British Journal of Pharmacology (1995) 116, 2771-2777

Cerebrovascular consequences of repeated exposure to N^G-nitro-L-arginine methyl ester

[1]Paul A.T. Kelly, Isobel M. Ritchie & Fiona M. Collins

Department of Clinical Neurosciences, University of Edinburgh, Edinburgh EH4 2XU

1 Acute treatment with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) produces cerebral oligaemia. The effects of repeated exposure to L-NAME upon cerebral blood flow were examined to determine whether the enhanced NOS inhibition reported following chronic treatment might reduce cerebral perfusion to ischaemic levels.

2 Rats were treated with L-NAME (75 mg kg^{-1}, i.p.) once daily for 10 days. Local cerebral blood flow and glucose utilization were measured by [^{14}C]-iodoantipyrine and [^{14}C]-2-deoxyglucose quantitative autoradiography respectively, either 1 h or 15 h after the last injection. A second group of rats was injected (i.p.) only once with L-NAME, either 1 h or 15 h prior to the measurement procedures.

3 Mean arterial blood pressure (MABP) was significantly increased (+35%) 1 h after a single injection of L-NAME. Although the hypertension was reduced 15 h after the injection (+13%), MABP remained significantly higher than control.

4 Local cerebral blood flow was significantly decreased 1 h after a single injection of L-NAME (ranging from −45% to −54%), and remained so even after 15 h (−39% to −48%). At neither time-point was there any change in glucose utilization.

5 At 15 h after the final injection of the chronic L-NAME treatment protocol, MABP was significantly elevated from control (+58%) and was also significantly higher than at 1 h following a single injection (+20%). There was no effect upon the established hypertension when rats treated chronically with L-NAME were challenged with a further injection of the drug and MABP was measured 1 h later, suggesting saturation of NOS inhibition.

6 Although reduced, cerebral blood flow was not significantly different from control when measured 15 h after the last injection of the chronic L-NAME treatment. When rats treated chronically with L-NAME were subjected to a further challenge with the drug, cerebral blood flow was reduced when measured 1 h after the acute injection (ranging from −34% to −41%). There was however evidence of some attenuation in the response when compared to that measured 1 h after a single injection of L-NAME (ranging from −45% to −54%). Thus, the cerebral circulation shows no evidence of either sustained L-NAME-induced vasoconstriction or saturated NOS inhibition following 10 daily injections of L-NAME. Chronic L-NAME treatment had no effect upon cerebral glucose use.

7 The trend towards re-establishment of cerebrovascular dilator tone and the normalization of cerebral flow-metabolism relationships could explain the lack of any ischaemic damage found in chronically treated rats, but the loss of an extended autoregulatory range afforded by acute L-NAME treatment may be responsible for an increased incidence of stroke.

Keywords: Chronic L-NAME; nitric oxide; nitric oxide synthase inhibition; cerebral blood flow; cerebral glucose utilization; quantitative autoradiography

Introduction

Decreases in basal cerebral blood flow (CBF) have been described following nitric oxide synthase (NOS) inhibition by a variety of N^G-substituted arginine analogues, including N^G-nitro-L-arginine (L-NOARG) (Kovách *et al.*, 1992; Wang *et al.*, 1992; Dirnagl *et al.*, 1993), N^G-monomethyl-L-arginine (Tanaka *et al.*, 1991; Kozniewski *et al.*, 1992), and N^G-nitro-L-arginine methyl ester (L-NAME) (Goadsby *et al.*, 1992; Northington *et al.*, 1992; Prado *et al.*, 1992; Iadecola *et al.*, 1993b; Pelligrino *et al.*, 1993). Where experiments have included a measure of cerebral metabolic activity, it has been found that these decreases in CBF occur in the absence of any underlying change in metabolic demand for glucose (Macrae *et al.*, 1993; Kelly *et al.*, 1994). Under most normal physiological conditions, CBF is closely coupled to the underlying metabolic demands of brain tissue (Sokoloff, 1981) and where this relationship is severely compromised, as with excitotoxins, irreversible damage to brain tissues is likely to occur (Celik *et al.*, 1982). Thus the cerebral oligaemia which is evident following acute NOS inhibition represents an inherently unstable physiological situation with obvious potential for pathlogical sequaelae.

Repeated treatment with NOS inhibitors produces an incremental decrease in neuronal NOS activity (Dwyer *et al.*, 1991), but effects upon endothelial NOS, as they are manifest in the haemodynamics of peripheral vascular beds, are complex. Chronic exposure to NOS inhibitors in drinking water results in the development of a level of hypertension which is more pronounced than that observed following acute treatment and which persists even after the treatment has ended (Morton *et al.*, 1993). However, this masks a much more dynamic cardiovascular response in which both alterations in cardiac output and regionally heterogeneous constriction of vascular smooth muscle over the period of chronic treatment may contribute to the observed blood pressure response (Gardiner *et al.*, 1992; 1993a).

Given that a single injection of the NOS inhibitor L-NAME produces reductions in cerebral blood flow (Kelly *et al.*, 1994), repeated treatment might be expected to result in even more profound cerebrovascular effects, possibly reducing CBF to ischaemic levels. The resultant pathological perturbation of the

[1]Author for correspondence at: Department of Clinical Neurosciences, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU.

cerebral circulation would be likely to confound the interpretation of, for example, behavioural studies of learning and memory in which daily injections of NOS inhibitors are a necessary part of the experimental design (Bannerman *et al.*, 1994). In the present study, the effects of repeated exposure to L-NAME upon local cerebral blood flow (LCBF), and the relationship to local cerebral glucose utilization (LCGU), were examined in parallel groups of similarly treated rats to determine whether chronic L-NAME treatment results in an enhanced and sustained cerebrovascular oligaemia. The quantitative autoradiographic methods used to assess LCBF and LCGU allow only a single measurement in the brain regions of interest, and so that we might be able to differentiate between sustained effects of chronic L-NAME and those which might arise acutely from the last injection, we measured LCBF and LCGU at 1 h and 15 h after the final injection of the chronic treatment protocol and compared these results with the effects at similar times after a single acute exposure. Moreover, this experimental design provided insight into the effects of a further challenge with L-NAME upon cerebrovascular phenomena already established by the chronic treatment, and allowed comparisons to be made with the hypertensive effects of the drug treatments. An abstract describing parts of this study has been published (Kelly & Ritchie, 1994).

Methods

Acute treatment protocols

Male Sprague-Dawley rats (250–275 g) were injected intraperitoneally on a single occasion with either saline ($n=8$) or the nitric oxide synthase inhibitor, L-NAME (75 mg kg^{-1}; $n=16$). Equal numbers of rats were used for the measurement of LCBF or LCGU either 1 h or 15 h after the injection.

Chronic treatment protocols

Male Sprague-Dawley rats (250–275 g at the outset) were injected intraperitoneally with L-NAME (75 mg kg^{-1}; $n=20$) or saline ($n=20$) once daily for ten consecutive days. On the day of the experiment equal numbers from each group were injected with either saline or L-NAME (75 mg kg^{-1}) 1 h prior to the measurement of either LCBF or LCGU. The final injection of the chronic treatment was given 15 h prior to the acute injection. Thus there were four treatment groups for each of the two measures ($n=5$ in each treatment group for each measure); chronic saline + acute saline, chronic saline + acute L-NAME, chronic L-NAME + acute saline, chronic L-NAME + acute L-NAME.

Animal preparation

On the day of the experiment the animals were prepared for the measurement of LCBF and LCGU as previously described (Kelly *et al.*, 1994), with the addition of an indwelling intraperitoneal cannula inserted via a trocar into the abdominal space under halothane anaesthesia (1% in a mixture of 70% nitrous oxide and 30% oxygen). All wounds were infiltrated with local anaesthetic (2% lignocaine spray) and covered with anaesthetic-soaked gauze. Rectal temperature, blood pressure and heart rate were monitored continuously throughout the experimental period. Blood gas status and plasma glucose levels were measured immediately before drug treatment and again before the initiation of blood flow or glucose use measurements. Rats were allowed to recover from anaesthesia for at least 2 h before experimentation began.

Measurement of local cerebral blood flow

Cerebral blood flow was measured using the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique (Sakurada *et al.*, 1978). The tracer (35 μCi in 0.5 ml saline) was infused intravenously at a constantly accelerating rate over 45 s. During the infusion period, blood was allowed to flow freely from one of the arterial cannulae and timed samples were collected intermittently onto preweighed filter discs. The timing of each sample was subsequently corrected to take account of the delay introduced by the flow characteristics of the cannula tubing. At 45 s, the animals were killed by decapitation, the brains dissected intact, and frozen in pre-cooled isopentane (−45°C) within 2 to 3 min of death. The filter discs were placed in scintillation vials and reweighed to determine the sample weight before being prepared for liquid scintillation analysis. Sample weights were converted to units of volume assuming a specific gravity of 1.01.

Measurement of local cerebral glucose utilization

Glucose use was measured in 20 rats using the fully quantitative [^{14}C]-2-deoxyglucose autoradiographic technique. The measurement was initiated with a 30 s intravenous injection of tracer (40 μCi in 0.75 ml saline). Over the subsequent 45 min, a total of 14 timed arterial blood samples were collected at predetermined intervals and centrifuged to separate plasma. Aliquots of each plasma sample were taken for the determination of ^{14}C concentrations (20 μl) and glucose levels (10 μl) by liquid scintillation analysis and semi-automated glucose oxidase assay (Beckman) respectively. At the end of the measurement period the rats were killed by decapitation and the brains prepared as for blood flow experiments.

Preparation and analysis of autoradiograms

Semi-serial cryostat sections (20 μm) were cut in the coronal plane from each brain, with three consecutive sections collected from every 200 μm of tissue. Autoradiograms were prepared by application of the sections to X-ray film (SB-5, Kodak) in a light-tight cassette for 7 days and the films processed according to the manufacturer's instructions. The resultant autoradiograms were analysed with a computer-based imaging system (Cambridge Instruments Quantimet 970). Isotope concentrations in brain sections were determined by densitometric analysis relative to precalibrated ^{14}C-containing standards (Amersham, U.K.) and LCGU or LCBF calculated with the operational equations for the techniques (Sokoloff *et al.*, 1977; Sakurada *et al.*, 1978).

Statistical analysis

Data are presented as mean ± s.d. Statistical analyses of the results from physiological measurements and independent LCBF and LCGU data were performed by analysis of variance followed by a *post hoc* Scheffé test to allow multiple comparisons. Acceptable levels of significance were set at $P<0.05$. To provide an initial assessment of differences between treatment groups in the relationship of LCBF to LCGU in each of the brain areas included in this study, the ratios of mean LCBF to mean LCGU were derived for each structure examined and compared by the Mann-Whitney U-test. More rigorous statistical analysis of the flow-metabolism relationship was performed by repeated measures analysis of variance (McCulloch *et al.*, 1982) using the BMDP/PC90 computerized statistical package (program 2V) (Jennrich *et al.*, 1990). The analysis was performed on the log transform of LCBF and LCGU data, the measurement type being regarded as a grouping factor, and brain areas regarded as a repeated measures trial factor.

Results

Physiological parameters

Physiological data were pooled from LCBF and LCGU experiments with equivalent treatments. Marked hypertension

(MABP = 170 ± 8 mmHg) was evident 1 h after a single acute injection of L-NAME, and although MABP was significantly lower by 15 h after the injection, and was more variable (143 ± 14 mmHg), it was still significantly higher than control (126 ± 7 mmHg) (Figure 1). With the exception of MABP, there were no significant differences between groups in any of the other physiological parameters, arterial pH, P_{CO_2}, P_{O_2} or rectal temperature.

A hypertensive response, similar to that found after a single injection of L-NAME, was evident following acute L-NAME treatment in rats previously treated chronically with saline. In these rats, MABP rose from 125 ± 6 mmHg before the injection of L-NAME to 165 ± 4 mmHg 1 h afterwards (Figure 2). However, in contrast to the effects of a single injection, 15 h after the last injection in the chronic L-NAME pretreatment protocol, MABP (198 ± 8 mmHg) was not only significantly higher than in the control group (+58%) but was also higher than that found 1 h after an acute injection of L-NAME (+20%) (Figure 2). A further acute challenge with L-NAME had no effect upon MABP in the chronic L-NAME-treatment group (MABP = 202 ± 6 prior to acute injection and 202 ± 9 after 1 h). With the exception of MABP, there were no significant differences in any of the other physiological parameters, arterial pH, P_{CO_2}, P_{O_2}, or rectal temperature, either between treatment groups, or when analysed within groups before and after acute treatments.

Effects of a single injection of L-NAME upon local cerebral blood flow

In keeping with previous results (Bannerman *et al.*, 1994), LCBF was significantly reduced from control values in all of the brain areas analysed 1 h after a single i.p. injection of L-NAME (Table 1). These reductions in flow were relatively constant throughout neocortical areas, ranging from −47% in occipital cortex to −51% in cingulate cortex, with similar effects in hippocampus (−45% to −54%) and striatum (−46%). Significant reductions in LCBF were also evident when the measurement was made 15 h after a single injection (Table 1), and although mean LCBF values were slightly higher than those measured at 1 h, the difference in response was minimal, ranging from 1 to 9%, and was not significant.

Effects of a single injection of L-NAME upon local cerebral glucose utilization and the flow metabolism relationship

A single injection of L-NAME had no significant effect upon LCGU measured at either 1 h or 15 h after the injection (Table 2). Decreases in LCBF in response to L-NAME in the absence of any changes of similar magnitude in LCGU, had profound effects upon the relationship between brain tissue perfusion and underlying metabolism. In control rats, there was clearly an association between mean LCBF and mean LCGU in the brain as a whole ($r = 0.92$) and the overall ratio between blood flow and glucose use, as illustrated by the slope of the best-fitting straight line, was 1.48 (Figure 3). In those animals treated with L-NAME, the relationship between LCBF and LCGU remained largely intact at both 1 h and 15 h after a single injection ($r = 0.91$; $r = 0.89$) (Figure 3). However, the overall ratios between LCBF and LCGU ($m = 0.78$; $m = 0.87$) were lower than in the control group (Figure 3).

An initial analysis using Mann-Whitney U-test revealed that the ratios of LCBF to LCGU were significantly lower than control following L-NAME treatment ($P < 0.004$), but that there was no difference between the ratios at the two time points. The more rigorous analysis afforded by repeated measures analysis of variance confirmed that following L-NAME treatment, the flow-metabolism relationship was significantly different from control ($P < 0.006$).

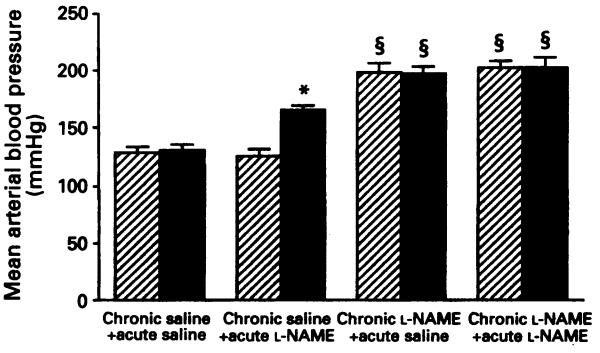

Figure 2 Effects of repeated i.p. injections of saline or L-NAME (75 mg kg^{-1}, once daily for 10 consecutive days) upon mean arterial blood pressure, measured before (hatched columns), and 1 h after (solid columns) a final acute challenge with saline or L-NAME. Data are presented as mean (mmHg) ± s.d. *Significantly different from pre-acute levels within groups; §significantly different from levels following a single L-NAME treatment (i.e. chronic saline + acute L-NAME) ($P < 0.05$; ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons).

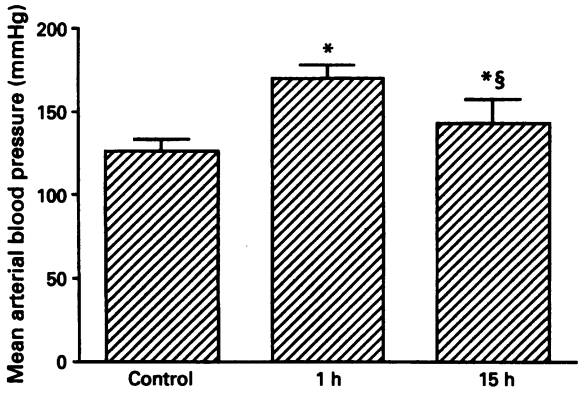

Figure 1 Effects of a single i.p. injection of saline or L-NAME (75 mg kg^{-1}) upon mean arterial blood pressure measured at 1 h and 15 h after treatment. Data are presented as mean (mmHg) ± s.d. for each group. *Significantly different from control and § significantly diffferent from measurement at 1 h ($P < 0.05$; ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons).

Table 1 Effects of a single injection of L-NAME upon local cerebral blood flow

	Control	Acute L-NAME 1 hour	15 hour
Neocortex			
Occipital	170 ± 12	90 ± 8*	96 ± 5*
Parietal	156 ± 7	79 ± 6*	85 ± 6*
Piriform	112 ± 6	58 ± 4*	62 ± 6*
Cingulate	161 ± 10	79 ± 6*	84 ± 8*
Hippocampus			
CA1	79 ± 6	39 ± 3*	41 ± 6*
CA2	93 ± 8	43 ± 5*	51 ± 4*
CA3	97 ± 7	53 ± 5*	59 ± 5*
Dentate gyrus	106 ± 8	55 ± 7*	56 ± 5*
Basal ganglia			
Striatum	152 ± 10	81 ± 7*	89 ± 4*

Data are presented as mean cerebral blood flow (ml $100\,g^{-1}$ min^{-1}) ± s.d. ($n = 5$ in each group). * Significantly different from saline-treated control group ($P < 0.05$; ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons). Blood flow was measured at either 1 h or 15 h after the injection of L-NAME.

Table 2 Effects of a single injection of L-NAME upon local cerebral glucose utilization

	Control	Acute L-NAME 1 hour	15 hour
Neocortex			
Occipital	87±5	91±5	95±4
Parietal	84±6	83±6	86±2
Piriform	50±6	57±4	59±2
Cingulate	101±10	108±6	108±7
Hippocampus			
CA1	47±4	48±5	53±4
CA2	47±3	50±4	54±4
CA3	60±4	62±2	66±3
Dentate gyrus	67±3	68±5	74±6
Basal ganglia			
Striatum	95±5	96±6	93±5

Data are presented as mean cerebral glucose utilization (μmol 100 g^{-1} min^{-1})±s.d. ($n=5$ in each group). No significant differences were detected (ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons). Glucose utilization was measured at either 1 h or 15 h after the injection of L-NAME.

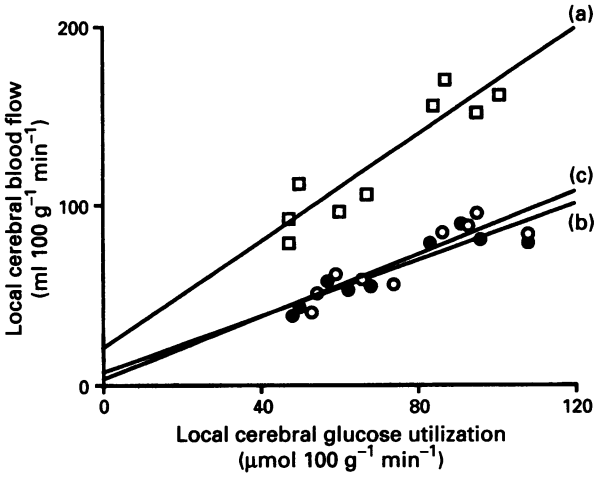

Figure 3 Effects of a single i.p. injection of saline or L-NAME (75 mg kg^{-1}) upon the relationship between local cerebral blood flow (LCBF) and local cerebral glucose utilization (LCGU) measured at 1 h and 15 h after treatment. Data are plotted as mean LCGU and mean LCBF values for each of the nine brain areas analysed. The best-fitting straight lines are shown for (a) saline controls (□), and L-NAME-treated rats measured (b) at 1 h (●) and (c) 15 h (○) after treatment.

Effects of repeated injections of L-NAME upon local cerebral blood flow

As expected, the acute response to L-NAME in rats previously treated for 10 days with saline, was both qualitatively and quantitatively similar to that found in the acute study (cf. Tables 1 and 3). Once again the reductions in flow were relatively constant throughout neocortical areas, ranging from −44% in occiptial cortex to −55% in cingulate cortex, with similar effects in hippocampus (−47% to −53%) and striatum (−54%) (Table 3). When measured 15 h after the last injection of the chronic L-NAME treatment protocol, LCBF was significantly lower than control only in CA1 (−20%) and dentate gyrus (−21%) of the hippocampus, and in the striatum (−22%), but in all areas of the brain, LCBF was significantly higher than following an acute injection of L-NAME (Table 3). When chronic L-NAME-treated animals were challenged with a further injection of L-NAME, LCBF (measured 1 h later) was significantly reduced when compared to controls and, with the exception of CA1 and CA2 hippocampal fields, was also significantly lower than with chronic treatment alone (Table 3). Although LCBF measured in this group was consistently higher than in the chronic saline with acute L-NAME treatment group, the conservative statistical analysis necessary for multiple pair-wise comparisons indcated significantly attenuated responses to L-NAME only in striatum and piriform cortex (Table 3).

Effects of repeated injections of L-NAME upon local cerebral glucose utilization and the flow-metabolism relationship

As in the acute study, there were no significant differences in LCGU between any of the chronic treatment groups (Table 4), and once again this absence of changes in LCGU whilst LCBF was decreased, resulted in a resetting of the flow-metabolism relationship in L-NAME-treated rats (Figure 4). The association between mean LCBF and mean LCGU found in rats treated chronically with saline ($r=0.93$) was maintained across all treatment groups whether treated acutely ($r=0.91$), or chronically with L-NAME ($r=0.90$), or challenged acutely following chronic treatment ($r=0.88$). However, the overall ratio between LCBF and LCGU, as illustrated by the slope of the best-fitting straight line, decreased from $m=1.51$ in rats chronically treated with saline, to $m=0.77$ in rats treated only

Table 3 Effects of repeated exposure to L-NAME upon local cerebral blood flow

	Chronic saline Acute saline	Acute L-NAME	Chronic L-NAME Acute saline	Acute L-NAME
Neocortex				
Occipital	164±18	92±11*	145±6§	100±14*#
Parietal	146±14	79±9*	139±9§	102±11*#
Piriform	114±11	51±5*	107±13§	72±5*§#
Cingulate	152±16	75±8*	136±10§	94±8*#
Hippocampus				
CA1	87±9	42±6*	70±8*§	57±6*
CA2	94±8	47±6*	81±8§	62±6*
CA3	96±13	51±6*	89±9§	63±6*#
Dentate gyrus	116±13	55±7*	92±10*§	71±8*#
Basal ganglia				
Striatum	166±13	77±4*	129±6*§	98±12*§#

Data are presented as mean cerebral blood flow (ml 100 g^{-1} min^{-1})±s.d. ($n=5$ in each group). *, Significantly different from saline-treated control groups; §, significantly different from acute L-NAME treatment; #, significantly different from chronic L-NAME treatment ($P<0.05$; ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons). Blood flow was measured at 1 h after the last injection of either saline or L-NAME.

Table 4 Effects of repeated exposure to L-NAME upon local cerebral glucose utilization

	Chronic saline		*Chronic L-NAME*	
	Acute saline	*Acute L-NAME*	*Acute saline*	*Acute L-NAME*
Neocortex				
Occipital	92±2	98±8	106±9	96±8
Parietal	81±5	83±9	83±11	75±5
Piriform	59±4	60±8	67±6	62±6
Cingulate	101±3	105±10	109±11	95±8
Hippocampus				
CA1	45±2	50±6	55±8	49±6
CA2	56±3	62±8	62±5	60±6
CA3	54±3	51±6	59±11	63±6
Dentate gyrus	65±2	66±7	73±8	67±8
Basal ganglia				
Striatum	84±5	93±9	90±7	85±4

Data are presented as mean cerbral glucose utilization (μmol 100 g^{-1} min^{-1}) ± s.d. ($n=5$ in each group). No significant differences were detected (ANOVA followed by *post hoc* Scheffé test to allow all possible pair-wise comparisons). Glucose utilization was measured at 1 h after the last injection of either saline or L-NAME.

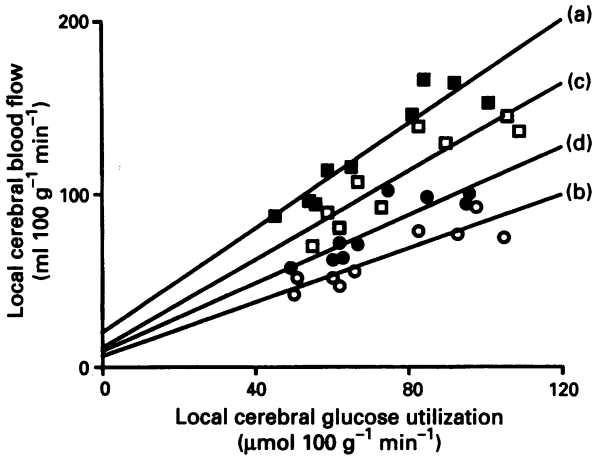

Figure 4 Effects of repeated i.p. injections of saline or L-NAME (75 mg kg^{-1}, once daily for 10 consecutive days) upon the relationship between local cerebral blood flow (LCBF) and local cerebral glucose utilization (LCGU) measured 1 h after a final challenge with saline or L-NAME. Data are plotted as mean LCGU against mean LCBF values for each of the nine brain areas analysed. The best-fitting straight lines are shown for (a) chronic saline + acute saline (■), (b) chronic saline + acute L-NAME (○), (c) chronic L-NAME + acute saline (□), and (d) chronic L-NAME + acute L-NAME-treated rats (●).

acutely with L-NAME (cf. $m=1.48$ and 0.78 following similar treatment in the acute study: Figures 3 and 4). In rats treated chronically with L-NAME, the overall ratio ($m=1.27$) was greater than that following acute L-NAME, but the ratio was decreased when chronically treated animals were challenged with a further L-NAME injection ($m=0.98$).

An intitial analysis using Mann-Whitney U-test revealed that the ratios of LCBF to LCGU were significantly different in all four groups ($P<0.008$), but the more rigorous (and conservative) analysis afforded by repeated measures analysis of variance indicated statistically significant differences only between the control group (chronic saline with acute saline) and the two acute L-NAME-treated groups ($P<0.01$).

Discussion

Exposure to NOS inhibitors administered in drinking water results in the relatively rapid development of hypertension in polydipsic Brattleboro rats (Gardiner *et al.*, 1990). In the acute phase of experiments such as these, peripheral vasoconstriction is accompainied by a significant decrease in cardiac output (Gardiner *et al.*, 1992). Although the blood pressure response is sustained with treatment periods extending to 72 h, levels of vasoconstriction, which are apparent acutely, do actually decline in some peripheral vascular beds (Gardiner *et al.*, 1993b). The maintenance of the blood pressure response despite reduced vasoconstriction may result from a restoration of cardiac output.

With longer treatment protocols, a similar hypertensive phenomenon has also been described in genetically normal rats, and chronic inhibition of NOS has been advanced as a useful model of arterial hypertension (Ribeiro *et al.*, 1992). Animals treated with NOS inhibitors over several weeks develop a marked hyptertension which persists even after treatment with the NOS inhibitor has ceased (Morton *et al.*, 1993). However, if the treatment is continued for a sufficiently long period (11 weeks), the incidence of mortality increases rapidly, due in the main to renal failure, but cerebrovascular accident in the spinal cord and brain may also be the cause of death (Blot *et al.*, 1994). Whilst stroke is an all too common outcome in chronically hypertensive patients, it is a surprising result in these rats given the effects upon the cerebrovasculature of acute NOS inhibition. Although drug-induced acute arterial hypertension in excess of around a mean pressure of 150 mmHg results in breakthrough of cerebrovascular autoregulation, acute L-NAME has been shown to extend the autoregulatory range to blood pressures considerably in excess of the normal upper limit of autoregulation (Kelly *et al.*, 1994; Talman *et al.*, 1995). It would appear therefore that the increased constrictor tone which results from acute L-NAME treatment, provides a degree of cerebrovascular protection against hypertension similar to that found with sympathetic stimulation (McKenzie *et al.*, 1979). It is a matter of interest therefore that the protection afforded by acute L-NAME treatment is apparently lost in the course of chronic treatment (Blot *et al.*, 1994), despite the fact that the peripheral effects, as reflected in vasoconstriction-induced hypertension, are maintained. In contrast to these previous studies, we used a parental intraperitoneal (i.p.) rather than enteral (via drinking water) route of L-NAME administration and much shorter treatment protocols (10 days as opposed to 11 weeks) so that direct parallels might be drawn between our examination of the cerebrovascular consequences of chronic NOS inhibition and previous behavioural studies (Bannerman *et al.*, 1994).

In this study, a single injection of L-NAME, either in previously naïve rats or rats treated chronically with saline, had similar effects in both peripheral and cerebral vessels when measured at 1 h. In the peripheral circulation, the constrictor effects were manifest in an increase in MABP of between 30 and 35%, whilst in the cerebral circulation significant vasoconstrictor-induced decreases in LCBF were measured. As-

suming no increase in intracranial pressure, decreased cerebral perfusion in the face of such a marked increase in perfusion pressure represents a considerable increase in cerebrovascular resistance. Interestingly, whilst the acute hypertensive effects of a single i.p. injection of L-NAME were significantly attenuated by 15 h, the effects upon the cerebral circulation remained almost unchanged from those measured at 1 h. Previous experiments using the intravenous route of administration have found reductions in LCBF to be maintained up to 3 h after a single bolus injection of L-NAME (Macrae *et al.*, 1993), but in that instance peripheral effects were of a similar duration. Whether the duration of the acute cerebrovascular response observed here represents a universal phenomenon, or is a function of dose and/or route of administration, remains to be determined.

Although acute NOS inhibition is followed by peripheral vasoconstriction, not all vascular beds are equally sensitive (Gardiner *et al.*, 1990) and neither is the response maintained for an equally long time with chronic treatment (Gardiner *et al.*, 1992). Thus the dissociation between cerebrovascular effects of L-NAME and those found in the peripheral circulation after a single dose of L-NAME should perhaps not be surprising. What is of interest however, is the observation that 15 h after the final injection of a chronic L-NAME treatment protocol, cerebral blood flow is found to be almost completely restored to control levels whilst the hypertensive response is not only sustained but also enhanced. It is not immediately apparent why dilator tone should return to cerebral blood vessels whilst those responsible for determining total peripheral vascular resistance should remain constricted, but differences in the response to chronic L-NAME between vascular beds have been reported previously (Bryant *et al.*, 1995).

Whilst the exact mechanisms by which dilator tone is re-established in the cerebral circulation, or the constriction induced by L-NAME is attenuated, may not be clear, what is clear is that the level of cerebral perfusion found after acute L-NAME treatment is considerably lower than would normally be expected for the given rate of glucose utilization, which despite the treatment, remains at control levels. It is possible that the imperative for fuelling the metabolic demands of brain tissue provides, over time, a sufficiently intense stimulus to overcome the constrictor effects of NOS inhibition and the effects upon LCBF may be interpreted as the re-establishment (by some mechanism as yet unknown) of flow-metabolism homeostasis to protect against potentially deleterious effects of sustained oligaemia. Interestingly however, there is no evidence in the brain of the reactive hyperaemia which might be expected to follow a period of oligaemia, although this could have occurred at an earlier time. Moreover, whilst in the periphery a further acute L-NAME challenge to previously L-NAME-treated rats has no further constrictor effect (i.e. no further increase in MABP), the cerebral circulation remains responsive, albeit to a somewhat attenuated extent. This would be compatible with an up-regulation of cerebral NOS production, providing additional NO-induced cerebrovascular dilatation with a return to normal perfusion levels, and additional targets for further L-NAME challenge, but current evidence points in the opposite direction towards a cumulative, near complete inhibition of cerebral NOS activity with chronic treatment.

The resolution of the acute L-NAME-induced cerebral oligaemia during the course of chronic treatment has clear parallels in behavioural studies of cognitive processes (Bannerman *et al.*, 1994). With the same dosing regime as used in this present study, L-NAME produced impaired performance in a visual discrimination task, but only on early trials. In later trials treated rats were indistinguishable from controls, despite a greater than 90% decrease in NOS activity in response to L-NAME. It is possible that the early deficits in learning capacity reflect an oligaemia-induced cerebral dysfunction, but with the subsequent attenuation of the blood flow effects over the course of chronic L-NAME treatment, the behavioural deficit is also resolved. Whether there is indeed a cause and effect relationship between cerebral oligaemia and learning deficits remains to be determined, but we do know that inappropriately low cerebral blood flow can lead to a confused state of mind.

The resolution of the acute L-NAME-induced oligaemia in animals treated chronically is not achieved without potential cost. The cerebrovascular constriction which results from acute NOS inhibition extends the autoregulatory range and thus provides the blood vessels of the brain with protection against the concomitant hypertension (Kelly *et al.*, 1994; Talman *et al.*, 1995). However in animals treated chronically, the protective effects of L-NAME upon the autoregulatory range will be lost as the hypertensive response is enhanced whilst cerebrovascular constriction appears to be attenuated and as a result the risk of hypertension-induced cerebrovascular accident must be increased. This would certainly explain previous reports of a complete absence of stroke at early time-points during chronic enteral treatment and the increased incidence at later times (Blot *et al.*, 1994). In an attempt to investigate whether such processes did indeed operate, we have attempted to lengthen the period of L-NAME treatment to 21 days. We found however, that the rats either displayed a similar pattern of LCBF responses to those found at 10 days (with no evidence of the hyperaemia which would indicate a breakthrough of autoregulatory function), died suddenly (usually overnight), or became progressively unwell and therefore could not be included in the study. These experiments were quickly abandoned as being ethically unacceptable.

In conclusion, the hypertension induced by chronic treatment with L-NAME is greater than that following a single acute injection, but appears to reach a point of saturation where further challenges with L-NAME do not add to the established hypertensive response. In contrast, reductions in cerebral blood flow which are apparent following a single acute injection of L-NAME appear to be attenuated following repeated exposure, in both magnitude and duration, but a residual capacity to respond to a further acute challenge remains in evidence. The complexities of NO action which underlie these responses have yet to be determined but these results, together with previous observations in the peripheral vasculature (Gardiner *et al.*, 1992), caution against the assumption of a generalized vasoconstriction to chronic NOS inhibition based merely upon the observation of a hypertensive response. Moreover, should NOS dysfunction underlie the vascular pathology of some disease states, it would appear that homeostatic mechanisms which may exist to protect the brain against the consequent oligaemia, may also render the brain more susceptible to hypertension-induced cerebrovascular accident.

This study was funded by the Wellcome Trust. F.M.C. was a Nuffield Foundation Vacation Scholar.

References

BANNERMAN, D.M., CHAPMAN, P.F., KELLY, P.A.T., BUTCHER, S.P. & MORRIS, R.G.M. (1994). Inhibition of nitric oxide synthase does not impair spatial learning. *J. Neurosci.*, **14**, 7404–7414.

BLOT, S., ARNAL, J.-F., XU, Y., GRAY, F. & MITCHELL, J.-B. (1994). Spinal cord infarcts during long-term inhibition of nitric oxide synthase in rats. *Stroke*, **25**, 1666–1673.

BRYANT, C.E., ALLCOCK, G.H. & WARNER, T.D. (1995). Comparison of effects of chronic and acute administration of N^G-nitro-L-arginine methyl ester to the rat on inhibition of nitric oxide-mediated responses. *Br. J. Pharmacol.*, **114**, 1673–1679.

CELIK, G., GRAHAM, D.I., KELLY, P.A.T. & MCCULLOCH, J. (1982). Kainic acid administration and the relationship between local cerebral glucose utilization and local cerebral blood flow. In *Cerebral Blood Flow: Effects of Nerves and Neurotransmitters*. ed. Heistad, D.D. & Marcus, M.L. pp. 249–255. New York: Elsevier.

DIRNAGL, U., LINDAUER, U. & VILLRINGER, A. (1993). Role of nitric oxide in the coupling of cerebral blood flow to neuronal activation. *Neurosci. Lett.*, **149**, 43–46.

DWYER, M.A., BREDT, D.S. & SYNDER, S.H. (1991). Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain in vitro and in vivo. *Biochem. Biophys. Res. Commun.*, **176**, 1136–1141.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Regional haemodynamic changes during oral ingestion of N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester in conscious Brattleboro rats. *Br. J. Pharmacol.*, **101**, 10–12.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1993a). Regional haemodynamics in Brattleboro rats during chronic ingestion of N^G-nitro-L-arginine methyl ester. *Blood Pressure*, **2**, 228–232.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1993b). Effects of chronic treatment with nitric oxide synthase inhibitors on regional haemodynamic responses to vasodilators in conscious Brattleboro rats. *Br. J. Pharmacol.*, **109**, 222–228.

GARDINER, S.M., KEMP, P.A., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1992). Nitric oxide synthase inhibitors cause sustained, but reversible, hypertension and hindquarters vasoconstriction in Brattleboro rats. *Eur. J. Pharmacol.*, **213**, 449–451.

GOADSBY, P.J., KAUBE, H. & HOSKIN, K.L. (1992). Nitric oxide synthesis couples cerebral blood flow and metabolism. *Brain Res.*, **595**, 167–170.

IADECOLA, C., ZHANG, F. & XU, X. (1993). Role of nitric oxide synthase-containing vascular nerves in cerebrovasodilation elicited from cerebellum. *Am. J. Physiol.*, **264**, R738–R746.

JENNRICH, R., SAMPSON, P. & FRANE, J. (1990). Analysis of variance and covariance with repeated measures. In *BMDP Statistical Software Manual, Vol. 1*. ed. Dixon, W.J., Brown, M.B., Engelman, L. & Jennrich, R.I. pp. 489–527. Berkeley: University of California Press.

KELLY, P.A.T. & RITCHIE, I.M. (1994). Local cerebral blood flow following repeated exposure to N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **113**, 78P.

KELLY, P.A.T., THOMAS, C.L., RITCHIE, I.M. & ARBUTHNOTT, G.W. (1994). Cerebrovascular autoregulation in response to hypertension induced by N^G-nitro-L-arginine methyl ester. *Neuroscience*, **59**, 13–20.

KOVÁCH, A.G.B., SZABÓ, C., BENYÓ, Z., CSÁKI, C., GREENBERG, J.H. & REIVICH, M. (1992). Effects of NG-nitro-L-arginine and L-arginine on cerebral blood flow in the cat. *J. Physiol.*, **449**, 183–196.

KOZNIEWSKI, E., OSEKA, M. & STYS, T. (1992). Effects of endothelium-derived nitric oxide on cerebral circulation during normoxia and hypoxia in the rat. *J. Cereb. Blood Flow Metab.*, **12**, 311–317.

MCKENZIE, E.T., MCGEORGE, A.P., GRAHAM, D.I., FITCH, W., EDVINSSON, L. & HARPER, A.M. (1979). Effects of increasing arterial pressure on cerebral blood flow in the baboon: Influence of the sympathetic nervous system. *Pflügers Arch.*, **378**, 189–195.

MACRAE, I.M., DAWSON, D.A., NORRIE, J.D. & MCCULLOCH, J. (1993). Inhibition of nitric oxide synthesis; Effects upon cerebral blood flow and gluscose utilization in the rat. *J. Cereb. Blood Flow Metab.*, **13**, 985–992.

MCCULLOCH, J., KELLY, P.A.T. & FORD, I. (1982). Effect of apomorphine on the relationship between local cerebral glucose utilization and local cerebral blood flow (with an appendix on its statistical analysis). *J. Cereb. Blood Flow Metab.*, **2**, 487–499.

MORTON, J.J., BEATTIE, E.C., SPEIRS, A. & GULLIVER, F. (1993). Persistent hypertension following inhibition of nitric oxide formation in the young Wistar rat: role of renin and vascular hypertrophy. *J. Hypertension*, **11**, 1083–1088.

NORTHINGTON, F.J., MATHERNE, G.P. & BERNE, R.M. (1992). Competitive inhibition of nitric oxide synthase prevents the cortical hyperaemia associated with peripheral nerve stimulation. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6649–6652.

PELLIGRINO, D.A., KOENIG, H.M. & ALBRECHT, R.F. (1993). Nitric oxide synthesis and regional cerebral blood flow responses to hypercapnia and hypoxia in the rat. *J. Cereb. Blood. Flow Metab.*, **13**, 80–87.

PRADO, R., WATSON, M.D., KULUZ, J. & DIETRICH, W.D. (1992). Endothelium-derived nitric oxide synthase inhibition. Effects on cerebral blood flow, pial artery diameter, and vascular morphology in rats. *Stroke*, **23**, 1118–1124.

RIBEIRO, M.O., ANTUNES, E., DE NUCCI, G., LOVISOLO, S.M. & ZATZ, R. (1992). Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension*, **20**, 298–303.

SAKURADA, O., KENNEDY, C., JEHLE, J., BROWN, J.D., CARBIN, G.L. & SOKOLOFF, L. (1978). Measurement of cerebral blood flow with iodo-[^{14}C]-antipyrine. *Am. J. Physiol.*, **234**, H59–H66.

SOKOLOFF, L. (1981). Relationships among local functional activity, energy metabolism and blood flow in the central nervous system. *Fed. Proc.*, **40**, 2311–2316.

SOKOLOFF, L., REIVICH, M., KENNEDY, C., DES ROSIERS, M.H., PATLAK, C.S., PETTIGREW, K.D., SAKURADA, O. & SHINOHARA, M. (1977). The [^{14}C]-deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anaesthetized albino rat. *J. Neurochem.*, **28**, 897–916.

TALMAN, W.T. & DRAGON, D.N. (1995). Inhibition of nitric oxide synthesis extends cerebrovascular autoregulation during hypertension. *Brain Res.*, **672**, 48–54.

TANAKA, K., GOTOH, F., GOMI, S., TAKASHIMA, S., MIHARA, T., SHIRAI, T., NOGAWA, S. & NAGATA, E. (1991). Inhibition of nitric oxide synthesis induces a significant reduction in local cerebral blood flow in the rat. *Neurosci. Lett.*, **127**, 129–132.

WANG, Q., PAULSON, O.B. & LASSEN, N.A. (1992). Effects of nitric oxide blockade by N^G-nitro-L-arginine on cerebral blood flow response to changes in carbon dioxide tension. *J. Cereb. Blood Flow Metab.*, **12**, 591–601.

(Received May 31, 1995
Revised July 27, 1995
Accepted July 31, 1995)