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Anti-ischaemic efficacy of a nitric oxide synthase inhibitor and a N-methyl-D-aspartate receptor antagonist in models of transient and permanent focal cerebral ischaemia

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1 We have recently developed a new model of transient focal ischaemia in the rat utilising topical application of endothelin-1 to the left middle cerebral artery (MCA). In order to validate this approach the present study assessed the neuroprotective efficacy of the NMDA receptor antagonist dizocilpine (MK-801) in the endothelin-1 model. The anti-ischaemic efficacy of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was subsequently evaluated, and contrasted with its efficacy against permanent focal ischaemia, to determine the utility of the endothelin-1 model for identification of novel pharmacoprotective agents.

2 MK-801 (0.12 mg kg^{-1} bolus, 108 $\mu g\ kg^{-1}\ h^{-1}$ infusion i.v., either 1 or 2.5 h pre-transient MCA occlusion (MCAO)) induced hypotension that persisted for approximately 1.5 h so that mean arterial blood pressure (MABP) at the time of MCAO was significantly lower in the 1 h group compared with control (MABP: 86 ± 11, 68 ± 6 and 84 ± 4 mmHg (mean ± s.d.) for saline, 1 h MK-801 and 2.5 h MK-801 groups respectively). The 2.5 h pretreatment schedule resulted in significant reduction (71%) in the volume of hemispheric damage (assessed 4 h post onset of ischaemia) while the 1 h pretreatment schedule did not (volumes of hemispheric damage: 59 ± 38, 51 ± 51 and 17 ± 28 mm^3 for saline, 1 h and 2.5 h MK-801 groups).

3 Thus the considerable neuroprotective effect of MK-801 in the endothelin-1 model of transient focal cerebral ischaemia was highly sensitive to drug-induced hypotension. This result is in contrast to previous studies of permanent MCAO where MK-801-induced hypotension did not compromise its neuroprotective action.

4 L-NAME (3 mg kg^{-1}, i.v. 30 min pre-MCAO) moderately, but significantly, reduced (16%) the volume of ischaemic damage 4 h post-permanent MCA occlusion, whereas the 29% reduction in volume of damage achieved in the model of transient focal ischaemia did not attain significance due to the greater variability associated with this model. L-NAME did not significantly alter MABP in either model.

5 The modest neuroprotection achieved with NO synthase inhibition suggests NO is of relatively minor importance as a mediator of neurotoxicity following permanent focal cerebral ischaemia. In addition the comparable efficacy of L-NAME against transient focal ischaemia suggests the presence of reperfusion does not enhance the contribution of NO to neuronal injury in the acute (4 h) phase following a focal ischaemic insult.

Keywords: Transient focal ischaemia; glutamate; MK-801; nitric oxide; L-NAME; endothelin-1

Introduction

Animal models of permanent middle cerebral artery (MCA) occlusion have been used extensively to study focal cerebral ischaemia. In particular the repeated demonstration of the anti-ischaemic efficacy of glutamate receptor antagonists in these models has led to the elucidation of the important role of excitotoxic mechanisms in mediating focal cerebral ischaemic damage. However, in man spontaneous resolution of embolic stroke and increased use of thrombolytic therapy means reperfusion following stroke is more common than persistence of the occlusion (Ringelstein *et al.*, 1992). Thus models of transient focal ischaemia have now been developed (see Macrae, 1992, for review) to assess the physiological significance of reperfusion to cerebral injury. We have previously characterized a novel model of transient MCA occlusion in the rat that utilises the potent vasoconstrictor peptide, endothelin-1, to induce temporary occlusion of the MCA (Macrae *et al.*, 1993b). Our model involves direct topical application of endothelin-1 to the exposed proximal portion of the MCA, a procedure that results in profound ischaemia followed by slow, progressive reperfusion that has been characterized over the first 4 h following endothelin-1 application (Macrae *et al.*, 1993b; Gartshore *et al.*, 1994). This model should not be confused with an alternative approach that uses intraparenchymal injection of endothelin-1 to induce cerebral ischaemia (Sharkey *et al.*, 1993). This alternative model (Sharkey *et al.*, 1993) is associated with a different temporal profile of blood flow changes (i.e. profound ischaemia sustained for over 3 h) and as such is more comparable to permanent than transient focal ischaemia.

The development of the endothelin-1 model of transient focal ischaemia and also other models of transient ischaemia means pharmacotherapies with proven efficacy against permanent focal ischaemic damage can now begin to be evaluated in the more clinically relevant context of ischaemia-reperfusion. However, models of transient focal ischaemia are by their very nature associated with greater variability than permanent ischaemia since the incorporation of a period of reperfusion into the design introduces an additional level of complexity above that associated with the ischaemic phase. Thus alterations in key physiological parameters such as blood pressure and cerebral blood flow (CBF) during both the ischaemic and reperfusion phases may impact on final outcome, and it may prove more difficult to demonstrate

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neuroprotective efficacy in the context of transient compared with permanent MCA occlusion. We therefore wished to compare the efficacy of different treatment strategies in permanent and transient MCA occlusion. The endothelin-1 model of transient focal ischaemia was first validated by assessing the efficacy of the N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (MK-801), a drug with proven efficacy against permanent focal ischaemic damage (see McCulloch *et al.*, 1991 for review). The efficacy of a low dose of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) was then compared in our models of permanent and transient MCA occlusion.

It has recently been proposed that glutamatergic excitotoxicity is mediated by NO. Inhibitors of NO synthase antagonize glutamate-induced neuronal injury both *in vitro* (Dawson *et al.*, 1991) and *in vivo* (Fujisawa *et al.*, 1993), but reports of their efficacy in models of permanent focal ischaemia are contradictory (Buisson *et al.*, 1992; Dawson *et al.*, 1992; Yamamoto *et al.*, 1992). The failure to observe neuroprotection consistently with NO synthase inhibitors *in vivo* may partly derive from the dose-dependent cerebral hypoperfusion induced by these drugs (Tanaka *et al.*, 1991; Macrae *et al.*, 1993a). We have previously demonstrated that a relatively high dose of L-NAME (2×30 mg kg^{-1}) has no significant effect on volume of ischaemic damage following permanent MCA occlusion (Dawson *et al.*, 1992). Therefore in the present study the efficacy of a lower dose of L-NAME (3 mg kg^{-1}) was assessed.

Methods

Surgical preparation and induction of ischaemia

Adult male Sprague-Dawley rats were anaesthetized with halothane (5%) in nitrous oxide:oxygen (70:30). Anaesthesia was subsequently maintained with 1% halothane. A tracheostomy was performed and the rats were artificially ventilated via a small respirator pump. The femoral arteries and veins were cannulated for blood sampling, continuous monitoring of blood pressure, and drug administration. Rectal temperature was maintained around 37°C by means of a heating blanket.

For both permanent and transient MCA occlusion, the left MCA was exposed using the sub-temporal approach previously described (Tamura *et al.*, 1981) with the exception that the zygomatic arch was left intact. A fine temperature probe was inserted into the ipsilateral temporalis muscle to estimate brain temperature during exposure and occlusion of the MCA. For permanent MCA occlusion the artery was occluded by bipolar diathermy from where it crossed the inferior cerebral vein to proximal to the origin of the lenticulo-striate branch(es). The artery was then transected to confirm complete occlusion and prevent recanalisation. The method for induction of transient MCA occlusion has been described previously in detail (Macrae *et al.*, 1993b). Briefly the arachnoid membrane overlying the MCA was opened, then endothelin-1 (2.5 nmol in 25 µl water) was applied topically to the proximal portion of the artery. Constriction of the main artery and the lenticulo-striate branch(es) was verified via the operating microscope. Following induction of permanent or transient MCA occlusion, the temporalis muscle temperature probe was removed and the wound closed with sutures.

Quantification of ischaemic damage

Four hours following the onset of permanent or transient MCA occlusion the animals were perfusion-fixed with 40% formaldehyde:acetic acid:methanol (1:1:8) and the brains processed for histological quantification of ischaemic damage (Osborne *et al.*, 1987). Haematoxylin and eosin stained sections at 8 pre-selected stereotactic levels were examined under a light microscope. Regions showing ischaemic cell change and evidence of early infarction (Brown, 1977) were transcribed onto scale drawings normalised to the mean hemisphere volume (570 mm^3) for Sprague-Dawley rats of the weight used. Areas of damage on the line drawings were measured using an image analyser (Quantimet 970, Cambridge Instruments) and converted by integration to the total volume of ischaemic damage.

Experimental groups

In experiment 1, rats underwent transient MCA occlusion with MK-801 pretreatment. MK-801 was administered as a bolus dose (0.12 mg kg^{-1}, i.v.) followed by continuous intravenous infusion (108 µg kg^{-1} h^{-1} at 0.6 ml h^{-1}) throughout the remainder of the experimental period. MK-801 treatment was initiated either 1 h (1 h group, $n = 9$) or 2.5 h (2.5 h group, $n = 9$) prior to endothelin-1 application to the MCA. The control group ($n = 10$) received bolus injection of saline (1 mg kg^{-1}, i.v.) followed by continuous saline infusion (0.6 ml h^{-1}) initiated 1 h pre-MCA occlusion. In experiment 2, L-NAME (3 mg kg^{-1} i.v., $n = 6$) or an equivalent volume of saline ($n = 5$) was administered 30 min prior to permanent MCA occlusion. In experiment 3, L-NAME (3 mg kg^{-1}, i.v., $n = 14$) or saline ($n = 14$) were given 30 min prior to transient MCA occlusion.

Chemicals

N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) was purchased from Sigma Chemical Co., MK-801 (dizocilpine) was a generous gift from Merck, Sharp and Dohme Research Laboratories. All drugs were dissolved in saline and administered in a volume of 1 ml kg^{-1}.

Statistical analysis

Statistical comparison of blood pressure data was performed using repeated measure 2 way analysis of variance (ANOVA), with subsequent pairwise comparisons by Student's *t* tests with a Bonferroni correction factor for multiple comparisons. For experiment 1 (MK-801), volumes of ischaemic damage were compared by one way ANOVA with subsequent individual comparisons by Dunnett's *t* tests. For experiments 2 and 3 (L-NAME), volumes of ischaemic damage were compared with Student's *t* tests.

Results

Physiological variables

Physiological variables from the 3 separate experiments are shown in Table 1. Values for respiratory parameters were in the normal physiological ranges for all groups. Rectal and temporalis muscle temperatures at the time of MCA occlusion did not differ between the drug and relevant control groups.

Effect of MK-801 on blood pressure and volume of ischaemic damage

MK-801 induced significant hypotension ($P < 0.001$) that persisted for approximately 1.5 h, resulting in significantly lower mean arterial blood pressure (MABP) at the time of MCA occlusion for the group in which MK-801 treatment was initiated 1 h pre-MCA occlusion (Figure 1, Table 1). A similar level of hypotension was induced by MK-801 in the 2.5 h group, but the longer pretreatment interval meant that MABP had returned to control level in this group by the time of MCA occlusion (Figure 1, Table 1).

Table 1 Physiological variables for MK-801 or N^G-nitro-L-arginine methyl ester (L-NAME) pretreatment in reversible or permanent focal cerebral ischaemia

	Group 1			*Group 2*				*Group 3*		
	MCAO	*+1 h*	*+4 h*		*MCAO*	*+1 h*	*+4 h*	*MCAO*	*+1 h*	*+4 h*
MABP (mmHg)				MABP (mmHg)						
Control	86±3	86±2	88±2	Control	90±3	88±2	89±3	85±2	86±3	88±1
MK-801 (1h)	68±2	80±2	88±2	L-NAME	93±5	87±4	90±1	85±2	82±2	89±1
MK-801 (2.5h)	84±1	85±1	86±1							
$PaCO_2$ (mmHg)				$PaCO_2$ (mmHg)						
Control	39±1	37±1	38±1	Control	37±1	40±2	37±1	39±1	37±1	37±1
MK-801 (1h)	37±1	37±1	37±1	L-NAME	36±2	35±2	35±1	35±1	38±1	37±1
MK-801 (2.5h)	38±1	36±1	37±1							
PaO_2 (mmHg)				PaO_2 (mmHg)						
Control	177±8	174±5	184±8	Control	212±14	209±9	220±14	171±8	177±7	184±6
MK-801 (1h)	176±7	180±9	185±5	L-NAME	204±10	199±13	208±10	167±5	174±7	178±7
MK-801 (2.5h)	179±10	177±9	174±3							
Rectal Temp. (°C)				Rectal temp (°C)						
Control	37.0±0.1	37.0±0.1	37.0±0.1	Control	37.0±0.1	37.1±0.1	37.0±0.0	37.2±0.1	37.1±0.1	37.0±0.0
MK-801 (1 h)	37.1±0.1	36.9±0.2	37.1±0.1	L-NAME	37.3±0.1	37.0±0.1	37.0±0.0	37.1±0.1	37.0±0.1	37.0±0.0
MK-801 (2.5h)	37.1±0.0	37.0±0.1	37.0±0.0							
Temporalis temp (°C)				Temporalis temp (°C)						
Control	36.7±0.1			Control	36.5±0.3			36.6±0.1		
MK-801 (1h)	36.7±0.1			L-NAME	36.8±0.2			36.5±0.1		
MK-801 (2.5h)	36.4±0.1									

Physiological variables for (1) MK-801 pretreatment prior to transient middle cerebral artery (MCA) occlusion (2) L-NAME pretreatment prior to permanent MCA occlusion and (3) L-NAME pretreatment prior to transient MCA occlusion. Physiological variables (except temporalis muscle temperature) were measured at the time of MCA occlusion (MCAO), and 1 h and 4 h post-MCA occlusion. Data are presented as mean ± s.e. mean.

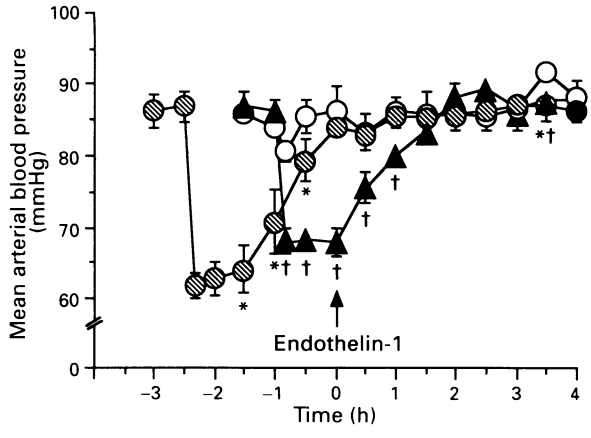

Figure 1 Time course for alterations in mean arterial blood pressure following MK-801 administered prior to transient middle cerebral artery (MCA) occlusion. MK-801 was administered as a bolus (0.12 mg kg^{-1}, i.v.) followed by continuous infusion (108 μg kg^{-1} h^{-1} at 0.6 ml h^{-1}). Treatment was initiated either 2.5 h (hatched symbols) or 1 h (solid symbols) prior to transient MCA occlusion induced by application of endothelin-1 to the exposed MCA. Data are presented as means ± s.e. mean. *$P<0.05$, †$P<0.05$ comparison with saline (open symbols) control at same time point for 2.5 h and 1 h groups respectively.

MK-801-treatment initiated 2.5 h pre-transient MCA occlusion markedly, and significantly, reduced the volumes of ischaemic damage in the whole hemisphere and caudate nucleus by 71% and 85% respectively (Figure 2). The volume of ischaemic damage in the cerebral cortex was reduced by 61% but this just failed to reach statistical significance at the 5% level. In contrast MK-801-treatment initiated 1 h pre-MCA occlusion did not significantly alter the volume of ischaemic damage in either whole hemisphere, cortex or caudate nucleus (Figure 2).

Effect of L-NAME on blood pressure and volume of ischaemic damage

Blood pressure was not significantly altered by administration of L-NAME so that MABP for L-NAME and saline groups were comparable at all time points including MCA occlusion (Figure 3, Table 1).

L-NAME (3 mg kg^{-1}, 30 min pre-MCA occlusion) significantly ($P<0.05$) reduced the volume of ischaemic damage measured 4 h post-permanent MCA occlusion in the whole hemisphere and cerebral cortex compared to saline control (Figure 4). The volume of ischaemic damage in the caudate nucleus was not significantly altered by L-NAME. Although significant, the neuroprotective effect obtained with L-NAME was relatively small with only a 16% reduction in the total volume of ischaemic damage.

In contrast L-NAME (3 mg kg^{-1}, 30 min pre-MCA occlusion) did not significantly reduce the volume of ischaemic damage assessed 4 h post-onset of transient MCA occlusion (Figure 4). There was however an obvious neuroprotective trend, with the volumes of damage reduced by 29%, 26% and 33% respectively in the whole hemisphere, cortex and caudate nucleus.

Discussion

Efficacy of MK-801 against transient focal cerebral ischaemia

The present study demonstrates that MK-801 pretreatment is neuroprotective in the endothelin-1 model of transient MCA occlusion. However, in contrast to permanent MCA occlusion, it appears that drug-induced hypotension present at the onset of ischaemia results in complete loss of the neuroprotective effect since the infusion of MK-801 initiated 2.5 h prior to MCA occlusion significantly reduced the volume of ischaemic damage while the infusion initiated 1 h pre-MCA occlusion did not. The dosing schedule employed in the current study attains a steady state plasma level of MK-801

within 60 min (Gill *et al.*, 1991; Willis *et al.*, 1991), therefore plasma concentrations of MK-801 at the time of MCA occlusion should have been equivalent in both drug groups, the only obvious difference being the relative blood pressures. Thus the lower blood pressure at onset of MCA occlusion in the 1 h MK-801 group appears to have increased the severity of the ischaemic insult and directly counteracted the neuroprotective action of the drug.

In addition to its peripheral action on blood pressure, MK-801 has marked effects on CBF which differ in conscious and anaesthetized animals (Park *et al.*, 1989; Nehls *et al.*, 1990; Roussel *et al.*, 1992) and which could potentially influence outcome following cerebral ischaemia. In conscious rats, MK-801 increases local CBF in specific regions including neocortex and caudate nucleus (Nehls *et al.*, 1990; Roussel *et al.*, 1992), while in artificially-ventilated, anaesthetized rats MK-801 induces widespread homogeneous reductions in CBF (Park *et al.*, 1989). However MK-801 does not induce further reductions in CBF in the ischaemic core region following permanent MCA occlusion in halothane-anaesthetized animals (Park *et al.*, 1989; Greenberg *et al.*, 1991). The cerebrovascular effects of MK-801 are therefore likely to have less impact on outcome following experimental ischaemia in which halothane anaesthesia is maintained (as in the present study) compared with studies in which animals are allowed to regain consciousness. Previous studies have

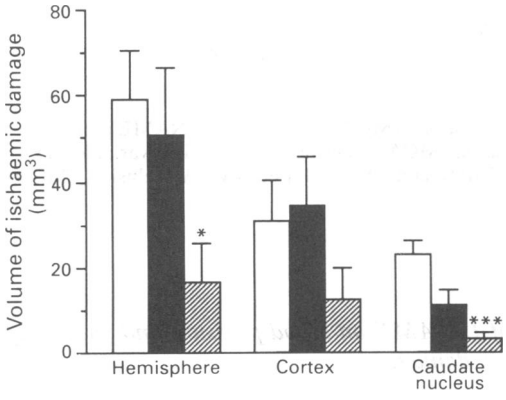

Figure 2 Effect of MK-801 on the volume of ischaemic damage induced by transient middle cerebral artery (MCA) occlusion. MK-801 pretreatment (0.12 mg kg^{-1} bolus, then 108 μg kg^{-1} h^{-1} at 0.6 ml h^{-1} i.v.) started 2.5 h prior to MCA occlusion (hatched column) significantly reduced the volume of ischaemic damage in the cerebral hemisphere and caudate nucleus. In contrast MK-801 treatment initiated only 1 h prior to MCA occlusion (solid column) did not significantly alter the ischaemic damage. Data are presented as means ± s.e. mean. *$P<0.05$, ***$P<0.001$ compared with saline control group (open column) respectively.

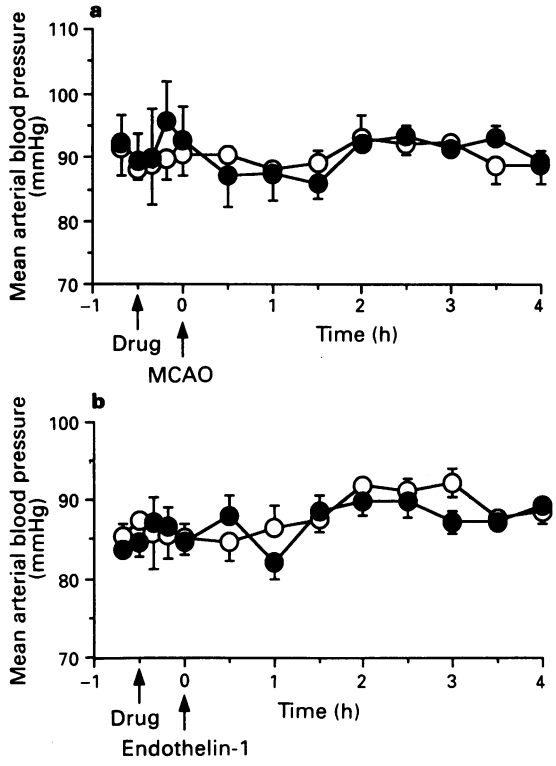

Figure 3 Mean arterial blood pressure following N^G-nitro-L-arginine methylester (L-NAME) administered 0.5 h prior to permanent or transient middle cerebral artery (MCA) occlusion. L-NAME (3 mg kg^{-1}, i.v., ●) pretreatment did not significantly alter blood pressure compared to the saline control group (○) for either (a) permanent MCA occlusion (MCAO) or (b) transient MCA occlusion induced by application of endothelin-1 to the MCA. Data are presented as means ± s.e. mean.

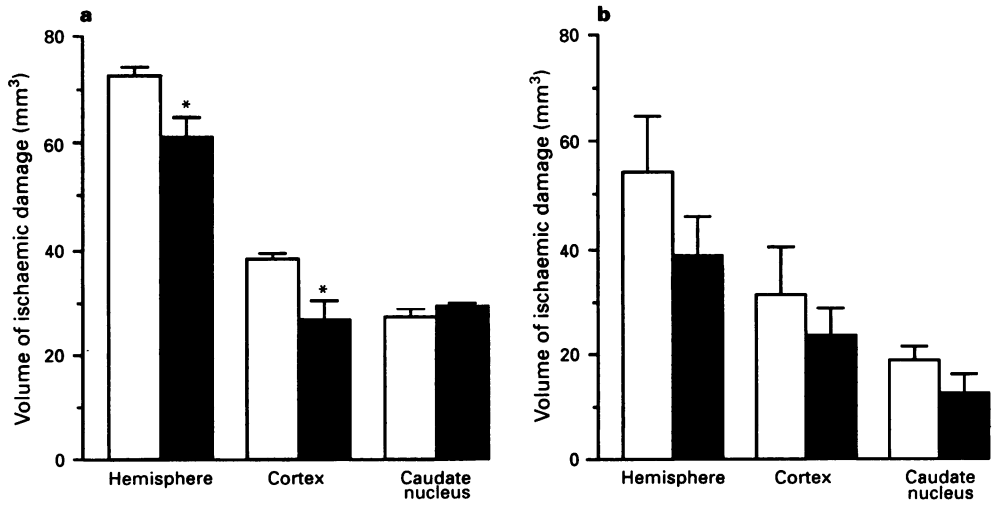

Figure 4 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on the volume of ischaemic damage induced by permanent and transient middle cerebral artery (MCA) occlusion. (a) L-NAME (3 mg kg^{-1}, i.v., solid columns) pretreatment significantly reduced the volume of ischaemic damage in the cerebral hemisphere and cortex following permanent MCA occlusion. (b) In contrast, L-NAME (3 mg kg^{-1}, i.v., solid columns) did not significantly alter the volume of ischaemic damage induced by transient MCA occlusion, although there was a moderate neuroprotective trend in both the cerebral hemisphere and caudate nucleus. Data are presented as means ± s.e. mean. *$P<0.05$ compared with relevant saline control group (open columns).

reported a beneficial action of MK-801 treatment initiated prior to or during the ischaemic phase of transient MCA occlusion in the normotensive rat (Yang *et al.*, 1991; Buchan *et al.*, 1992). However in these models the rats were conscious for most of the ischaemic period and the reduction in damage observed with MK-801 has been attributed to an increase in CBF in the ischaemic core region rather than a direct anti-excitotoxic action (Buchan *et al.*, 1992). Therefore to the best of our knowledge the present study represents the first demonstration of a significant neuroprotective action of MK-801 in a model of transient MCA occlusion in the normotensive rat, that is independent of the cerebrovascular effects of the drug and probably mediated by a direct anti-excitotoxic action.

Comparative efficacy of MK-801 in models of permanent and transient focal cerebral ischaemia

MK-801 administered pre- or up to 30 min post-permanent MCA occlusion significantly reduces the volume of ischaemic damage assessed at 4 h (Park *et al.*, 1988; Gill *et al.*, 1991) and significant neuroprotection can be achieved despite the presence of drug-induced hypotension at the time of MCA occlusion (Park *et al.*, 1988; Gill *et al.*, 1991). These results are in contrast to the present study where a similar degree of hypotension resulted in complete abolition of the neuroprotective effect of MK-801. Thus although we have demonstrated, in agreement with others (Dezsi *et al.*, 1992; Buchan *et al.*, 1992), that MK-801 can give significant neuroprotection against focal ischaemia-reperfusion injury, the most important aspect of our findings is that the observed neuroprotective effect is more highly sensitive to drug-induced hypotension than in permanent focal ischaemia. The outcome following transient MCA occlusion also appears more sensitive to alterations in other key physiological parameters such as temperature (Morikawa *et al.*, 1992a) than similar models of permanent MCA occlusion.

In models of permanent MCA occlusion the neuroprotective effect of MK-801 is predominantly restricted to the cerebral cortex with no (Gill *et al.*, 1991) or very minor (Park *et al.*, 1988) reduction in damage in the caudate nucleus due to the absence of collateral supply to this region (Gill *et al.*, 1991). In contrast in the present study MK-801 significantly, and markedly, reduced the volume of ischaemic damage in the caudate nucleus following transient MCA occlusion. Thus the restoration of CBF to the caudate nucleus following transient ischaemia renders this region potentially amenable to pharmacological intervention with efficacious drugs such as MK-801.

The role of nitric oxide in focal cerebral ischaemic damage

Permanent focal cerebral ischaemia As investigation into the biological roles of NO proceeds, it is becoming increasingly evident that NO influences a variety of physiological parameters that have the potential to either ameliorate or exacerbate cerebral ischaemic damage. To further complicate the situation, NO is now known to exist in 2 inter-changeable redox forms with distinct pro-excitotoxic (free radical form of NO) and anti-excitotoxic (nitrosonium ion form) actions (Lipton *et al.*, 1993). Endothelial-derived NO, produced immediately after the onset of ischaemia (Tominaga *et al.*, 1993) may be beneficial to outcome by inducing cerebral vasodilatation and improving CBF in the ischaemic region. This effect can be augmented by exogenously-derived NO that improves CBF following permanent MCA occlusion and significantly reduces infarct size (Morikawa *et al.*, 1992b,c; Zhang & Iadecola, 1993). In contrast non-selective inhibition of NO synthase in the immediate post-ischaemic period may induce cerebral hypoperfusion and increase the severity of an ischaemic insult. This may explain why we and others have previously reported no beneficial effect of relatively high doses of NO synthase inhibitors administered solely prior to or immediately following permanent MCA occlusion (Dawson *et al.*, 1993; Yamamoto *et al.*, 1993; Zhang & Iadecola, 1993).
($3\ mg\ kg^{-1}$), that does not significantly alter local CBF in the cortex or caudate nucleus (Macrae *et al.*, 1993a), induced only a relatively small, although significant, reduction in the volume of ischaemic damage assessed 4 h post-permanent MCA occlusion. This result suggests that NO is not a major mediator of neuronal injury in the acute (4 h) phase of permanent focal ischaemia and is supported by the observation that NO levels are only transiently elevated following permanent MCA occlusion, reaching a peak within 5–10 min of the onset of ischaemia and declining to basal levels within 1 h (Kader *et al.*, 1993; Malinski *et al.*, 1993).

The modest neuroprotective effect of L-NAME in the present study (16% reduction in the volume of ischaemic damage) is in direct contrast to the dramatic reductions in lesion size (50%) reported for repeated dosing of NOS inhibitors following permanent MCA occlusion in rats and mice (Nowicki *et al.*, 1991; Buisson *et al.*, 1992). However the apparent neuroprotective efficacy of NO synthase inhibitors in these studies may in fact reflect reduced NO-dependent oedema formation rather than neuronal survival *per se* since the method of quantification of ischaemic damage used in these studies was not corrected for brain swelling and NO synthase inhibitors have been shown to reduce oedema 48 h post-permanent MCA occlusion (Nagafuji *et al.*, 1992). Furthermore NOS inhibitors induce significant hypothermia in conscious rats (Macrae *et al.*, 1993a) which may also contribute to the apparent neuroprotective efficacy of NOS inhibition in conscious animals.

Transient focal cerebral ischaemia In contrast to permanent focal ischaemia, the restoration of CBF following transient focal ischaemia allows a more sustained increase in NO production during the reperfusion phase (Malinski *et al.*, 1993). Thus the potential contribution of NO to neuronal injury may be greater in the context of transient focal ischaemia. However, we were unable to demonstrate greater neuroprotection with L-NAME in transient compared with permanent focal ischaemia. Indeed, under broadly similar experimental conditions, the neuroprotective effect of L-NAME was comparable in both models (total volumes of tissue salvaged were $16\ mm^3$ and $12\ mm^3$ for transient and permanent MCA occlusion respectively), the only difference being that the reduction in tissue damage in the transient ischaemia model did not attain statistical significance due to the greater variability inherent in models of transient versus permanent MCA occlusion. Furthermore the efficacy of L-NAME was poor compared with MK-801 pretreatment in the same model (29% and 71% reductions in volume of damage for L-NAME and MK-801 respectively). These results suggest the contribution of NO to neuronal injury in the *acute* phase following a transient ischaemic insult is moderate and similar to that observed for permanent focal ischaemia. However, our results do not exclude the possibility that NO contributes to the maturation of the lesion in the late reperfusion phase since we have previously demonstrated that lesion volume continues to increase in our endothelin-1 model beyond the 4 h time point used in the present study (Dawson *et al.*, 1993). Indeed preliminary results from other laboratories would suggest that this is indeed the case (Cole *et al.*, 1993).

Several of the consequences of NO synthase inhibition may specifically detract from the inherent neuroprotective efficacy of L-NAME against transient focal ischaemic injury. For example L-NAME-induced vasoconstriction and enhancement of leucocyte adherence to vascular endothelium (Ma *et al.*, 1993) may impair restoration of CBF to previously ischaemic regions, while L-NAME may also increase extracellular glutamate levels during the reperfusion phase (Zhang *et al.*, 1993). Indeed in some methods of transient MCA occ-

lusion (e.g. the intraluminal thread model) L-NAME pretreatment can exacerbate infarct volume (Kuluz et al., 1993). However, L-NAME-induced exacerbation of endothelial damage induced by insertion and retraction of the intraluminal thread may be a major contributory factor to the poor outcome in this particular case. One of the main advantages of our endothelin-1 model of transient MCA occlusion is that mechanical damage to the MCA is avoided, allowing the demonstration of a small (although non-significant) neuroprotective effect of L-NAME pretreatment in the present study.

From this discussion it is evident that NO synthase inhibitors induce a range of physiological responses that can influence outcome following either transient or permanent focal ischaemia. The disparate findings reported in the literature concerning the neuroprotective efficacy of both NO synthase inhibitors and NO donors probably reflects differential interaction between these various factors, the balance of which is dependent on the particular experimental design utilised. However, the anti-ischaemic efficacy of NO synthase inhibitors may be improved in the near future with the development of selective NO synthase inhibitors (Moore et al., 1993) that would allow inhibition of neuronal and inducible NO synthase (putative mediators of excitotoxicity and oedema formation) while conserving endothelial NO synthase activity (to maintain CBF).

Variability in lesion size induced by transient MCA occlusion

In the present study the percentage reduction in ischaemic damage achieved with L-NAME in the transient MCA occlusion model was comparable to that observed in the permanent MCA occlusion model. However this result failed to attain statistical significance. This discrepancy is not surprising since all models of transient focal ischaemia are associated with greater variability than comparable methods of permanent focal ischaemia due to the incorporation of a period of reperfusion into the design (Macrae, 1992). Power analysis calculations ($\alpha = 0.05$, $\beta = 0.2$) have revealed that group sizes of approximately 100 animals would be necessary to detect the observed 29% reduction in total lesion volume demonstrated with L-NAME pretreatment against transient MCA occlusion. The time and expenditure necessary to conduct such a large study make it impractible to carry out. Furthermore we do not think completion of a larger study would improve the information gained from the present study with smaller group sizes, since it is evident from the results presented herein that, as for permanent MCA occlusion, the efficacy of L-NAME as a neuroprotective agent against acute ischaemic damage is considerably less than that of MK-801.

Although all models of transient focal ischaemia are more variable than their permanent counterparts, this comparative lack of power must be balanced against the maximum degree of pharmacoprotection that could potentially be demonstrated in the two model types. For permanent MCA occlusion only the penumbra region, where CBF is less severely reduced, will be amenable to pharmacological intervention. This means that even the most efficacious drugs such a MK-801 can only protect approximately 50% of the total tissue at risk (see Bullock & Fujisawa, 1992). In contrast, for transient MCA occlusion the restoration of CBF (providing it is initiated before ischaemic damage becomes irreversible) renders 100% of the tissue potentially salvagable. Thus in the present study, tissue damage in the caudate nucleus, an end artery region, was successfully reduced by MK-801 pretreatment whereas previous studies have shown that in the presence of permanent MCA occlusion this region is more resistant to neuroprotective agents. Thus highly efficacious drugs such as MK-801 which have the potential to reduce damage substantially (e.g. by over 70% as in the present study) will be able to achieve significant neuroprotection in models of transient focal ischaemia without resorting to large experimental groups.

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