Nitric oxide inactivation in brain by a novel O_2-dependent mechanism resulting in the formation of nitrate ions

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In order for nitric oxide (NO) to function as a biological messenger it has to be inactivated, but little is known of how this is achieved. In cells from the brain, we have recently shown the existence of a powerful NO sink that 'shapes' NO signals for targeting its receptor, soluble guanylate cyclase, whilst simultaneously preventing NO rising to toxic concentrations [Griffiths and Garthwaite (2001) J. Physiol. (Cambridge, U.K.) **536**, 855–862]. In the present study, the properties of this sink were investigated further. Inactivation of NO was preserved in rat brain homogenates. In both cerebellar cell suspensions and brain homogenates, NO inactivation required O_2 and, from measurements in homogenates, the principal end-product was NO_3^-, which is also the main product of endogenously formed NO *in vivo*. Direct chemical reaction with O_2, superoxide anions or haemoglobin was not responsible. Consumption of NO was, however, inhibited by heat or protease treatment. Pharmacological tests were negative for several candidate enzymes, namely cytochrome *c* oxidase, H_2O_2-dependent haem peroxidases, prostaglandin H synthase, 12/15-lipoxygenase and a flavohaemoglobin-like NO dioxygenase. The capacity of the NO sink in cells was limited because regeneration of the activity was slow (2 h). It is concluded that NO is consumed in the brain through a novel protein, ultimately forming NO_3^-, and that the slow regeneration of the activity provides a scenario for NO to become toxic.

Key words: cGMP, neurodegeneration, respiration.

INTRODUCTION

Nitric oxide (NO) is an intercellular signalling molecule that performs a variety of roles throughout the body. Many of the physiological effects of NO are mediated by binding to its receptor, soluble guanylate cyclase (sGC), resulting in cGMP synthesis. The cGMP signalling pathway in cells is engaged by low NO concentrations, with half-maximal activation of sGC occurring at 2–20 nM [1,2]. Higher NO concentrations (approx. micromolar) are known to have pathological consequences, including lipid peroxidation, DNA damage and the inhibition of respiration [3]. Thus the NO concentration is critical in determining whether it acts as a physiological signal or pathological agent. Tissue NO concentrations reflect the balance between the rates of NO synthesis and breakdown. To date, much has been learnt about the enzymic synthesis of NO [4], but little is understood of how it is inactivated. Without a biological termination mechanism, NO would be susceptible to the vagaries of chemical decay, which is an unlikely fate.

It has been known for many years that the decay of NO is accelerated in *in vitro* biological systems, an effect that is partially attributable to reaction with superoxide anions [5,6]. Another well known reaction is between NO and haemoglobin in circulating red blood cells, but the extent to which this contributes to the biological inactivation of NO remains uncertain [7,8]. An O_2-dependent NO degrading activity has been reported in isolated liver cells [9] and very recently, various cell lines were found to consume NO in a reaction that required O_2 and was inhibited by haem poisons, suggesting the operation of an enzyme analogous to the bacterial flavohaemoprotein, NO dioxygenase [10].

The brain is particularly enriched in the NO signalling pathway and we have recently found that cells from one brain area, the cerebellum, are equipped with a powerful NO consuming activity [2]. The activity could not be attributed to reaction with superoxide ions or contaminating red blood cells. Moreover, the properties of the sink suggested that it would impose on NO a half-life ($t_{1/2}$) of approx. 100 ms, a duration comparable with that of some other neural signalling molecules, such as noradrenaline and dopamine, and with the $t_{1/2}$ of NO estimated for liver [9] and heart [11]. Functionally, evidence was obtained that the NO sink in the brain serves as a device for 'shaping' NO signals for targeting sGC whilst, at the same time, providing protection against the damaging effects of sustained NO production [2].

In addition to flavohaemoglobin, several enzymes have been reported to influence NO breakdown *in vitro*, including cytochrome *c* oxidase [12], lipoxygenases [13,14], peroxidases [15] and prostaglandin H synthase [16]. The aim of the present study was to investigate further the properties of the NO sink in brain tissue and determine if any of these suggested mechanisms could account for the activity.

EXPERIMENTAL

Experiments used brain tissue from 8-day-old Sprague–Dawley rats. The animals were anaesthetized, perfused transcardially with PBS to flush out red blood cells, and then killed by decapitation and associated exsanguination as approved by the U.K. Home Office and the local ethics committee. Homogenates of whole brain were made in 20 mM Tris/HCl buffer (pH 7.4 at

Abbreviations used: DPI, diphenylene iodonium; ETYA, eicosatetraynoic acid; NO, nitric oxide; DETA/NO, diethylenetriamine/NO adduct; PROLI/NO, proline/NO adduct; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; $t_{1/2}$, half-life.

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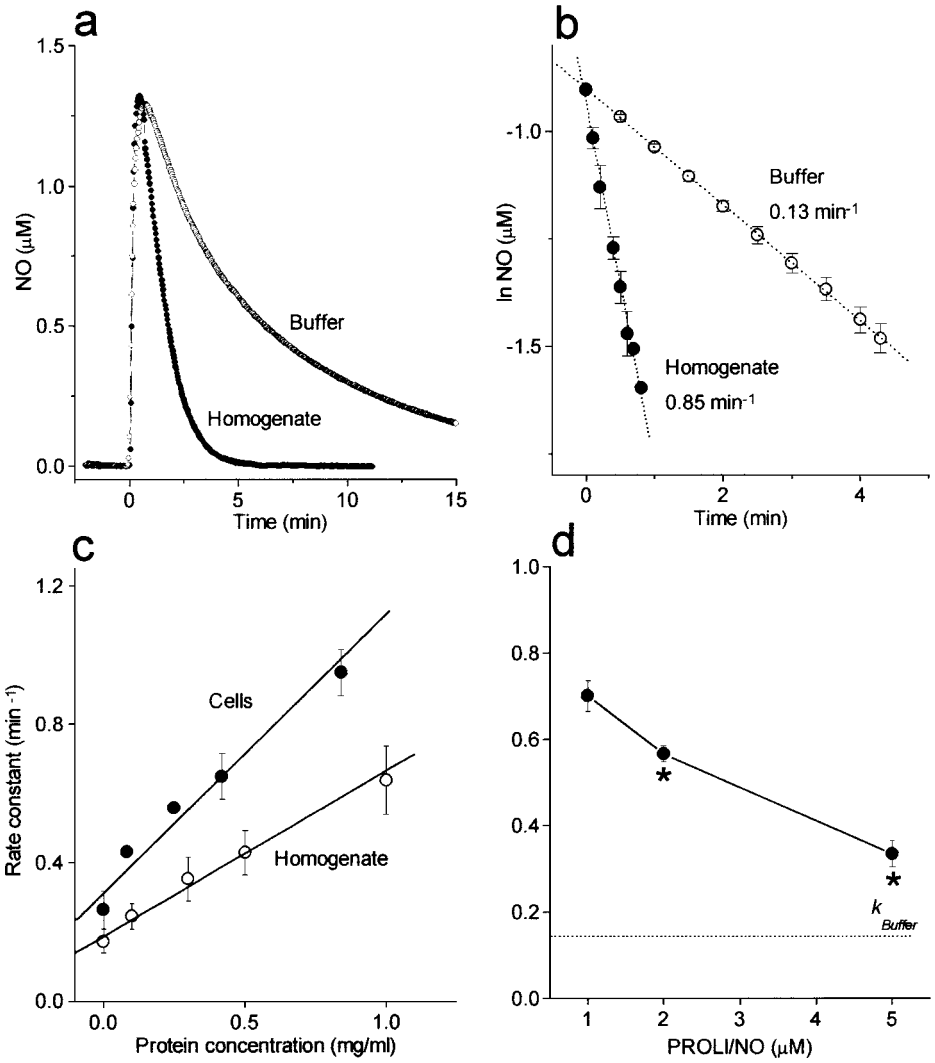

Figure 1 Acceleration of the decay of NO in rat brain tissue

(**a**) The profiles of NO over time are shown following the addition of 1 μM PROLI/NO to buffer [20 mM Tris/HCl (pH 7.4)] or homogenate (1.5 mg/ml protein). Data are the mean of three individual traces. (**b**) Data from (**a**) at 0.2–0.4 μM NO are plotted semi-logarithmically to obtain rate constants. (**c**) Rate constants obtained following the addition of 1 μM PROLI/NO to various concentrations of cerebellar cell suspensions and brain homogenates are plotted against protein concentration. (**d**) Rate constants obtained following the addition of PROLI/NO to brain homogenates (1.0 mg/ml protein) are plotted against PROLI/NO concentration. The line marked k_{buffer} is the rate constant in buffer alone. Data in (**b**–**d**) are means $\pm$ S.E.M. ($n = 3$). $^*P < 0.05$ compared with 1 μM PROLI/NO.

4 °C). Cell suspensions were prepared as described previously [17], except that the pups were not pretreated with hydroxyurea. The cell-incubation medium contained: 130 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 15 mM Tris/HCl and 11 mM glucose, and was adjusted to pH 7.4 at 37 °C. Unless otherwise stated, all experiments contained superoxide dismutase (SOD, 100–1000 units/ml). Protein concentrations were measured by the bicinchoninic acid method, and cGMP by RIA.

For measurements of NO and O_2 concentrations, samples (1 ml) of cell suspension were incubated in a sealed, stirred vessel (at 37 °C) equipped with an oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.) and an NO electrode (Iso-NO; World Precision Instruments, Stevenage, Herts., U.K.). Two NONOate donors were used to supply NO: proline/NO adduct (PROLI/NO) and diethylenetriamine/NO adduct (DETA/NO), both from Alexis, Nottingham, U.K. Stock solutions were made in 10 mM NaOH and kept on ice until use. To describe the decay of NO quantitatively following addition of PROLI/NO, the rate of decline over the concentration range 0.2–0.4 μM was analysed by plotting the natural logarithm of the concentration against time. Over the range measured this was linear, and the rate constant was calculated from the gradient. In order to deplete O_2, samples were incubated with 1 unit/ml ascorbate oxidase and 1 mM ascorbate in the sealed incubation chamber and the O_2 concentration was monitored. Experiments began only after the O_2 concentration within the chamber was stable at approx. 0 μM for > 3 min. The formation of the NO breakdown products NO_2^- and NO_3^- from 1 μM PROLI/NO was determined by allowing the donor to decompose in incubation buffer or homogenate at various protein concentrations for 30 min at 37 °C, and then measuring by chemiluminescence the concentrations of NO_2^- and, following treatment with NO_3^- reductase, NO_2^- plus NO_3^- [18]. Where proteinase K was used, samples were supplemented

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with 1 mM $CaCl_2$ and incubated with 0.1 mg/ml proteinase K immobilized on acrylic beads (Sigma, Poole, Dorset, U.K.) for 30 min at 37 °C. The beads were removed by centrifugation at 2300 *g* for 1 min and the activity of the supernatant was assessed. To verify protein digestion, samples were separated by SDS/PAGE [10 % (w/v) gel] and the proteins were detected with a Silver Staining Kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Data are presented as means ± S.E.M., with each determination (n) being an individually prepared and treated sample. Statistical comparisons were carried out using Student's t test.

RESULTS

Measurements of NO consumption initially used the NONOate PROLI/NO (1 μM), which has a $t_{1/2}$ of 1.8 s at 37 °C [19]. In buffer alone, the peak NO concentration (approx. 1.25 μM, truncated by the electrode response time) was followed by a gradual decline to approx. 0.25 μM over 10 min (Figure 1a). When the experiment was repeated using whole brain homogenate (1.5 mg/ml protein), NO inactivation was accelerated such that no detectable NO remained after 6 min (Figure 1a). Approx. 20 s after addition of PROLI/NO more than 99 % of the donor has decomposed and the rate of NO disappearance corresponds to the rate of NO consumption, provided the electrode response time is not limiting. The rate of decline of NO over the concentration range 0.2–0.4 μM was selected for quantification on the basis of these considerations and, as before [2], apparent first-order kinetics was observed (Figure 1b). The inactivation rate constant was 0.13 ± 0.01 min^{-1} ($n = 3$) and in whole brain homogenate it increased approx. 7-fold to 0.85 ± 0.07 min^{-1} ($n = 3$). A similar value for the homogenate (0.81 ± 0.08 min^{-1}; $n = 3$) was obtained in PBS rather than the usual Tris/HCl buffer. At equivalent protein concentrations, the rate of NO consumption by the brain homogenate measured using this method was approximately half that found in intact cerebellar cells, and in both preparations, the activity varied in proportion to protein content (Figure 1c). As the concentration of PROLI/NO was increased above 1 μM, the NO inactivation rate constant decreased (Figure 1d), consistent with the mechanism being saturable (see below).

Inactivation of NO by liver cells [9] and some cell lines [10] has been reported to be O_2-dependent. Similarly, in the present experiments, depletion of O_2 to below detection limits (< 2 μM O_2) reduced NO inactivation in both cerebellar cells and brain homogenate to the rate found in buffer (Figure 2a). Homogenate inactivation of NO was not attributable to the reaction of NO with superoxide ions, as neither increasing the SOD concentration 5-fold (to 5000 units/ml) nor omitting it altogether had any effect on the rate of inactivation (results not shown). Inclusion of catalase (500 units/ml) in the homogenate (containing SOD) had no significant effect on the NO-degrading activity (0.97 ± 0.05 min^{-1} versus 0.81 ± 0.06 min^{-1} for the control, $n = 3$). In contrast, the homogenate activity was greatly reduced by boiling for 10 min (0.25 ± 0.06 min^{-1}, $n = 3$).

To obtain information on the underlying chemistry, the degradation products of NO derived from 1 μM PROLI/NO were analysed by chemiluminescence (Figure 2b). In buffer, NO_2^- plus NO_3^- totalled 1.6 ± 0.5 μM ($n = 3$), of which the contribution by NO_3^- was not significant, as expected for autoxidation [20]. In brain homogenates, the sum of NO_2^- and NO_3^- recovered was the same as in buffer. With increasing homogenate concentration, however, the concentration of NO_2^- progressively diminished to a final value (at 3–10 mg/ml protein) that was approx. 25 % of the total. Thus the major end-product of NO degradation in the brain homogenate was NO_3^-.

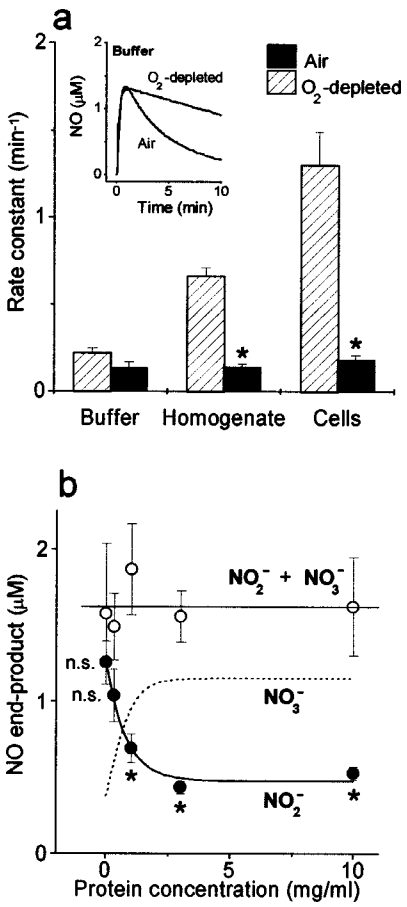

Figure 2 Brain NO sink is O_2-dependent and generates NO_3^-

(**a**) Rate constants for NO decay were measured in buffer [20 mM Tris/HCl (pH 7.4)], cerebellar cell suspensions (20×10^6 cells/ml; 0.84 mg/ml protein) and whole brain homogenates (1.0 mg/ml protein) in air-equilibrated and O_2-depleted solutions. Inset shows representative recordings of the profile of the NO concentration over time in buffer before and after O_2 depletion. (**b**) Following the addition of 1 μM PROLI/NO to buffer or homogenate (1–10 mg/ml), the concentrations of NO_2^- (●) and $NO_2^- + NO_3^-$ (○) were determined by chemiluminescence. Data are means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with the corresponding concentration of $NO_2^- + NO_3^-$. The difference between the two sets of data (broken line) gives the concentration of NO_3^-.

To compare how brain homogenates and intact cerebellar cells handle a more physiological rate of NO production, we employed another donor, DETA/NO, whose long $t_{1/2}$ (20 h) means that, at a given concentration, NO will effectively be generated at a constant rate for prolonged periods. Previously, it was found that NO consumption by cerebellar cells served to transform different rates of NO production from DETA/NO into proportionate steady-state concentrations [2]. In agreement, in the present experiments, a plateau NO concentration of 22 ± 5 nM was formed by the cells in response to an NO production rate of 90 nM/min (100 μM DETA/NO; Figure 3a). The plateau lasted for approx. 8 min before the mechanism became saturated, as evidenced by a secondary rise in the NO concentration (Figure 3a). Brain homogenates retained the capacity to generate a plateau NO concentration. The amplitude was similar to that found with living cerebellar cells (16 ± 4 nM; Figure 3a), but the duration was approximately twice as long.

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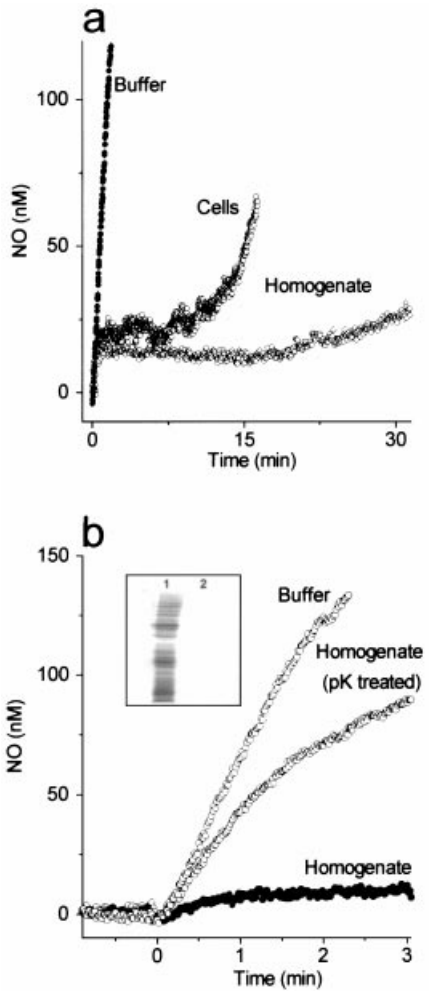

Figure 3 NO inactivation generates a protease-sensitive concentration clamp

(**a**) DETA/NO (100 μM) was added to cells (20×10^6 cells/ml; 0.84 mg/ml protein), homogenate (1.0 mg/ml protein) and buffer at $t = 0$ min and the NO concentration was determined. Each trace is representative of at least three experiments. (**b**) The effect of protein digestion on the NO clamp formed in homogenates in response to DETA/NO was tested by preincubation with proteinase K (pK). Each trace is representative of at least three experiments. Protein degradation was verified by gel electrophoresis and silver staining (inset: lane 1, control homogenate; lane 2, homogenate + proteinase K).

Preservation of this property in brain homogenates provided the opportunity to investigate the underlying mechanism. Complete digestion of homogenate protein by incubation with proteinase K (verified by gel electrophoresis; Figure 3b, inset) abolished the ability of the homogenate to clamp the NO concentration at low levels (Figure 3b). The rate of rise of the NO concentration in the digest was slightly slower than in buffer, however, indicating a minor residual NO consumption.

Pharmacological agents were used to investigate the role of several proteins suggested previously to consume NO. As a simple assay, the NO concentration attained 2 min after addition of 100 μM DETA/NO provided a sensitive index of NO consuming activity. An involvement of mitochondrial cytochrome *c* oxidase [12] was examined using 1 mM cyanide. Despite completely inhibiting cellular O_2 consumption (results not shown), cyanide failed to influence the NO clamp (Table 1). Azide

Table 1 Effect of inhibitors on NO inactivation in brain homogenate

Buffer or brain homogenate (approx. 1.0 mg/ml of protein) incubated with or without the compounds listed was exposed to 100 μM DETA/NO, and the NO concentration measured 2 min later. SOD (1000 units/ml) was included throughout. Results are means ± SEM (n = 3–6). $^*P < 0.05$ compared with homogenate. Treatment with inhibitors did not significantly alter the response when compared with homogenate alone. Abbreviation: KCN, potassium cyanide; NaN_3, sodium azide.

Additions	Preincubation period (min)	NO (nM)
Buffer	–	218 ± 11*
Homogenate	–	11 ± 4
+ KCN (1 mM)	10	21 ± 9
+ NaN_3 (10 mM)	10	6 ± 1
+ Indomethacin (20 μM)	10	11 ± 1
+ ETYA (100 μM)	10	15 ± 2
+ ODQ (30 μM)	10	8 ± 2
+ 1% (v/v) DMSO	60	8 ± 1
+ DPI (30 μM) in 1% (v/v) DMSO	60	8 ± 1

(10 mM), another cytochrome *c* oxidase inhibitor was similarly ineffective. The lack of effect of cyanide is also evidence against the involvement of a flavohaemoglobin-like NO dioxygenase [10]. A further test of this possibility was made using the flavoenzyme inhibitor, diphenylene iodonium (DPI), at a high concentration (30 μM) and following a prolonged preincubation period (60 min). The amplitude of the NO clamp was unaffected (Table 1). Another haemoprotein reported to catalyse NO consumption is prostaglandin H synthase and, in platelets, this activity can be inhibited by indomethacin [16]. When tested on brain homogenates at a concentration (20 μM) previously found to block platelet NO consumption [16], however, indomethacin had no effect (Table 1). NO consumption by the non-haem iron-containing enzymes, 12/15-lipoxygenases, is inhibited by the compound eicosatetraynoic acid (ETYA) [13,14], but this compound had no effect on NO degradation in the brain homogenate (Table 1). Finally, the primary physiological target for NO, sGC, has been suggested to reduce NO concentrations by binding NO [21], although this reversible binding would not be expected to result in NO degradation. In accordance, the haem-site sGC inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) [22], had no affect on the NO-clamping mechanism (Table 1).

The ability of both intact cells and homogenate to inactivate NO in the presence of persistent NO release was limited (Figure 3a), possibly because the mechanism is slow to regenerate. Use of DETA/NO for investigating this property has the disadvantage that once added it cannot be readily removed. The rate of reactivation was instead tested using PROLI/NO. The activity of both cells and homogenate decreased progressively during four successive applications of 1 μM PROLI/NO, although the inactivation rate at the end remained higher than in buffer (Figure 4a). A similar degree of saturation was achieved in cells if a single bolus of PROLI/NO was delivered and then the rate of NO inactivation assessed by subsequent delivery of 1 μM PROLI/NO. Using increasing PROLI/NO concentrations it was found that addition of 30 μM PROLI/NO (giving a peak NO concentration of approx. 19 μM) achieved supramaximal saturation of NO consumption (results not shown). The 'saturated' cells were then allowed to rest for various intervals (0–2 h) and the rate of inactivation was determined. Recovery was slow, requiring 2 h before the initial rate of NO consumption was regained (Figure 4b).

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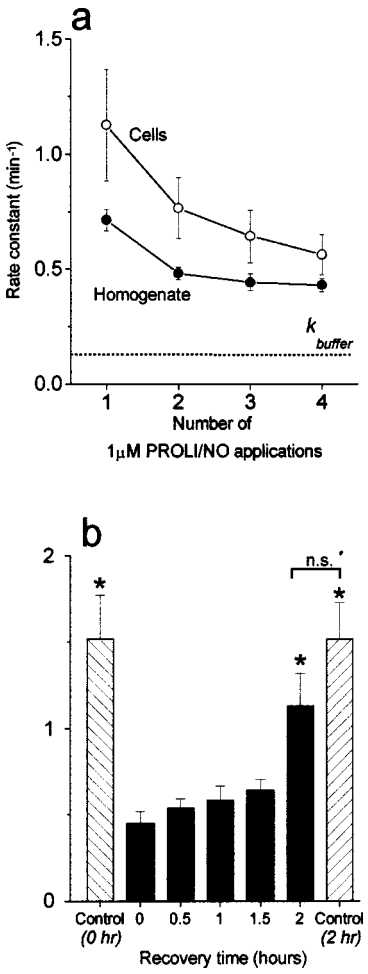

Figure 4 Mechanism of NO inactivation is saturable and slow to regenerate

(**a**) PROLI/NO (1 μM) was applied to either whole brain homogenate (1 mg/ml protein) or a cerebellar cell suspension (20×10^6 cells/ml; 0.84 mg/ml protein) and the NO concentration monitored. Once the NO had completely decayed, 1 μM PROLI/NO was reapplied three times, with NO being allowed to decay completely between each application. The rate constant of inactivation was measured for each addition and plotted in comparison with the value in buffer alone (k_{buffer}). (**b**) NO inactivation mechanism in cells (20×10^6 cells/ml; 0.84 mg/ml protein) was saturated by a single application of PROLI/NO (30 μM). Recovery was assessed by leaving the cells for various time intervals (0–2 h) at 37 °C and then measuring the rate of NO inactivation with a single application of 1 μM PROLI/NO ($n = 3$–6; solid bars). Control cells at the start ($t = 0$) and end ($t = 2$ h) of the experiment are shown for comparison (hatched bars). *$P < 0.05$ compared with 'saturated' cells at $t = 0$; n.s., not significant.

DISCUSSION

Cerebellar cells possess a powerful NO sink that can shape NO signals [2]. In the present study, we demonstrate that this NO sink is retained in brain homogenates, which has allowed a more direct investigation of its properties and of the participation of several candidate proteins suggested to consume NO in mammalian cells.

Firstly, several chemical reactions can be excluded. Simple reaction with O_2 (autoxidation) in the aqueous phase cannot account for the activity, as this would be unaffected by the presence of tissue and is negligible at the low clamped NO concentrations generated in response to DETA/NO. Accelerated autoxidation resulting from the partitioning of NO and O_2 into lipid [23] or hydrophobic protein domains [24] is also not responsible, because it would be much too slow at the tissue concentrations used in the present study. For example, taking the extreme case of all the added tissue behaving as lipid [23], accelerated autoxidation would contribute $< 5\%$ of the total measured activity. Reaction with superoxide anions is ruled out as the tissue activity was unaffected by SOD. NO scavenging by haemoglobin from any lysed red blood cells present in the homogenate cannot account for the activity, because the rate of this reaction is much too rapid [25].

The finding that NO consumption was virtually eliminated by protease treatment, or by boiling, provides strong evidence for the involvement of a protein. However, none of the proteins suggested so far to consume NO appears to participate. The lack of effect of cyanide and azide eliminates cytochrome *c* oxidase [12]; the lack of effect of cyanide and DPI rules out a flavohaemoglobin-like enzyme [10]; the lack of effect of azide and catalase also eliminates H_2O_2-dependent haem peroxidases [15]; and the lack of effect of indomethacin or ETYA suggests that prostaglandin H synthase [16] and 12/15-lipoxygenase [13,14] do not contribute. Cytochrome *c* has also been shown to increase NO decay [26], but the rate of this reaction is too slow to account for the NO sink described in the present study.

These negative findings indicate that a novel protein is responsible for the NO sink in brain tissue. Nevertheless, the mechanism does have some similarities to NO-consuming activity in other cells, notably in its dependence on O_2 [9,10] and in the formation of NO_3^- as an end-product [10]. The answer to whether NO_3^- is formed primarily by the NO-consuming reaction or secondarily by conversion of other species into NO_3^- [27] must await identification and purification of the protein. Nevertheless, it is interesting to note that NO_3^- is also the major end-product of NO breakdown in the brain [18] and in the body as a whole [28] *in vivo*. An established mechanism for generating NO_3^- from NO is via reaction with ferrous-oxy haem proteins [27]. This possibility cannot be excluded, but the rate of NO oxidation would have to be much lower than that of the archetypal haem protein, haemoglobin [25], to account for the NO inactivation rates observed in the present study. The protein would also need to be insensitive to the classical haem poisons, cyanide and azide.

A characteristic of the NO inactivation mechanism in brain cells [2], but apparently not in liver cells [9], is its saturability. This property was also found in brain homogenates and it was evident that the NO clamp formed in the homogenates in response to DETA/NO was more persistent (and tended to be of lower amplitude) than in intact cells, suggesting enhanced NO degrading activity. This may reflect a greater availability of cofactors as a result of cell disruption. On the other hand, the NO decay rate measured following delivery of PROLI/NO was higher in the living cells than in the homogenate. However, this latter protocol used only a relatively small portion of the NO decay curve following a bolus NO challenge, during which the NO concentration constantly changes, which may explain the discrepancy. Nevertheless, examination of the rate of recovery of NO consumption in intact cells following a bolus of NO indicates that the property of saturability is a reflection of a remarkably slow (2 h) regeneration of the NO-consuming activity. Possibly, NO is a 'suicide' substrate for an enzyme and the recovery of activity depends on synthesis of new protein. Alternatively, slow replenishment of a cofactor may be the explanation. Regardless, the potential functional importance of this property is that it may explain how NO, when synthesized for prolonged periods, can become toxic to cells. As we have seen previously [2], while it lasts, the NO sink in brain cells translates physiologically relevant rates of NO synthesis into steady-state NO concentrations tuned to the requirements of the primary NO target, sGC. Exhaustion

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of the sink permits the NO concentration to rise to concentrations capable of exerting deleterious effects, such as respiratory inhibition.

Clearly, further progress in understanding the biodegradation of NO in the brain (and elsewhere) and its roles in physiology and pathology will be facilitated by the identification of the underlying molecular mechanism. Unlike the presumed flavohaemoglobin operating in some cell lines [10], the activity of the NO sink in the brain persists in homogenates, thereby providing the necessary starting point for its purification, identification and characterization.

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