; complex II–III, 10.6 ± 1.4 nmol/min/mg; complex IV, 2.23 ± 0.26 k/min/mg. For astrocytes: complex I, 60.0 ± 5.6 nmol/min/mg; complex II–III, 10.1 ± 1.2 nmol/min/mg; complex IV, 2.15 ± 0.11 k/min/mg, where k is the first rate order constant. *Statistically different ($P < 0.05$) from the corresponding control values. Data obtained with permission from Bolaños et al. (1995).

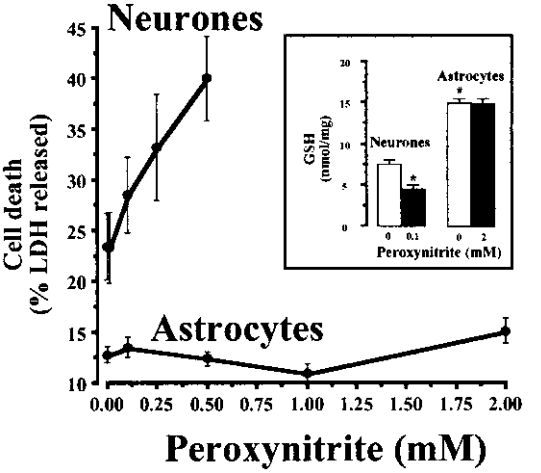

Fig. 3. Peroxynitrite causes cell death in neurones, but not in astrocytes. Neurones or astrocytes were incubated with peroxynitrite at the indicated concentrations for 5 min, but cells were further incubated for 24 h in DMEM. The medium was collected for the measurement of lactate dehydrogenase (LDH) activity released, which was expressed as a percentage of total (cells plus medium) LDH and used as an index of cell death. Cells were collected for GSH determination by HPLC with electrochemical detection (inset). Peroxynitrite caused a dose-dependent increase in LDH release and GSH depletion in neurones. However, LDH released and GSH from astrocytes were unaffected after incubation with peroxynitrite up to 2 mM. *Statistically different ($P < 0.05$) from 0 mM. #Statistically different ($P < 0.05$) when compared with control neurone values. Data obtained with permission from Bolaños et al. (1995).

as reduced glutathione (GSH) (Clancy et al., 1994). Thus, increases in GSH concentration are accompanied by a reduction in $ONOO^-$-mediated complex I–III damage in brain submitochondrial particles (Lizasoain et al., 1996). In the intact cell systems used in our work (Bolaños et al., 1995), $ONOO^-$ caused mitochondrial damage in neurones, but not astrocytes, which is consistent with a different cellular thiol composition. Thus, GSH contents in neurones are much lower than in astrocytes (Fig. 3, inset) (Raps et al., 1989; Sagara et al., 1993; Makar et al., 1994; Bolaños et al., 1995), supporting the notion that the absence of any effect of $ONOO^-$ on mitochondrial function in intact astrocytes may be due to the rapid clearance of $ONOO^-$ brought about by astrocytic GSH. In contrast, the lower GSH concentration found in neurones seems to be insufficient to scavenge exogenously added $ONOO^-$ and, therefore, mitochondrial damage occurs (Bolaños et al., 1995). Interestingly, isolated brain mitochondria (hence lacking the $ONOO^-$ scavenging potential of cytosolic GSH) exposed to $ONOO^-$ for 5 min displayed a similar pattern of mitochondrial respiratory chain inhibition to that shown by intact neurones (Bolaños et al., 1995).

The role of GSH in dictating cell susceptibility to oxidative stress has long been known. Thus, alteration in GSH concentrations in the same cell type causes mitochondrial damage in a number of

cell systems (Benzi et al., 1991; Jain et al., 1991; Benzi and Moreti, 1995; Heales et al., 1995; Walker et al., 1995; Niknahad and Obrien, 1996). In cultured neurones, GSH depletion by incubation with L-buthionine sulphoximine, an inhibitor of GSH biosynthesis, is accompanied by inhibition of all components of the mitochondrial respiratory chain (Bolaños et al., 1996). Whether mitochondrial damage after GSH depletion is due to increased nitric oxide biosynthesis, as observed in vivo (Heales et al., 1995), remains to be elucidated. In astrocytes, the activity of complexes I and II–III are decreased by $ONOO^-$ only after GSH depletion, but not under normal conditions (Barker et al., 1996). This observation strongly suggests that the cell glutathione status (Raps et al., 1989; Sagara et al., 1993), or the ability to newly synthesise glutathione (Makar et al., 1994; Dringen et al., 1997) may play a relevant role in $^{\bullet}NO$-mediated neurotoxicity (Barker et al., 1996; Bolaños et al., 1996).

Astrocytic-released arginine as a potential protective mechanism against peroxynitrite-mediated neurotoxicity

As mentioned above, astrocytes can be considered as the brain's GSH reservoir (Raps et al., 1989; Sagara et al., 1993; Makar et al., 1994; Bolaños et al., 1995). Thus, we have shown that the neuronal GSH concentration is doubled after a 24-h incubation period with astrocytes (Bolaños et al., 1996); this doubled concentration is thought to protect neurones against oxidative stress. However, free arginine is also predominantly located in astrocytes (Aoki et al., 1991; Pow, 1994; Kharazia et al., 1997), which have the y^+ cationic amino acid-carrier system for arginine plasma membrane transport (Schmidlin and Wiesinger, 1994). Neurones are able to uptake this amino acid because they have the same carrier system (Westergaard et al., 1993). However, unlike in astrocytes, the arginine content in neuronal cells is very low and is limiting for nNOS activity (Garthwaite et al., 1989; Culcasi et al., 1994). Under these conditions, or in the absence of sufficient superoxide dismutase activity (Schmidt et al., 1996), glutamate-receptor stimulation actively produces $ONOO^-$ in neurones. Since this compound is highly liposoluble (Beckman et al., 1990; Radi et al., 1991), its diffusion between neurones and astrocytes is quite conceivable in the in vivo situation.

Grima et al. (1998) have recently demonstrated that the rate of nNOS activity in neurones depends on the supply of arginine from glial cells. Furthermore, glial-derived arginine is increased upon activation of non-NMDA ionotropic glutamate receptors (Grima et al., 1997), although the precise nature of the molecules involved in the intracellular signalling cascade leading to arginine release is not known. In this context, we have shown that $ONOO^-$ and $O_2^{\bullet -}$, but not $^{\bullet}NO$ donors or H_2O_2, stimulate arginine release in cultured rat astrocytes (Fig. 4) (Vega-Agapito et al., 1999). Moreover, whereas the effect of $O_2^{\bullet -}$ was non-specific, the effect of $ONOO^-$ appeared to be mediated by arginine transporter activation. Thus, incubation of cells with L-lysine, one of the most potent inhibitors of arginine transport in glial cells (Schmidlin and Wiesinger, 1994), prevents $ONOO^-$-mediated arginine release in a concentration-dependent way (Fig. 4). This suggests that the effect of $ONOO^-$ on arginine release would be mediated by the y^+ arginine transporter.

Since neurones are able to efficiently take up arginine from the extracellular fluid (Westergaard et al., 1993), a replenishment of cytosolic arginine could facilitate the formation of physiological $^{\bullet}NO$ instead of neurotoxic $ONOO^-$ by neurones. If so, this neuronal–glial interaction could represent a neuroprotective mechanism brought about by astrocytes aimed at preventing the propagation of neuronal death upon excess glutamate receptor activation.

Neuroprotective role of glucose metabolism against peroxynitrite-mediated glutathione depletion

The mitochondrial inhibition caused by iNOS induction in astrocytes is accompanied by increased glucose consumption and lactate accumulation (Fig. 5) (Bolaños et al., 1994), suggesting an enhanced glycolytic rate. Moreover, this phenomenon is completely prevented by NMMA and SOD/CAT (Fig. 5), strongly suggesting that this is a $^{\bullet}NO$-/$ONOO^-$-mediated effect. Glycolytic activation may be a response aimed at compensating the inhibition of mitochondrial ATP synthesis (Fig. 1) and is possibly brought about by a transient decrease

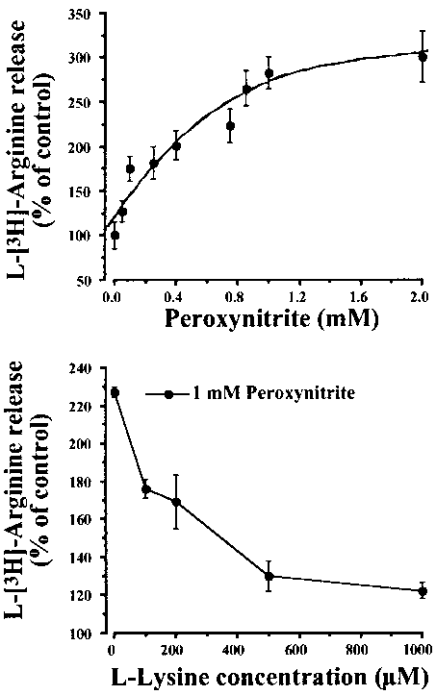

Fig. 4. Peroxynitrite stimulates L-arginine release from astrocytes. Astrocytes were loaded with L-[2,3,4,5-^{3}H]arginine for 30 min, washed, and further incubated for 20 min with a buffer change every 5 min until the basal radioactivity released was constant. At minute 25, peroxynitrite (0.05–2 mM) or degraded peroxynitrite (control cells) were added; cells were incubated for a further 5 min and the radioactivity released was quantified. The stimulated release of arginine in the presence of peroxynitrite was expressed as percentages as compared to control cells (100% value). As shown, incubation with peroxynitrite stimulated L-arginine release from astrocytes in a dose-dependent fashion (top panel). Furthermore, peroxynitrite (1 mM)-stimulated L-arginine release was dose-dependently prevented by the competitive y^+ carrier inhibitor L-lysine, which was present throughout the experiment (bottom panel). Control release was 1184 ± 61 cpm/10^6 cells (top panel) and 1086 ± 31 cpm/10^6 cells (bottom panel). Data obtained with permission from Vega-Agapito et al. (1999).

in the ATP/ADP ratio, a well-known phosphofructokinase 1 activation factor. However, this particular issue remains to be fully elucidated. Interestingly, LPS-mediated iNOS induction in astrocytes is accompanied by a concomitant increase in glucose uptake, possibly due to induction of the GLUT-1 glucose transporter (P. Cidad, P. García-Nogales and J.P. Bolaños, submitted), in agreement with the idea that increased intracellular glucose availability may be beneficial against nitric oxide toxicity.

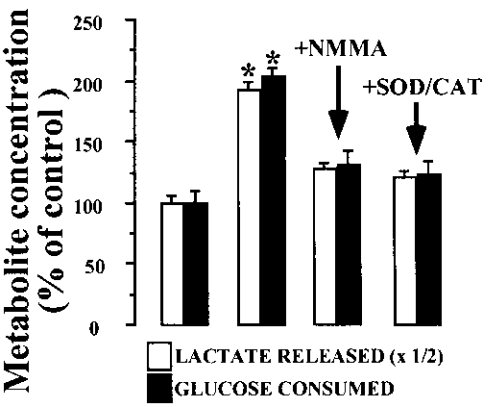

Fig. 5. Induction of iNOS is accompanied by the stimulation of lactate release and glucose consumption in astrocytes. Incubation of astrocytes with LPS/IFN-γ (1 μg/ml plus 100 U/ml, respectively) over 18 h was accompanied by a stimulation of lactate release and glucose consumption, an effect which was fully prevented by NMMA (1 mM), or SOD/CAT (250 U/ml each). Control values were as follows: lactate released, 25.9 ± 1.9 μmol/mg; glucose consumed, 14.9 ± 1.4 μmol/mg. *Statistically different ($P < 0.05$) from the corresponding control values. Data obtained with permission from Bolaños et al. (1994).

In spite of glycolysis, alternative pathways for glucose utilisation in protecting the brain against nitric oxide-mediated mitochondrial damage cannot be ruled out. Thus, glucose metabolisation through the pentose-phosphate pathway (PPP) activity accounts for an important cellular supply of NADPH (Kletzien et al., 1994). Since NADPH is a necessary factor for GSH to be regenerated from GSSG, PPP activity may be an important factor involved in the regulation of GSH contents. Thus, glucose efficiently protects neurones against neurotoxicity and mitochondrial damage due to glutamate receptor stimulation in primary neurones, possibly through glucose metabolisation through the PPP for NADPH production and GSH regeneration from GSSG (M. Delgado-Esteban, A. Almeida and J.P. Bolaños, submitted). Moreover, iNOS induction in astrocytes by LPS (1 μg/ml) is accompanied by increased transcriptional expression of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting step in the PPP in a time-dependent fashion (Figs. 6 and 7) (García-Nogales et al., 1999). This effect is accompanied by the induction of enzyme activity (Fig. 6) and also of PPP activity (Fig. 9), suggesting a possible functional role for G6PD. Inhibition of iNOS activity

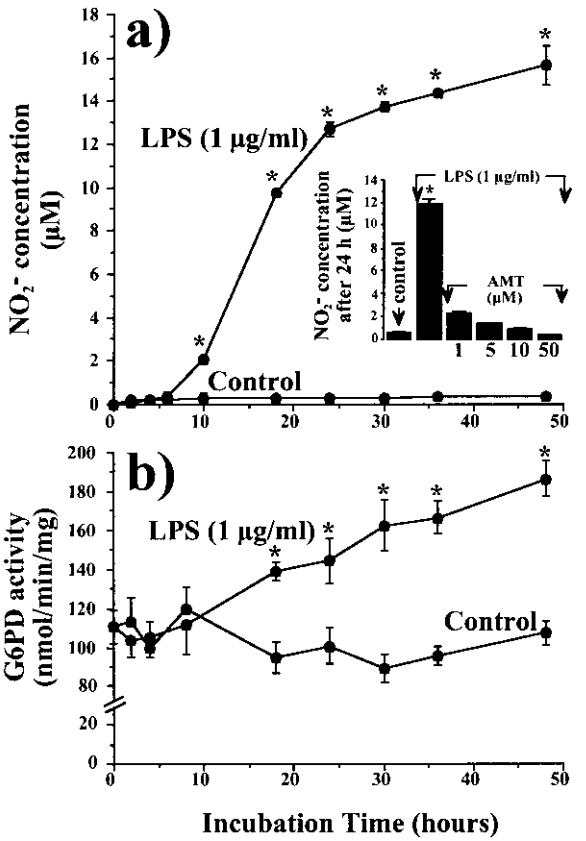

Fig. 6. iNOS induction is accompanied by a time-dependent increase in glucose-6-phosphate dehydrogenase (G6PD) activity in astrocytes. Astrocytes were incubated in the presence of LPS (1 μg/ml) in DMEM for the indicated time periods. Media were removed for NO_2^- analysis and cells collected for enzyme activity analysis. The inset in a shows the effect of the selective iNOS inhibitor AMT in preventing the LPS (1 μg/ml)-mediated increase in NO_2^- concentrations in the medium. *Statistically different ($P < 0.05$) from the corresponding control values. Data obtained with permission from García-Nogales et al. (1999).

with 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT), a selective iNOS inhibitor (Nakane et al., 1995) does not alter LPS-mediated increased G6PD mRNA expression (Fig. 7). By contrast, blockade of NF-κB activation with *N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal (ZIE) completely prevents LPS-mediated G6PD mRNA expression (Fig. 7). Since the activation of NF-κB is an essential requirement for LPS-mediated iNOS transcription (Lowenstein et al., 1993; Xie et al., 1994), our results strongly suggest that both iNOS and G6PD are co-induced through a common transcriptional pathway involving NF-κB activation.

Due to its co-expression with iNOS, a possible role for G6PD induction in protecting astrocytes against •NO-mediated cellular damage might be advanced. In fact, treatment of astrocytes with LPS and/or certain cytokines triggers the production of large amounts of •NO, with no apparent signals of cytotoxicity (Galea et al., 1992; Simmons and Murphy, 1992; Bolaños et al., 1994). As mentioned in the above sections, the intracellular content of GSH may be a key factor in dictating different cellular susceptibilities to •NO/$ONOO^-$-mediated mitochondrial dysfunction and cell death (Bolaños et al., 1995, 1996; Clementi et al., 1998). We therefore investigated the possible role of G6PD induction in maintaining GSH levels in •NO-synthesising astrocytes. LPS treatment increased intracellular NADPH concentrations after 24 h of incubation, an effect which was abolished by the inhibition of PPP activity with the G6PD inhibitor dehydroepiandrosterone (DHEA) (Fig. 8) (Tian et al., 1998). Moreover, inhibition of G6PD activity with DHEA in LPS-treated astrocytes increased GSSG concentrations by 2-fold after 24 h of incubation (Fig. 9), resulting in a fall in the GSH/GSSG ratio from 15.9 to 7.8. These results suggest that induction of G6PD by LPS in astrocytes may be involved in supplying NADPH for GSH regeneration from GSSG (Kletzien et al., 1994). Interestingly, the presence of AMT abolished the DHEA-mediated decrease in the GSH/GSSG ratio (Fig. 9) highlighting the important role of increased G6PD activity in protecting astrocytes against •NO-mediated alterations in glutathione metabolism. These results suggest that G6PD induction may contribute to the high resistance of astrocytes to •NO/$ONOO^-$-mediated cellular damage. Since the activity of other putative enzymes, such as malic enzyme or isocitrate dehydrogenase, are also responsible for intracellular NADPH generation, further work is required to elucidate the relative importance of glucose-metabolising pathways within the overall defense machinery of the brain under conditions of excess •NO biosynthesis, such as those occurring in certain neurodegenerative diseases.

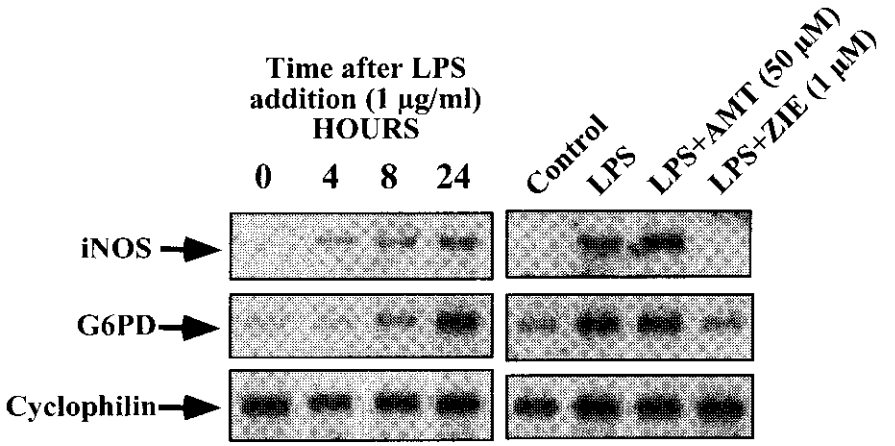

Fig. 7. iNOS induction is accompanied by a time-dependent, NF-κB-mediated increase in glucose-6-phosphate dehydrogenase mRNA levels. Astrocytes were incubated in DMEM containing LPS (1 μg/ml) alone for the indicated time periods (left panel), or in combination with the nuclear factor κB (NF-κB) inhibitor ZIE (1 μM) or iNOS inhibitor AMT (50 μM) for 24 h. After incubation, the medium was immediately removed and total RNA was extracted from the cells for G6PD and iNOS mRNA analysis by Northern blot using the appropriate cDNA probes. Cyclophilin was used as an mRNA loading marker. Figure obtained with permission from García-Nogales et al. (1999).

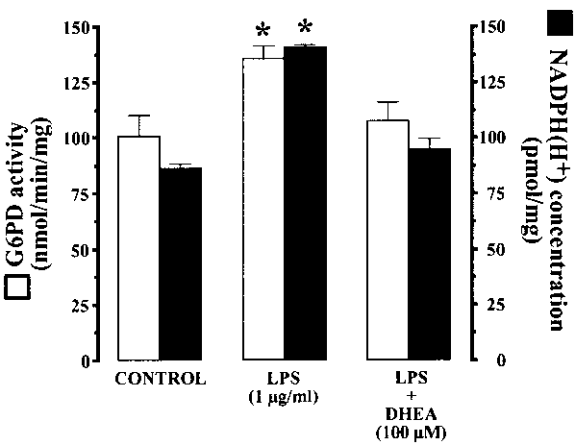

Fig. 8. G6PD induction is accompanied by an increase in NADPH concentration. Astrocytes were incubated in DMEM containing the indicated compounds for 24 h. For G6PD activity determinations, cells were trypsinized, pelleted, homogenized in 0.1 M potassium phosphate buffer (pH 7.0), and frozen (−20ºC) until enzyme activity analysis. For NADPH determination, cells were digested in 0.5 M KOH in 50% (v/v) ethanol, neutralized and used immediately for NADPH analysis. DHEA, dehydroepiandrosterone. *Significantly different from the corresponding control value. Data obtained with permission from García-Nogales et al. (1999).

Concluding remarks

The mechanisms responsible for the interference of nitric oxide with mitochondrial function in brain cells, and the potential neuroprotective role of glucose metabolism are reviewed. In brief, physiological •NO may regulate mitochondrial activity through its competition with oxygen for complex IV of the mitochondrial respiratory chain in astrocytes. In contrast, excessive or inappropriate •NO formation reacts with $O_2^{\bullet -}$ to form $ONOO^-$, which irreversibly damages the mitochondrial respiratory chain in both astrocytes and neurones. Whilst the former seems to be a physiological mechanism designed to regulate mitochondrial activity, the latter may be responsible for the development of certain neuropathological situations. On the other hand, glucose uptake and metabolism seem to be key factors in protecting neural cells against excess •NO. Thus, iNOS induction in astrocytes is accompanied by a concomitant upregulation of glucose uptake, a phenomenon that is accompanied by an increased rate of glycolysis and glucose-6-phosphate dehydrogenase activity, the first rate-limiting step in the pentose phosphate pathway. Therefore, glycolytic ATP generation compensates for mitochondrial inhibition, while NADPH formation is useful for glutathione regeneration under conditions of excess $ONOO^-$ formation. In conclusion,

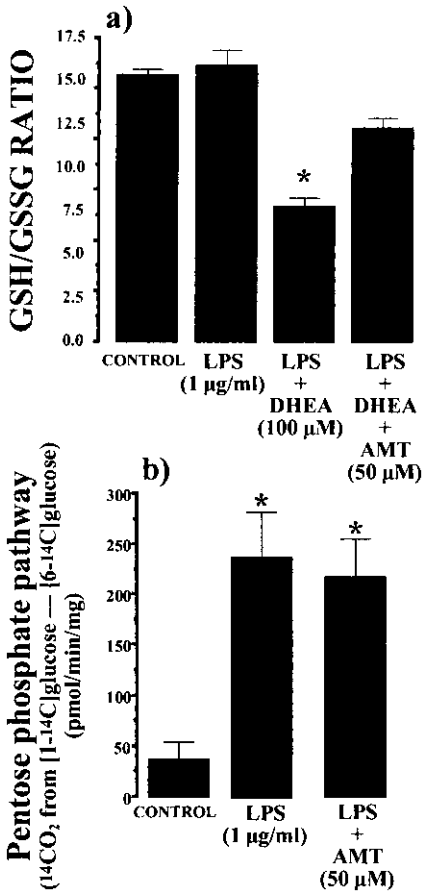

Fig. 9. Increased pentose-phosphate pathway activity prevents glutathione oxidation in astrocytes. Astrocytes were incubated in DMEM containing the indicated compounds for 24 h and cells were scraped with 1% (w/v) sulphosalicylic acid for glutathione determinations. In parallel experiments, cells were resuspended in an O_2-saturated incubation buffer (pH 7.4) and incubated in the presence of 5.5 mM D-glucose and the inhibitors, where appropriate, with either D-[1-^{14}C]glucose (0.5 μCi) or D-[6-^{14}C]glucose (2 μCi) in sealed-Erlenmeyer flasks at 37ºC. After 20 min, incubation was stopped by injection of $HClO_4$ through the cap, and CO_2 was collected in benzethonium hydroxide placed in central wells. Radioactive $^{14}CO_2$ was quantified by liquid scintillation counting. The pentose-phosphate pathway (PPP) was calculated as the $^{14}CO_2$ collected from D-[1-^{14}C]glucose minus the $^{14}CO_2$ collected from D-[6-^{14}C]glucose. *Significantly different from the corresponding control value. Data obtained with permission from García-Nogales et al. (1999).

glial cells have efficient mechanisms aimed at preventing $ONOO^-$-mediated cellular damage. Whether these mechanisms operate in neurones remains to be elucidated. In any case, these results suggest that up-regulation of glucose metabolism in neuronal cells may be helpful in protecting cells against $ONOO^-$-mediated glutathione depletion and neurotoxicity.

Abbreviations

AMT	2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine
CAT	catalase
CNS	central nervous system
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
e, i or nNOS	endothelial, inducible or neuronal nitric oxide synthase
G6PD	glucose-6-phosphate dehydrogenase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
IFN-γ	interferon-γ
NF-κB	nuclear factor κB
NMDA	*N*-methyl-D-aspartate
NMMA	*N*-monomethylarginine
PPP	pentose-phosphate pathway
SOD	superoxide dismutase
ZIE	*N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal

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