



# Cytochrome P450 oxidoreductase participates in nitric oxide consumption by rat brain

Catherine N. HALL<sup>1,2</sup>, Robert G. KEYNES<sup>1</sup> and John GARTHWAITE

Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6BT, U.K.

In low nanomolar concentrations, NO (nitric oxide) functions as a transmitter in brain and other tissues, whereas near-micromolar NO concentrations are associated with toxicity and cell death. Control of the NO concentration, therefore, is critical for proper brain function, but, although its synthesis pathway is well-characterized, the major route of breakdown of NO in brain is unclear. Previous observations indicate that brain cells actively consume NO at a high rate. The mechanism of this consumption was pursued in the present study. NO consumption by a preparation of central glial cells was abolished by cell lysis and recovered by addition of NADPH. NADPH-dependent consumption of NO localized to cell membranes and was inhibited by proteinase K, indicating the involvement of a membrane-bound protein. Purification of this activity yielded CYPOR (cytochrome P450 oxidoreductase). Antibodies against CYPOR

inhibited NO consumption by brain membranes and the amount of CYPOR in several cell types correlated with their rate of NO consumption. NO was also consumed by purified CYPOR but this activity was found to depend on the presence of the vitamin E analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), included in the buffer as a precaution against inadvertent NO consumption by lipid peroxidation. In contrast, NO consumption by brain membranes was independent of Trolox. Hence, it appears that, during the purification process, CYPOR becomes separated from a partner needed for NO consumption. Cytochrome P450 inhibitors inhibited NO consumption by brain membranes, making these proteins likely candidates.

**Key words:** brain, cytochrome P450 oxidoreductase (CYPOR), NADPH, nitric oxide.

## INTRODUCTION

NO (nitric oxide) is an intercellular signalling molecule with a role in several neurophysiological functions, including the acute modulation of neuronal excitability, the longer-term synaptic changes associated with learning, and the development of the nervous system [1]. Its major physiological receptor is the NO<sub>GC</sub> (NO-activated guanylyl cyclase, also known by its homogenate-based name, soluble guanylyl cyclase), through which it stimulates the production of the second messenger cGMP. cGMP has numerous targets, including cyclic nucleotide gated ion channels, protein kinases and phosphodiesterases, mediating the short- and long-term modulations of neuronal function [2,3].

These physiological pathways are engaged by low nanomolar concentrations of NO. The dynamic range of the NO<sub>GC</sub> receptor, as measured in intact cells, is between 0.1 and 10 nM NO [4–6], suggesting that this is the range of NO concentrations normally experienced by cells. Indeed, electrical stimulation of cerebellar brain slices yielded 4 nM NO, as measured by electrodes positioned at the slice surface [7]. Even lower NO concentrations may also be physiologically relevant, as NO-dependent phosphorylation events have been reported after exposure to sub-nanomolar NO concentrations [6].

At higher concentrations, NO may be linked with pathophysiology. NO inhibits the respiratory chain enzyme, cytochrome *c* oxidase, with an IC<sub>50</sub> of 60–120 nM at physiological oxygen concentrations [4,8], and micromolar NO levels can produce cell damage via reaction with superoxide and production of the highly oxidising species peroxynitrite [9].

Control of the amplitude and duration of changes in NO concentration is therefore likely to critically affect both the manner in which NO can act physiologically and also whether it has any pathological effects. The NO concentration experienced by a cell will be determined by the relative rates of NO synthesis and breakdown but, although the mechanism of NO synthesis from L-arginine is relatively well characterized, there is no known dedicated consumption pathway for NO in the brain, although a number of enzymes have been proposed to fulfil this function in other tissues [10–14]. One such protein is CYPOR (cytochrome P450 oxidoreductase), which is involved in an extremely avid NO consumption by a colorectal cancer cell line [15]. A process with similar properties [membrane localization and NAD(P)H dependence] has also been reported in cultured endothelial cells [16].

Previous work has revealed that brain tissue actively consumes NO [17–19]. In dissociated brain cells, part of the NO consumption was found to be caused by lipid peroxidation, which is likely to be of particular relevance to pathology, but inhibition of lipid peroxidation unmasked another consumption process [18]. The present study aimed to identify this mechanism.

## MATERIALS AND METHODS

All compounds were purchased from Sigma (Poole, U.K.) unless otherwise stated. All tissue culture media components were purchased from Invitrogen (Paisley, U.K.).

Abbreviations used: CYPOR, cytochrome P450 oxidoreductase; DETA/NO, diethylenetriamine NONOate; DHEA, dehydroepiandrosterone; DPI, diphenyleneiodonium chloride; DTPA, diethylenetriaminepentaacetic acid; L-NNA, L-nitroarginine; NO, nitric oxide; NO<sub>GC</sub>, NO-activated guanylyl cyclase; NOS, NO synthase; SOD, superoxide dismutase; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed (email [catherine.hall@ucl.ac.uk](mailto:catherine.hall@ucl.ac.uk)).

## NO measurement

For NO measurements, samples (1 ml) were incubated in an open stirred vessel at 37°C equipped with an NO electrode (ISO-NOP, World Precision Instruments, Stevenage, U.K.). NO was delivered using DETA/NO [diethylenetriamine NONOate (diazoniumdiolate); Alexis Biochemicals, Nottingham, U.K.]. Stock solutions of DETA/NO were prepared in 10 mM NaOH, kept on ice, and diluted 100-fold or more into the experimental solution.

## Tissue preparation

Animals were killed by decapitation and associated exsanguination, before removal of the brains, except when blood was taken. In this case, rats were anaesthetised with 5% isoflurane in oxygen at 2 litres/min, and were bled by cardiac puncture before being killed by cervical dislocation. All procedures were in accordance with the U.K. Home Office guidelines and approved by the local ethics committee.

## Glia

Glial cultures were prepared as described in [18]. Cultures were used after 6–10 days *in vitro* at which stage they were fully confluent. Immunohistochemical staining indicated that 77% of the cells were astrocytic, 7% neuronal and 16% microglial (results not shown).

To prepare the suspension of mixed glia for studies of NO consumption, dishes were washed with ~100 ml of cell incubation buffer (20 mM Tris/HCl, 130 mM NaCl, 5 mM KCl, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub> and 11 mM glucose, adjusted to pH 7.45 at 37°C) and incubated with 30 ml of 0.05% (w/v) trypsin, 0.53 mM EDTA in HBSS (Hanks balanced salt solution) for 15 min at 37°C to dissociate the cells, which were washed and resuspended at 3 × 10<sup>6</sup> cells/ml in incubation buffer. Cell viability was verified at more than 95% based on Trypan Blue staining.

## Platelets and white blood cells (used in Figure 2A)

Platelets and white blood cells were prepared from adult Sprague–Dawley rat blood. Whole blood was collected into acid citrate dextrose solution (12.5%) and centrifuged at 300 g for 10 min at 20°C. The platelet-rich plasma was removed and the centrifugation repeated to eliminate residual red and white blood cells from the supernatant. White cells were aspirated from the top of the pellet and suspended in platelet buffer containing 137 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 0.55 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 25 mM Hepes and 5.6 mM glucose, pH 7.45 at 37°C. The platelet-rich plasma was then centrifuged for 10 min at 2000 g at 20°C and the platelet pellet resuspended in platelet buffer. Both platelets and white cells were suspended in a small volume, the protein determined and then each sample was diluted to the appropriate concentration (usually 1 mg/ml). White cells were counted using a haemocytometer to determine the relationship between their number and protein concentration (1 mg/ml protein corresponded to 48 million white cells/ml).

## Synaptosomes

Crude synaptosomes were prepared by homogenizing adult rat forebrains into 0.25 M sucrose with 8 strokes of a Potter–Elvehjem homogeniser (Braun Melsungen, Melsungen, Germany). Homogenates were then centrifuged at 1000 g for 10 min and the pellet discarded. The supernatant was spun for a further 15 min at 46 000 g and the resulting supernatant

discarded. The synaptosome pellet was resuspended in incubation buffer using 8 strokes of the Potter homogenizer.

## T47D cells

Control T47D cells and those that had previously been transfected to overexpress CYPOR [20] were a generous gift from Kaye Williams (School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, U.K.). They were cultured in RPMI medium containing 10% foetal calf serum, supplemented with 2 mM glutamine. When confluent, cells were harvested in the same manner as cultured glia.

## Lysates and cell fractionation

Lysates were prepared from the intact suspensions by freezing to –20°C, thawing and brief sonication. Lysis was verified visually under a light microscope.

Membrane and cytosolic fractions were prepared by centrifugation at 53 000 rev./min using a TLA-100.2 rotor (Beckman Instruments) for 1 h. The supernatant was retained and membranes were resuspended in incubation buffer by sonication. Protein concentrations of membranes refer to the concentration of protein in the pre-spun sample.

## Whole brain homogenates and membranes

Membranes from forebrains from 8–12-day-old Sprague–Dawley rat pups were homogenized in 25 mM Tris/HCl, pH 7.45 using an Ultraturrax homogenizer. Crude membranes were prepared by centrifugation of homogenate at 5 mg protein/ml at 53 000 rev./min using a TLA-100.2 rotor for 1 h. To prepare a purer preparation, homogenates were centrifuged for 1 h at 50 000 g, the supernatant discarded and the membranes resuspended and centrifuged for a further 1 h at 50 000 g, before final resuspension in 25 mM Tris/HCl, pH 7.45, at ~10 mg/ml protein. This dual spin was found to increase the recovered activity more than the single 53 000 rev./min (using a TLA-100.2 rotor) spin (results not shown). Membranes were then stored at –80°C for later use.

## Solubilization and chromatography

Forebrain membranes (0.8 mg/ml protein) were solubilized by gentle agitation for 1 h at 4°C in buffer containing 25 mM Tris/HCl, pH 7.45, 100 mM KCl, 10% (v/v) glycerol and 3 mM dodecyl maltoside (Calbiochem, Nottingham, U.K.). Any remaining particulate material was removed by centrifugation at 53 000 rev./min using a TLA-100.2 rotor for 1 h. Chromatography was conducted using the ÄKTA FPLC system (GE Healthcare UK, Amersham, Bucks. U.K., who also provided all the columns used). Fast flow Q cationic ion exchange columns were used for the initial capture step. Solubilized membranes (150 ml) were loaded onto 4 × 5 ml columns and the protein was eluted with a gradient of 100–500 mM KCl over 200 min, with a flow rate of 1 ml/min. The fractions that showed the most NADPH-dependent NO consumption were combined and desalted into 100 mM KCl (4 × 5 ml Hi Trap columns at a flow rate of 1–1.3 ml/min), then loaded onto a Mini Q cationic ion-exchange column, before elution with a further 100–500 mM KCl gradient over 2 h, with a flow rate of 0.2 ml/min. The most active fractions were combined and 1 ml was loaded on a Superdex 200 gel filtration column and eluted with a flow rate of 0.25 ml/min. The molecular mass eluted at each volume of eluate from the Superdex 200 column was calibrated according to standards (BioRad, Hemel Hempstead, U.K.). Samples from each fraction were concentrated by spinning

through a 5 kDa cut-off filter (Ultrafree-MC, Millipore, MA, U.S.A.), boiled for 10 min with SDS and separated using gel electrophoresis (45 min at 200 V on 4–15 % Ready Gel Tris-HCl gels; BioRad Laboratories) before staining using Colloidal Blue or silver stain. Colloidal Blue-labelled bands were excised and sent for protein identification (York Proteomics Services, University of York, U.K.).

### Quantification of CYPOR activity

The rate of cytochrome *c* reduction is an indicator of CYPOR activity [21]. Cytochrome *c* reduction was followed by measuring absorbance at 550 nm every 10–12 s for 10 min after addition of 100  $\mu$ M NADPH to a reaction mix containing cell membranes, 37  $\mu$ M cytochrome *c*, 100  $\mu$ M sodium cyanide (NaCN; to block any cytochrome oxidase activity), 100  $\mu$ M DTPA (diethylenetriaminepentaacetic acid), 100  $\mu$ M Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; not included in some cases) and 1000 units/ml SOD (superoxide dismutase), in 25 mM Tris/HCl buffer or cell incubation buffer. The CYPOR activity of membranes was determined by comparison to the rate of cytochrome *c* reduction by purified CYPOR standards (Sigma).

Experiments were carried out in the presence of 1000 units/ml SOD and 100  $\mu$ M DTPA (intact and lysed glia) and also 100  $\mu$ M Trolox (all experiments, unless otherwise stated) to block any lipid peroxidation. Protein concentrations were determined by the bicinchoninic acid method.

### Statistics

Unless otherwise stated, data represent means  $\pm$  S.E.M. Statistical analyses were carried out using SPSS for Windows 11.5 (SPSS U.K., Woking, U.K.).

## RESULTS

### Intact cerebellar glia inactivate NO

The NO-consuming activity of brain tissue was assessed by comparing the NO concentration profile in control buffer and various preparations of brain tissue on application of the NO donor DETA/NO. Since this compound releases NO with a half-life of 20.5 h, the rate of release is essentially constant over the time-course of these experiments. The NO concentration initially rises but reaches a steady concentration when the rate of release equals the rate of breakdown. In control buffer, NO is consumed by reaction with O<sub>2</sub> (autooxidation; [22]), but in the presence of glia cultured from the cerebellum, NO reached a lower steady-state concentration, signifying faster consumption (Figure 1A; [18]).

Pharmacological inhibitors of previously proposed breakdown pathways for NO were used to try to identify the consumption process in cerebellar glia. NOS (NO synthase) inhibition using L-NNA (L-nitroarginine; 100  $\mu$ M) had no effect on NO consumption (control NO plateau following 250  $\mu$ M DETA/NO = 208  $\pm$  37 nM, plus L-NNA = 166  $\pm$  28 nM;  $P$  = 0.37 using Student's *t* test), nor did the prostaglandin H synthase inhibitor indomethacin (control NO = 211  $\pm$  21 nM; 20  $\mu$ M indomethacin, NO = 226  $\pm$  19 nM;  $P$  = 0.90 using Student's *t* test). The standard inclusion of 1000 units/ml SOD in the reaction mix rules out a contribution of reaction with superoxide (doubling the concentration to 2000 units/ml had no additional effect; results not shown). The vitamin E analogue Trolox (100  $\mu$ M), which was included throughout to inhibit inadvertent lipid peroxidation,

also inhibits lipoxygenases [23], thereby ruling out these enzymes as well.

### NO inactivation depends on a membrane-bound flavoprotein

The consumption of NO by cerebellar glia was inhibited by DPI (diphenyleneiodonium chloride), an inhibitor of flavoproteins (Figure 1A, steady-state after 100  $\mu$ M DETA/NO: control cells = 105  $\pm$  8 nM; 0.5  $\mu$ M DPI = 192  $\pm$  15 nM;  $P$  = 0.007 using Student's *t* test,  $n$  = 3).

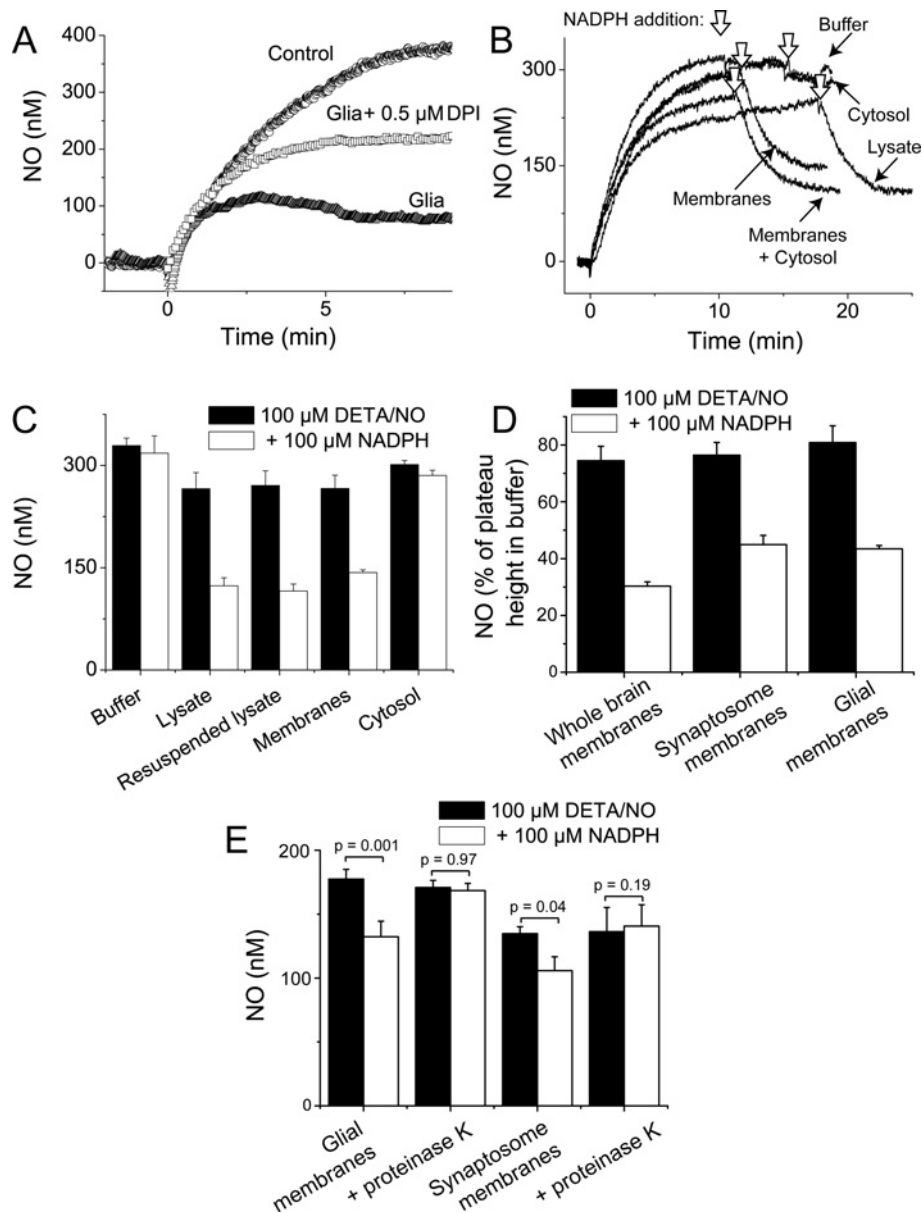
Inhibition of NADPH synthesis by 100  $\mu$ M DHEA (dehydroepiandrosterone; which typically reduces intracellular NADPH levels by less than half [24,25]) also produced a significant decrease in the NO-consuming activity of intact glia (control plateau after 100  $\mu$ M DETA/NO = 106  $\pm$  6 nM; plus DHEA = 141  $\pm$  6 nM;  $P$  = 0.011, Student's *t* test,  $n$  = 3). NO consumption by the glia was destroyed by cell lysis but could be recovered by addition of 100  $\mu$ M NADPH (Figures 1B and 1C), suggesting the same process is responsible for NO consumption in both intact and lysed glia.

NADPH-dependent NO consumption localized to the membrane fraction following high-speed centrifugation of lysed glia (Figures 1B and 1C). Membranes from forebrain synaptosomes and whole brain homogenates also both exhibited a similar NADPH-dependent NO consumption (Figure 1D). As in intact glia, this NADPH-dependent NO consumption could be inhibited by DPI (Figure 2D). Incubation of synaptosome or glial membranes with proteinase K degraded all protein and abolished NADPH-dependent NO consumption (Figure 1E), indicating that a protein is required. It seems, therefore, that brain membranes possess a flavin-dependent protein that is involved in NO consumption.

Solubilization of brain membranes with 3 mM dodecylmaltoside preserved NADPH-dependent NO consumption (but see below), allowing purification of this activity by chromatography (see the Materials and methods section). Two major protein products were identified after three purification steps: the calcium-binding protein calnexin and the NADPH-dependent flavoprotein CYPOR (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/419/bj4190411add.htm>). The NADPH-binding properties of CYPOR made it a good candidate for mediating NADPH-dependent NO consumption by brain membranes. Indeed CYPOR has already been implicated in the extremely avid NAD(P)H-dependent NO consumption in a colorectal cancer cell line [15].

### Cytochrome P450 oxidoreductase content correlates with NO consumption activity

We quantified the CYPOR activity in membranes of glia, whole brain, and several other cell types, by measuring the rate of reduction of cytochrome *c*. The CYPOR content of the different cell types correlated with the NO consumption activity, expressed as the change in NO on application of NADPH as a percentage of the NO plateau after DETA/NO addition (Figure 2A). Cell types previously found to have no detectable NO-consuming activity when intact (platelets and white blood cells; [18]) also showed no NADPH-dependent NO consumption by their membranes, and had a low CYPOR content. A breast cancer cell line (T47D) showed some NO consumption when intact, even at a very low cell density (0.06 mg/ml protein; plateau NO = 82 % of that in buffer,  $P$  = 0.018 compared with buffer,  $n$  = 6), and also demonstrated NADPH-dependent membrane consumption and some CYPOR content (Figure 2A). NO consumption by intact T47D cells and by their membranes was higher when CYPOR was constitutively overexpressed (Figure 2A; plateau NO in intact cells = 43 % of buffer,  $P$  < 0.0001 compared with buffer,  $n$  = 6).



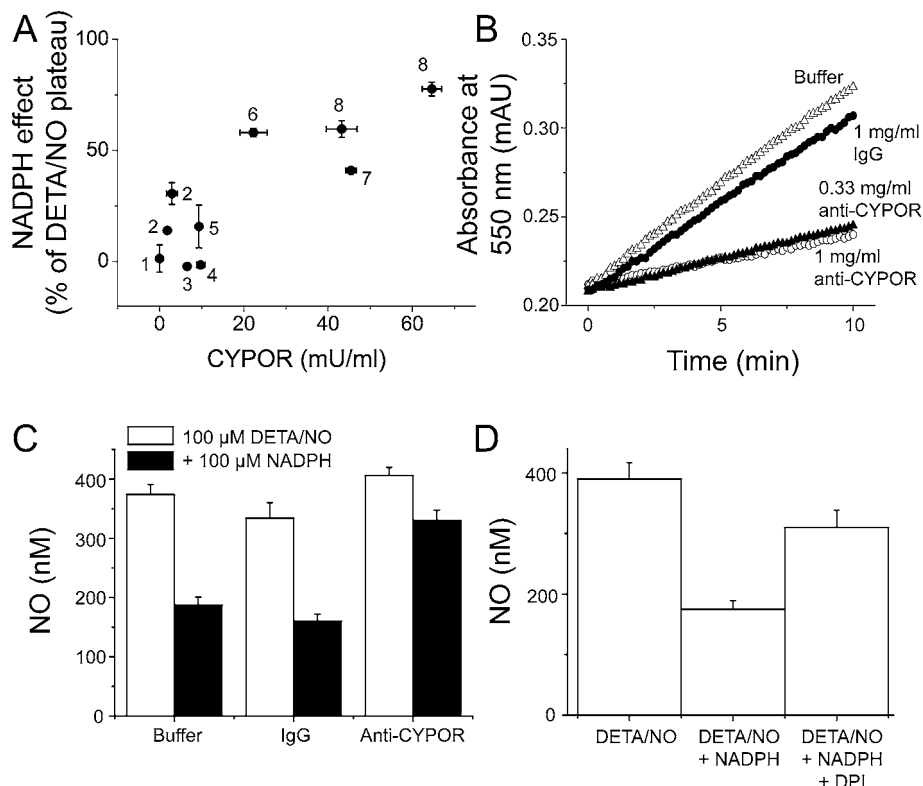
**Figure 1** NO inactivation by glia requires NADPH and is dependent on a membrane protein

(A) Typical NO profiles on addition of 100 μM DETA/NO to buffer alone or 1 mg/ml cultured cerebellar glia ± 0.5 μM DPI. (B) Representative traces of NO accumulation following sequential applications of 100 μM DETA/NO (at  $t = 0$ ) and 100 μM NADPH (open arrows) to buffer, glial lysate and, following centrifugation at 53000 rev./min using a TLA-100.2 rotor for 1 h, cytosolic supernatant plus membrane pellet, the membrane pellet and supernatant alone. Lysed cells only inactivate NO after addition of NADPH. This activity remains in the pellet after high-speed centrifugation. (C) Summary of results from (B). Buffer and cytosol are not significantly different from each other, but were different from the membrane pellet, resuspended lysate and control lysate (repeated measures using ANOVA with Tukey *post hoc* tests,  $P < 0.001$ ;  $n = 4$ ). (D) Summary of results when 100 μM DETA/NO and 100 μM NADPH were added to membranes from glia, synaptosomes and whole brain (all at 1 mg/ml). Results are normalized to the NO concentration reached on each experimental day when 100 μM DETA/NO was added to buffer alone ( $n = 4$ ). (E) NO levels following addition of 100 μM DETA/NO and 100 μM NADPH to synaptosome and glial membranes at 1 mg of protein/ml before and after incubation at 37 °C for 30 min ± 0.5 mg/ml proteinase K. NADPH decreased NO levels in controls but not in proteinase K-treated membranes ( $P = 0.20$ ; glial membranes:  $n = 8$ ; synaptosome membranes  $n = 3$ ). The incubation at 37 °C has caused some decrease in the activity of the controls compared with that observed in (C).

### Anti-CYPOR antibodies inhibit NO consumption by brain membranes

Inhibition of CYPOR activity in brain membranes was achieved by incubation of brain membranes (1 mg protein/ml) with an antibody against CYPOR ( $\alpha$ -CYPOR, Abcam, Cambridge, U.K.; 0.33 or 1 mg/ml) for 2 h on ice. Unlike after incubation with 1 mg/ml of control  $\gamma$ -immunoglobulins or buffer alone, both antibody concentrations dramatically reduced the activity of

CYPOR, as assessed by the ability of brain membranes to reduce cytochrome *c* (Figure 2B). Similarly, NADPH-dependent NO consumption was reduced by incubation with 0.33 mg/ml  $\alpha$ -CYPOR, but not by  $\gamma$ -immunoglobulins or buffer (Figure 2C), indicating that NO consumption by brain membranes is dependent on CYPOR activity. The degree of inhibition by  $\alpha$ -CYPOR was similar to that produced by DPI (Figures 2C and 2D), as would be expected if both are targeting the same protein (CYPOR).



**Figure 2** NADPH-dependent NO consumption involves CYPOR

(A) 300  $\mu$ M DETA/NO was added to membranes from different cell types (as labelled) in cell incubation buffer, with DTPA but no Trolox. 1 Buffer alone; 2, T47D wild-type cells (0.05 mg of protein/ml); 3, white blood cells (2 mg of protein/ml); 4, platelets (2 mg of protein/ml); 5, crude brain (0.5 mg protein/ml); 6, partially purified brain (0.42 mg protein/ml); 7, glia (1 mg of protein/ml); 8, T47D transfected cells (0.05 mg of protein/ml). Note that the experiment with T47D wild-type and transfected cells was carried out on two separate occasions, so there are duplicates of the data from these cell types. The change in NO level on subsequent addition of NADPH was plotted against CYPOR content. CYPOR content significantly correlates with NO consumption (Pearson's  $r = 0.85$ , which is significantly different from zero;  $P = 0.002$ ,  $n = 3-6$ ). (B) To assay cytochrome *c* reduction by CYPOR, absorbance at 550 nm was followed with time. After incubation for 2 h on ice with buffer (closed circles), absorbance increased linearly. The rate was only slightly slower when incubated with 1 mg/ml IgG (open triangles), but was greatly reduced by incubation with 0.33 or 1 mg/ml of a CYPOR antibody (filled triangles and open circles, respectively). (C) NO concentration reached after 100  $\mu$ M DETA/NO addition to brain membranes at 1 mg of protein/ml, following incubation for 2 h on ice with control buffer, 0.33 mg/ml of  $\gamma$ -IgG or 0.33 mg/ml of  $\alpha$ -CYPOR, before (open bars) and after (solid bars) addition of 100  $\mu$ M NADPH. The change in NO on NADPH addition was significantly less following incubation with  $\alpha$ -CYPOR (one-way ANOVA with Tukey *post-hoc* tests;  $P < 0.001$  compared with buffer and IgG;  $n = 4$ ), but was not affected by incubation with  $\gamma$ -IgG ( $P > 0.05$  compared with buffer). (D) NO concentration reached after sequential additions of 100  $\mu$ M DETA/NO, 100  $\mu$ M NADPH and 50  $\mu$ M DPI to synaptosome membranes (1 mg protein/ml). DPI significantly inhibits NADPH-dependent NO consumption (one-way ANOVA with Tukey *post-hoc* tests: NADPH compared with DPI,  $P < 0.01$ ; DPI compared with DETA/NO,  $P > 0.05$ ;  $n = 6$ ). DPI and  $\alpha$ -CYPOR inhibit the NADPH effect to the same extent (Student's *t* test: 100  $\mu$ M DETA/NO plus 100  $\mu$ M NADPH plus 50  $\mu$ M DPI compared with 100  $\mu$ M DETA/NO plus 100  $\mu$ M NADPH plus 0.33 mg/ml of  $\alpha$ -CYPOR;  $P = 0.41$ ,  $n = 4-6$ ).

### CYPOR requires a partner to consume NO

As CYPOR seems to be involved in NO consumption by brain membranes, we tested whether purified CYPOR could also consume NO in an NADPH-dependent manner. In contrast with brain membranes, purified CYPOR did not consume NO upon addition of NADPH, unless Trolox and DTPA (100  $\mu$ M) were present in the reaction mix (Figure 3A). Further study revealed that the critical component was Trolox, which concentration-dependently enabled NO consumption by purified CYPOR, whereas DTPA did not (Figures 3B and 3C). Other antioxidants, edaravone (500  $\mu$ M) and phenothiazine (200  $\mu$ M), could not sustain NO consumption by CYPOR (results not shown), indicating that this is a special property of Trolox rather than a general antioxidant effect.

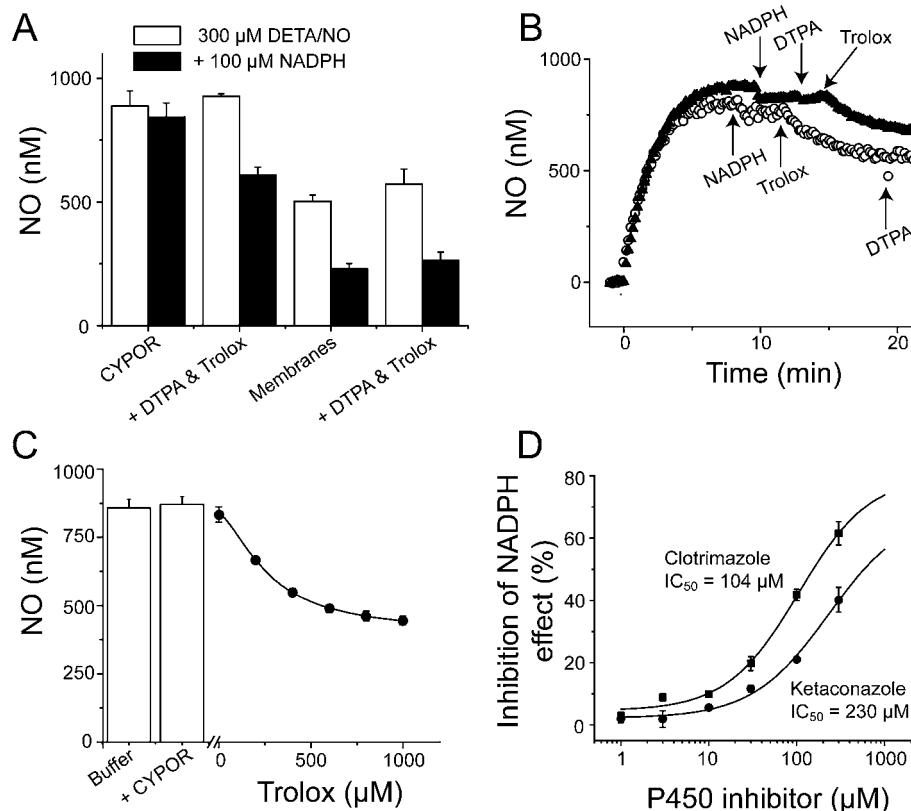
Solubilization of brain membranes by incubation with the detergent dodecylmaltoside (3 mM), 100 mM KCl and 10% (w/v) glycerol reduced NADPH-dependent NO consumption but this could be recovered by addition of Trolox (change in NO concentration on addition of 100  $\mu$ M NADPH to 300  $\mu$ M DETA/NO without Trolox =  $38 \pm 13$  nM, with Trolox =  $89 \pm 8$  nM; Stu-

dent's *t* test:  $P = 0.03$ ,  $n = 3$ ). Solubilized membranes in the presence of Trolox could then be further purified to yield purified CYPOR, as described above. This suggests that the solubilization process may physically separate CYPOR from a partner which is also involved in NADPH-dependent NO consumption and for which Trolox can substitute.

A major role of CYPOR is to maintain cytochrome P450s in the reduced state. We tested two well-known cytochrome P450 inhibitors with distinct inhibitory profiles towards subtypes of this enzyme family, namely clotrimazole and ketoconazole [26]. The compounds (200  $\mu$ M) did not affect CYPOR activity in the presence of Trolox (results not shown) but they both concentration-dependently reduced NADPH-dependent NO consumption by brain membranes (Figure 3D), suggesting that one or more cytochrome P450s may be involved.

### DISCUSSION

In this study we present evidence that brain cells consume NO by a membrane-localized process that involves NADPH oxidation



**Figure 3** CYPOR requires a partner to inactivate NO

(A) Plateau NO concentrations following application of NADPH to 300  $\mu$ M DETA/NO and purified CYPOR (100 m-units/ml) or brain membranes (2 mg of protein/ml)  $\pm$  DTPA and Trolox (both 100  $\mu$ M). In the case of purified CYPOR, NADPH reduced the NO concentration (and therefore increased NO consumption) only when DTPA and Trolox were present, whereas DTPA and Trolox had no effect on NADPH-dependent NO consumption by brain membranes.  $n = 4$ . (B) Example traces showing the NO profile when 300  $\mu$ M DETA/NO was added to 100 m-units/ml purified CYPOR. Trolox, but not DTPA (both 100  $\mu$ M), was required for NADPH-dependent NO consumption. (C) The effect of 100  $\mu$ M NADPH addition to 300  $\mu$ M DETA/NO  $\pm$  100 m-units/ml CYPOR and NADPH plus different Trolox concentrations. A logistic fit to the data (continuous line) gives an  $EC_{50}$  for Trolox of 270  $\mu$ M.  $n = 4$ . (D) The decrease in NO concentration on the addition of NADPH to brain membranes (2 mg/ml) plus 300  $\mu$ M DETA/NO was reduced by the cytochrome P450 inhibitors clotrimazole (squares) and ketoconazole (circles).  $IC_{50}$  values were calculated from logistic fits to the data (solid lines).  $n = 4$ .

by CYPOR, a microsomal protein that transfers electrons from NADPH to an acceptor, classically a haem-containing cytochrome P450 [27]. Cytochrome P450 inhibitors decreased NO consumption by brain membranes, suggesting that reduction of these proteins by CYPOR underlies NO consumption.

Cytochrome P450s perform hydroxylation reactions which, in the brain, are involved in diverse functions including steroid hormone synthesis, cholesterol homeostasis and vitamin, eicosanoid and xenobiotic metabolism [28,29]. Both CYPOR and several members of the cytochrome P450 family are expressed in brain, though at much lower levels than in the liver (1–10%; [30]). Interestingly, NO has been shown to bind and inhibit several cytochrome P450s [31,32], making them intriguing candidates for NO consumption by brain membranes. Indeed, the reductase domain of NOS is very similar to that of CYPOR and performs an analogous function, donating electrons to the haem-containing oxygenase domain [27]. NO binding to the NOS haem domain inhibits its catalytic activity and NO can then be consumed here by reaction with  $O_2$  [33].

The CYPOR/cytochrome P450 system has been previously linked with NO consumption in a colorectal cancer cell line (CaCo-2 cells; Hallstrom et al. [15]) in which the authors suggest one of the following reaction schemes: 1) NO binding to cytochrome P450 haem- $Fe^{2+}$  to form  $Fe^{2+}$ -NO, which reacts with  $O_2$  to form nitrate and haem- $Fe^{3+}$ , or 2)  $O_2$  binding to the same

haem- $Fe^{2+}$ , which reacts with NO to form nitrate and haem- $Fe^{3+}$ . In both schemes, NADPH/CYPOR then reduces the haem back to  $Fe^{2+}$  to continue the reaction cycle. Our finding that brain contains a similar NADPH/CYPOR-dependent NO consumption process suggests that this general mechanism plays a role in normal (non-cancer) cells.

The sensitivity of brain membranes to imidazole-derived cytochrome P450 inhibitors suggests that the operative cytochrome P450 is not likely to be CYPs 3A4, 2A6 or 2C19, which are much more sensitive to ketoconazole and clotrimazole [26]. The inhibition profiles found here are instead similar to those for CYP2D6 or CYP1A2 which are less sensitive to imidazole inhibition and are both expressed in the brain [26,34,35]. On a cautionary note, however, both clotrimazole and ketoconazole may affect proteins other than cytochrome P450s (e.g.  $K^+$  channels; [36]).

As CYPOR was able to consume NO in the presence of the soluble vitamin E analogue Trolox (but not other antioxidants), it is also possible that vitamin E is an additional endogenous partner for CYPOR in brain membranes. Vitamin E is highly lipid-soluble and its membrane location would be appropriate for interacting with CYPOR, a membrane protein. Unfortunately, the high lipid solubility of vitamin E makes it very difficult to explicitly test for this relationship. Attempts to incorporate vitamin E and CYPOR into brain membranes and reconstituted phospholipid vesicles

**Table 1** Rate constants for NO consumption by brain tissue

The rate of NO consumption at steady-state is equal to the rate of NO release from DETA/NO and is therefore given by the equation:

$$\text{Rate of NO consumption} = 1.6 \times [\text{DETA/NO}] \times \frac{\ln 2}{t_{1/2}}$$

The stoichiometry of NO release from DETA/NO is 1.6, as measured in this laboratory [19] and  $t_{1/2}$  is the half-life of DETA/NO (20.5 h at 37°C). The mean rate constant is  $0.59 \pm 0.06 \text{ min}^{-1}$  per mg of protein.

Figure	DETA/NO concentration ( $\mu\text{M}$ )	Rate of NO consumption ( $\mu\text{M}/\text{min}$ )	Tissue concentration (mg/ml)	Steady state NO concentration ( $\mu\text{M}$ )	Rate constant for NO consumption ( $\text{min}^{-1}$ per mg of protein)
1(C)	100	0.09	1	0.12	0.75
2(C)	100	0.09	1	0.19	0.47
2(D)	100	0.09	1	0.17	0.53
3(A)	300	0.27	2	0.23	0.59

were not effective in enhancing NO consumption activity (results not shown), but we were unable to positively control for their successful incorporation, so cannot exclude a role for vitamin E. Endogenous vitamin E levels can be manipulated by dietary means, but this procedure also affects levels of cytochrome P450s and other proteins [37], so would not clarify the issue.

The mechanism by which Trolox, CYPOR and NADPH together support NO consumption is unclear. Flavins in the nitric oxide synthase reductase domain (which is analogous to CYPOR) can pass electrons to molecular  $\text{O}_2$  to produce superoxide [38]. In the bulk solution this will be dismutated by SOD to hydrogen peroxide but it is possible that at the active site, NO could react with superoxide to form peroxynitrite, a very reactive molecule that could oxidize the protein structure. Possibly Trolox could then recover enzymatic activity by reducing the relevant residues. Alternatively, Trolox can be oxidized to a quinone in the presence of hydrogen peroxide [39], produced by SOD, and Trolox-quinone can then be reduced by NADPH to a semiquinone which readily reduces molecular oxygen to superoxide [40]. As above, superoxide could react directly with NO or alternatively, NO itself may be directly reduced by quinones or flavins. Which, if any, of these mechanisms underlies the Trolox-dependent NO consumption remains unclear. Before pursuing it further, the physiological relevance of this process to that observed in brain membranes and intact cells needs to be clarified, bearing in mind that the lack of Trolox-dependence in brain membranes indicates that the Trolox effect may be an artifact.

NADPH-dependent NO consumption in brain is somewhat less active than in CaCo-2 cells. In the latter, the rate of NO consumption at  $1 \mu\text{M}$  NO was  $4 \text{ nmol}/\text{min}$  per mg of protein, or  $4 \mu\text{M}/\text{min}$  at  $1 \text{ mg}$  of protein/ml. In brain membranes, the rate constant for NO consumption is  $0.59 \text{ min}^{-1}$  per mg of protein (see Table 1 and calculations in the legend), so at  $1 \text{ mg}$  of protein/ml and  $1 \mu\text{M}$  NO, the rate of NO consumption is  $0.59 \mu\text{M}/\text{min}$ , almost 7-fold lower than in CaCo-2 cells. That CaCo-2 cells are more active than brain is consistent with the relative expression levels of the CYPOR/cytochrome P450 systems: low in brain [30] but relatively high in the gastrointestinal tract [41] and in cancer cells and cell lines [42,43]. This pattern is consistent with the results showing a correlation between CYPOR activity and NO consumption in different cell types (Figure 2A).

In conclusion, CYPOR-dependent NO consumption appears important in shaping the NO concentration in both brain homogenates and intact brain cells. An additional component, possibly a cytochrome P450-like protein or a vitamin E-like compound, also appears to be needed. The absence of an effect of DPI on cGMP levels and, by extension, NO levels in slices of rat cerebellum [44] may suggest a limited role for this process in

intact cerebellum. However, seeing that the NADPH-dependent responses in cerebellar glia are smaller than in forebrain synaptosomes (see Figures 1C and 1D), regional or cellular differences may exist. Examination of this possibility and more direct measurement of NO levels within intact brain tissue are needed to clarify the role of the NO inactivation pathway *in vivo*.

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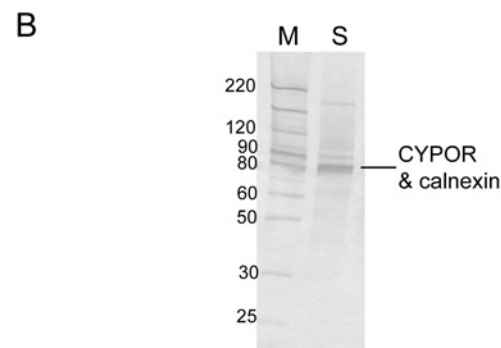
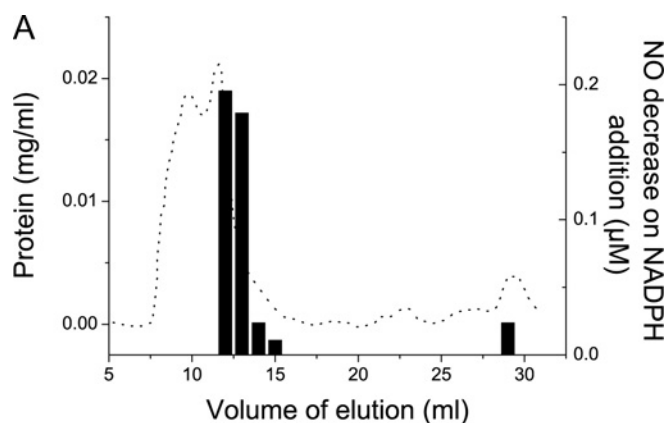


## SUPPLEMENTARY ONLINE DATA

# Cytochrome P450 oxidoreductase participates in nitric oxide consumption by rat brain

Catherine N. HALL<sup>1,2</sup>, Robert G. KEYNES<sup>1</sup> and John GARTHWAITE

Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6BT, U.K.



**Figure S1 Purification of NADPH-dependent NO consumption**

(A) Chromatogram showing elution of protein from a Superdex-200 gel filtration column (broken line) and NADPH-dependent NO consumption of samples at different volumes of elution (black bars). The second protein peak is the only one that is active. (B) Silver-stained gel showing protein size markers (M; with values in kDa to the left) and concentrated sample (S), which eluted from the Superdex-200 column at 12 ml. When stained with Colloidal Blue, only the labelled band at ~80 kDa was apparent. This was excised and, using peptide mass fingerprinting and combined MALDI (matrix-assisted laser-desorption/ionization)-MS and MS/MS (tandem MS) analysis (York Proteomics Services, University of York, U.K.), was found to contain calnexin (65 kDa) and CYPOR (76 kDa).

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<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed (email catherine.hall@ucl.ac.uk).