British Journal of Pharmacology (2002) 135, 823–829

www.nature.com/bjp

A single subcutaneous bolus of erythropoietin normalizes cerebral blood flow autoregulation after subarachnoid haemorrhage in rats

[1]Jacob Bertram Springborg, [2]XiaoDong Ma, [2]Per Rochat, [3]Gitte Moos Knudsen, [4]Ole Amtorp, [3]Olaf B. Paulson, [2]Marianne Juhler & *,[1]Niels Vidiendal Olsen

[1]Department of Neuroanaesthesia, The Neuroscience Center, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark; [2]Department of Neurosurgery, The Neuroscience Center, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark; [3]The Neurobiology Research Unit, The Neuroscience Center, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark and [4]Department of Pharmacology, Panum Institute, University of Copenhagen, Copenhagen, Denmark

1 Systemic administration of recombinant erythropoietin (EPO) has been demonstrated to mediate neuroprotection. This effect of EPO may in part rely on a beneficial effect on cerebrovascular dysfunction leading to ischaemic neuronal damage. We investigated the *in vivo* effects of subcutaneously administered recombinant EPO on impaired cerebral blood flow (CBF) autoregulation after experimental subarachnoid haemorrhage (SAH).

2 Four groups of male Sprague-Dawley rats were studied: group A, sham operation plus vehicle; group B, sham operation plus EPO; group C, SAH plus vehicle; group D, SAH plus EPO. SAH was induced by injection of 0.07 ml of autologous blood into the cisterna magna. EPO (400 iu kg^{-1} s.c.) or vehicle was given immediately after the subarachnoid injection of blood or saline. Forty-eight hours after the induction of SAH, CBF autoregulatory function was evaluated using the intracarotid ^{133}Xe method.

3 CBF autoregulation was preserved in both sham-operated groups (lower limits of mean arterial blood pressure: 91 ± 3 and 98 ± 3 mmHg in groups A and B, respectively). In the vehicle treated SAH-group, autoregulation was abolished and the relationship between CBF and blood pressure was best described by a single linear regression line. A subcutaneous injection of EPO given immediately after the induction of SAH normalized autoregulation of CBF (lower limit in group D: 93 ± 4 mmHg, NS compared with groups A and B).

4 Early activation of endothelial EPO receptors may represent a potential therapeutic strategy in the treatment of cerebrovascular perturbations after SAH.

British Journal of Pharmacology (2002) **135,** 823–829

Keywords: Autoregulation; cerebral blood flow; erythropoietin; subarachnoid haemorrhage

Abbreviations: ANOVA, analysis of variance; CBF, cerebral blood flow; EPO, erythropoietin; ICP, intracranial pressure; iNOS, inducible nitric oxide synthase; MABP, mean arterial blood pressure; NO, nitric oxide; SAH, subarachnoid haemorrhage; Xe, Xenon

Introduction

Erythropoietin (EPO) was originally defined as a hormonal erythroid growth factor produced in the foetal liver or adult kidney. Recent studies have established that neurons and astrocytes are capable of EPO production and that neurons, glial cells and brain capillary endothelial cells express specific EPO receptors (Bernaudin *et al*., 1999; 2000; Brines *et al*., 2000; Juul *et al*., 1998; Masuda *et al*., 1994; Morishita *et al*., 1997; Yamaji *et al*., 1996). Similar to the peripheral regulation, the expression of EPO receptors and the synthesis of EPO in the central nervous system are induced by hypoxia (Juul *et al*., 1998; Marti *et al*., 1996; Masuda *et al*., 1994). *In vivo* animal studies have demonstrated that intracerebroventricular administration of recombinant EPO protects against neuronal damage in models of stroke (Bernaudin *et al*., 1999; Calapai *et al*., 2000; Konishi *et al*., 1993; Sadamoto *et al*., 1998; Sakanaka *et al*., 1998). Until recently it has been assumed that the brain and the peripheral EPO systems were parallel and separate, based on the view that the blood-brain barrier is largely impermeable to large glycosylated molecules such as EPO. However, also systemic administration of recombinant EPO protects against neuronal damage and improves survival after focal brain ischaemia (Brines *et al*., 2000; Calapai *et al*., 2000; Siren *et al*., 2001) and subarachnoid haemorrhage (Alafaci *et al*., 2000; Buemi *et al*., 2000). A recent study indicated that the presence of EPO receptors in brain capillaries may provide a specific route for circulating EPO to enter the brain (Brines *et al*., 2000).

Acute subarachnoid haemorrhage (SAH) secondary to rupture of an intracranial aneurysm is a life-threatening condition that often strikes young and middle-aged adults. SAH often leads to vasomotor instability resulting in vasospasms and impaired autoregulation of cerebral blood flow (CBF) (Cesarini *et al*., 1999; Voldby *et al*., 1985). Cerebrovascular perturbations contribute significantly to the development of delayed cerebral ischaemia which is the major

*Author for correspondence at: Department of Neuroanaesthesia 2091, The Neuroscience Center, Copenhagen University Hospital, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark;
E-mail: fino@farmakol.ku.dk

cause of a poor clinical outcome in patients surviving the initial bleeding (Mendelow, 1988). The exact pathophysiologic background for changes in cerebrovascular tone after SAH is unknown. The development of delayed vasospasms is associated with an impaired endothelium-dependent, nitric oxide (NO) mediated vasodilatation, perhaps because of a diminished production of endothelium-derived NO in cerebral vessels (Faraci & Heistad, 1998; Sobey & Faraci, 1998). Other lines of evidence exist to indicate that an inflammatory response leading to expression of inducible NO synthase (iNOS) may result in decreased activity of endothelial NOS essential for normal endothelial function (Schwartz *et al*., 1997; Scott & McGormack, 1999; Sheehy *et al*., 1998). Recently, experimental SAH in rats was shown to involve an increased expression of iNOS that predominated in cerebrovascular tissue (Widenka *et al*., 1999; Sayama *et al*., 1998; 1999). Detectable presence of nitrotyrosin was co-located with the sites of iNOS expression, indicating concomitant formation of toxic levels of peroxynitrite (Sayama *et al*., 1999). Most probably, SAH triggers the onset of an inflammatory cascade with elevated levels of reactive oxygen species secondary to accumulation of haemoglobin, Fe^{++} and arachidonic acid metabolites, induction of iNOS and cytokines, increased activity of xanthinoxidase, and aggregation of leukocytes (Lipton, 1999; Sobey & Faraci, 1998). In line with this, increased cerebrospinal levels of proinflammatory cytokines have been measured in patients with SAH (Kikuchi *et al*., 1995; Mathiesen *et al*., 1997; Hirashima *et al*., 1997).

The mechanisms by which EPO mediates its neuroprotective effects are still unknown but may involve a modulation of apoptosis secondary to inhibition of iNOS and production of reactive oxygen species (Calapai *et al*., 2000; Sakanaka *et al*., 1998; Siren *et al*., 2001). A recent study by Squadrito *et al*. (1999) emphasized the therapeutic potential of recombinant EPO in the treatment of disorders involving a marked inflammatory response: In a model of circulatory shock induced by total splanchnic ischaemia/reperfusion in rats, intravenous treatment with EPO at the time of reperfusion in a dose-dependent manner improved survival and attenuated the increase in circulating levels of nitrite/nitrate and activity of iNOS in aortic smooth muscle. This study also demonstrated that recombinant EPO inhibits endotoxin-induced formation of NO in cultured macrophages (Squadrito *et al*., 1999). Moreover, recombinant EPO has been shown to inhibit the expression of iNOS after stimulation with the proinflammatory cytokine IL-1β in vascular smooth muscle cells (Akimoto *et al*., 1999; Kusano *et al*., 1999).

Thus, it remains possible that the neuroprotective effects of recombinant EPO obtained in animal models of ischaemic brain injury and SAH in part relies on a beneficial interaction of EPO in initial cerebrovascular inflammatory processes leading to ischaemic neuronal damage. If so, activation of endothelial EPO receptors may represent a potential therapeutic strategy in the treatment of secondary neuronal damage after SAH. Studies in our laboratory have demonstrated that CBF autoregulation is impaired after experimental SAH in rats (Ma *et al*., 2000; Rasmussen *et al*., 1992). In the present study, we tested the hypothesis that a single subcutaneous bolus of recombinant EPO given immediately after the induction of SAH protects against SAH-induced loss of CBF autoregulation.

Methods

Experimental animals and protocol

Male Sprague-Dawley rats weighing approximately 300 g were obtained from a breeding centre (Møllegaarden, Eiby, Denmark), and housed in a temperature and moisture controlled room with a 12 h light-dark cycle. The animals were allowed access to standard laboratory chow (Altromin Standard Diet 1324, Brogaarden, Denmark) and water *ad libitum*. Four different groups of animals were investigated: group A: sham operation plus vehicle; group B: sham operation plus EPO (400 iu kg^{-1} s.c.); group C: SAH plus vehicle; group D: SAH plus EPO (400 iu kg^{-1} s.c.)

All procedures were performed in accordance with the National Institute of Health's Guide for Care and Use of Laboratory Animals and the study was approved by the Committee on Animal Experimentation under the Ministry of Justice, Denmark. All surgical procedures were performed by the same investigator.

Induction of SAH and administration of EPO

The animals were prepared according to the method described by Delgado *et al*. (1985). The rats were anaesthetized with 4% isoflurane in a mixture of 70% N_2O and 30% O_2 and the isoflurane level was reduced to 1.75%. The atlanto-occipital membrane was exposed and a thin indwelling catheter was placed in the cisterna magna after which the skin was closed. SAH was induced by injection of 0.07 ml of autologous blood drawn from the left ventricle of the heart. Sham operated rats were prepared in a similar manner except that 0.07 ml of isotonic saline was injected instead of blood. Immediately after the subarachnoid injection of blood or saline, 400 iu kg^{-1} recombinant human EPO (Eprex™ 10,000 iu ml^{-1}, Janssen-Cilag, Denmark) in a total volume of 0.3 ml or vehicle (isotonic saline) in an equal volume was given subcutaneously in the neck. Thereafter, the animals were allowed to emerge from anaesthesia and were housed individually for 48 h.

Surgical preparation

CBF measurements were performed 48 h after the induction of SAH (Delgado *et al*., 1985; Rasmussen *et al*., 1992; Yamamoto *et al*., 1998). The animals were anaesthetized as described above and were connected to a respirator after tracheotomy. Anaesthesia was maintained with isoflurane 1.75% throughout the experiment. End-tidal CO_2 level was adjusted to normocapnic values by changing the inspiratory volume. A bolus of 3 mg of suxamethone followed by a continuous infusion (0.01 mg min^{-1}) was given intraperitoneally to achieve relaxation throughout the experiment. Body temperature was kept constant at $37 \pm 0.5^{\circ}C$ by the use of a heating table. Catheters were inserted into the femoral arteries for blood sampling and measurements of mean arterial blood pressure (MABP), and into the femoral veins for blood substitution and drug administration.

Measurements of CBF

CBF was measured using the method described by Hertz *et al*. (1984). The pterygopalatine artery and the branches of the

external carotid artery were ligated to minimize extracerebral distribution of radioactive xenon (^{133}Xe). A retrograde catheter was inserted into the external carotid artery with the tip just cranial to the bifurcation for administration of ^{133}Xe. After the injection of a single dose (0.04 to 0.1 ml) of ^{133}Xe dissolved in saline (555 MBq ml^{-1}, Du Pont Pharma, Brussels, Belgium) the activity was measured by a single collimated NaI crystal placed ipsilaterally over the exposed cranium between the eye and ear. CBF was calculated from the initial slope of the washout curve with a correction for background activity. This method allows rapid and repetitive unilateral measurements of CBF in mainly cortical gray matter (Olesen *et al*., 1971).

Time-dependent variation of CBF in the model was separately evaluated by repetitive measurements of CBF in three sham-operated, vehicle-treated rats. This time-course study was conducted under the same conditions and with the use of similar anaesthetic and surgical procedures as described above. Starting at 30 min after surgery, CBF was measured in duplicate every 30 min for 3 h.

CBF autoregulation studies

After surgery a period of 30 min was included to obtain a steady state. Thereafter, a minimum of three baseline measurements of CBF, temperature, intracranial pressure (ICP), MABP and blood gases were obtained. ICP was measured by the use of the catheter previously inserted into the cisterna magnum. After basline measurements and the confirmation of steady state, MABP was increased to 20 mmHg above baseline values by continuous intravenous administration of noradrenaline (0.4%, 0.6 to 5.5 ml h^{-1}). This use of noradrenaline to produce moderate arterial hypertension has been shown to induce only little, if any, changes in cerebral haemodynamics and metabolism (Olesew, 1972; Tuor *et al*., 1986). CBF was measured 5 min after a new stable value of MABP had been reached. Subsequently, MABP was decreased step-by-step, initially by reducing the infusion rate of noradrenaline and later by controlled bleeding produced by withdrawal of blood from the venous catheter. CBF, temperature and ICP were measured at every 10 mmHg decrement of MABP after a 5 min stabilization period at each level. After each measurement, pH, $PaCO_2$ and PaO_2 were analysed in an arterial blood sample of 100 μl by the use of a blood gas analyser (ABL-605™, Radiometer, Copenhagen, Denmark). All blood samples were immediately replaced with heparinized blood from a donor rat of the same strain. After the experiments the rats were given a lethal injection of pentobarbitone.

Calculations and data analysis

All baseline values are presented as means ± s.d. Baseline data were analysed by a one-way analysis of variance (ANOVA). In case of $P<0.05$, unpaired Student's *t*-tests with correction for multiple comparisons were used to analyse differences between groups. Statistical analyses were performed using Statistica for Windows 5.1 (StatSoft Inc., Tulsa, OK, U.S.A.).

In each animal, CBF values were expressed as a percentage of the baseline CBF (CBF%), and were plotted in CBF%/MABP curves for every single rat. CBF%/cerebral perfusion pressure curves were not plotted since values of ICP are not available from all measurements because of inadequate sensitivity of the equipment. A computer programme was used to evaluate autoregulation (Schmidt *et al*., 1990). The programme repetitively fits a sloped regression line and a horizontal line to the CBF%/MABP values from each animal. The sum of squares of the distance from all observed datapoints to the combined curve area are calculated and the combined curve with the lowest sum of squares is returned as the best fit (Schmidt *et al*., 1990). The lower limit of autoregulation is defined as the MABP value corresponding to the intersection of the two lines. In addition, a single linear regression line is fitted through the CBF%/MABP data from each rat and the sum of squares is compared with the sum of squares obtained by the combined curve. In this study, we accepted the identification of a lower MABP limit of autoregulation only if all of the following criteria were fulfilled: the MABP value of the lower limit identified by the computer was physiologically acceptable with a standard error of less than 25% of the lower limit itself; the minimum sum of squares obtained by the aforementioned two lines were lower than that obtained by the single linear regression line; the MABP value of the lower limit was at least 10 mmHg higher than the lowest MABP measured in the experiment; and the MABP interval measured in the experiment was $\geqslant$30 mmHg. Conversely, we defined the autoregulation as abolished if the criteria could not be fulfilled; the single linear regression line was identified as the autoregulation curve; and if CBF increased >10% per 30 mmHg increase in MABP. Mean CBF autoregulation curves in the four groups were calculated by the computer programme by pooling data from the individual animals as described previously (Schmidt *et al*., 1990). Differences between lower limits in the groups were evaluated using the Wilcoxon rank-sum test for unpaired data. The s.e. of the estimate of the curve was evaluated according to a *t*-statistic testing the intersection from two different lines (Kendall & Stuart, 1958; Welch, 1947).

Results

Baseline values obtained 48 h after the induction of SAH are shown in Table 1. ICP was higher and arterial pH was lower in the two SAH groups compared with group A. Administration of EPO had no effect on ICP or arterial pH. CBF, MABP, $PaCO_2$, PaO_2 and temperature did not differ between the four groups.

The time-course study showed that CBF in the present model remained stable over time: Means and ranges of CBF at 0, 30, 60, 90, 120, 150 and 180 min were 153 (143–171), 146 (136–153), 149 (143–155), 150 (143–159), 167 (148–177), 158 (153–162), and 158 (155–161) ml 100 g^{-1} min^{-1}, respectively. In the three animals, coefficients of variation were 10.0%, 7.3% and 8.8%, respectively. The mean bias ±2 s.d. between two consecutive measurements of CBF in the same animal was −3.4 ± 27.4 ml 100 g^{-1} min^{-1}.

Individual autoregulation curves are presented in Figure 1 and mean autoregulation curves in the four groups are presented in Figure 2. Throughout the autoregulation studies arterial blood gases and temperature remained constant with values of PaO_2 well above hypoxaemic levels and $PaCO_2$

Table 1 Baseline values in the four experimental groups

	Sham operated		*SAH*		
	Vehicle (Group A)	*EPO* (Group B)	*Vehicle* (Group C)	*EPO* (Group D)	P-*value* (ANOVA)
CBF (ml 100 g^{-1} min^{-1})	165±47	160±25	177±71	176±50	0.884
MABP (mmHg)	103±10	103±8	95±13	103±10	0.301
ICP (mmHg)	3.3±0.7	3.6±0.7	4.3±1.0*	4.3±0.8*	0.041
$PaCO_2$ (mmHg)	38.9±0.3	39.1±0.4	38.9±0.4	38.9±0.4	0.652
PaO2 (mmHg)	132.8±18.8	128.4±10.7	126.9±14.7	124.6±12.8	0.721
PH	7.46±0.01	7.46±0.02	7.43±0.03*	7.43±0.03*	0.024
Temperature (°C)	37.0±0.2	37.2±0.1	37.0±0.3	37.0±0.2	0.458

Values are means±s.d. $n=8$ in all groups. *$P<0.05$ compared with group A.

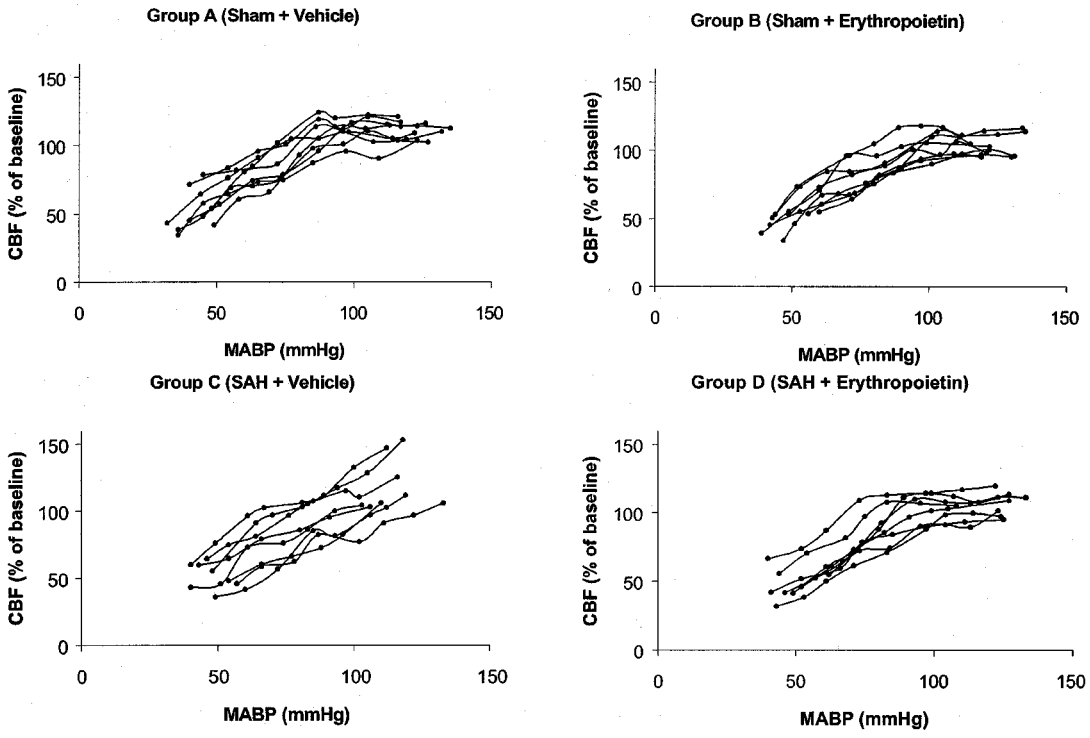

Figure 1 Individual CBF autoregulation curves in the four experimental groups.

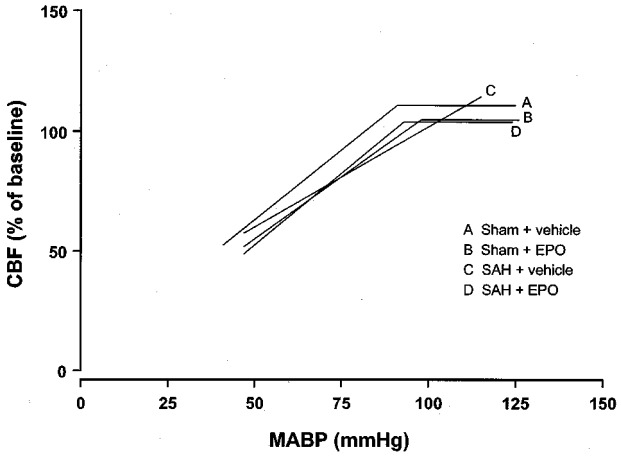

Figure 2 Mean CBF autoregulation curves in the four experimental groups. Highest and lowest values of MABP are means of the maximum and minimum values in the four groups.

within eucapnic values. Arterial pH also remained constant except at low MABP values where a small decline in arterial pH was observed in all four groups (data not shown). Consistent with previous studies (Paulson *et al*., 1990; Rasmussen *et al*., 1992), autoregulation in the vehicle treated control group was preserved in each individual animal with the autoregulation curves consisting of two parts: a plateau above the threshold MABP value (lower limit) and a sloping part below, where CBF declined with decreasing values of MABP. Pooled data from group A also showed preservation of autoregulation. In the EPO treated control group, autoregulation was also preserved in each individual animal and in the group as a whole and similar autoregulation curves were observed. In the vehicle treated SAH group, autoregulation was absent in each individual animal and in the group as a whole and a single linear regression line best described the relationship between CBF and MABP. Thus, a plateau of CBF or a lower limit of MABP could not be defined. However, in the EPO-treated SAH group, auto-

regulation in each individual animal and in the group as a whole was preserved with autoregulation curves showing a plateau and a lower limit. Lower limits of MABP and the CBF plateaus in the three groups with intact autoregulation did not differ (Table 2).

Discussion

In the present study, we have demonstrated for the first time that a single subcutaneous bolus of EPO injected immediately after the induction of SAH prevents SAH-induced impairment of CBF autoregulation. Baseline values of CBF and MABP in the four groups were comparable but the induction of SAH slightly increased ICP and lowered arterial pH. Because acute elevations of ICP up to 50 mmHg do not abolish CBF autoregulatory function (Hauerberg & Juhler, 1994), the small differences in ICP are assumed not to influence autoregulatory function. The $PaCO_2$ was maintained within eucapnic values and PaO_2 was well above hypoxaemic levels in all groups. Systemic arterial pH changes appear not to influence CBF at a constant $PaCO_2$ level (Lassen, 1968). Consequently, the minor drop in baseline arterial pH in the SAH groups is not likely to have influenced autoregulatory function. In this study, baseline CBF values were higher than those observed in previous studies in our laboratory (Ma *et al*., 2000; Rasmussen *et al*., 1992). This could be explained by the use of different anaesthetics. The present addition of nitrous oxide to isoflurane results in significantly higher CBF values than those measured with isoflurane alone, an effect not produced by the addition of nitrous oxide to halothane as used in previous studies (Hansen *et al*., 1989). However, autoregulation curves in the control groups were similar to those previously found indicating that the difference in baseline CBF did not influence CBF autoregulatory function. Moreover, the time-course study showed that values of CBF remained stable throughout the study period.

CBF autoregulatory function was normal in the two sham-operated groups. In the vehicle treated SAH group, CBF autoregulation was abolished and a lower limit and a CBF plateau could not be identified. However, in the EPO-treated SAH group CBF autoregulatory function was normalized with a CBF plateau and a lower limit of MABP not different from sham-operated animals. The present finding agrees with recent studies reporting a beneficial effect of recombinant EPO in experimental models of SAH. In rabbits, intraperitoneal injections of 1000 iu kg^{-1} started immediately after the induction of SAH improved survival and functional recovery at 72 h (Buemi *et al*., 2000) and reduced the amount of necrotic cortical neurons at 24 h (Alafaci *et al*., 2000). This administration of recombinant EPO in animals with SAH was reported to increase EPO concentrations in cerebrospinal fluid (Alafaci *et al*., 2000). Although it cannot be excluded that SAH-induced leakage of the blood-brain barrier allowed passage of EPO into the brain, as suggested by the authors (Alafaci *et al*., 2000), a translocation of EPO across the blood-brain barrier (Brines *et al*., 2000) may as well have contributed. In support of this, Brines *et al*. (2000) reported that intraperitoneal administration of EPO (5000 iu kg^{-1}) in normal rats within 30 min increased the concentration of EPO in cerebrospinal fluid. It is unknown whether the smaller dose of 400 iu kg^{-1} used in our study increased the intracerebral levels of EPO, and, furthermore, the present study was not designed to test the effect of recombinant EPO on neuronal damage and survival. In patients with SAH, the cause-and-effect relationship between cerebral ischaemia, arterial vasospasms, and impaired CBF autoregulatory function still remains unsettled. However, loss of autoregulation constitutes a potential threat against adequate cerebral perfusion, and in patients with SAH cerebrovascular dysfunction is associated with increased morbidity and mortality (Mendelow, 1988; Voldby *et al*., 1985). Further studies are needed to examine how the present beneficial effect of recombinant EPO on CBF autoregulation is related to brain ischaemic damage and outcome after SAH. In addition, it remains unknown whether EPO is equally efficacious to improve autoregulation if given by intrathecal administration after SAH.

Different lines of evidence now indicate that the neuroprotective and anti-inflammatory effects of EPO may involve several complex mechanisms. In neuronal-like cells and cultured hippocampal and cerebral cortical neurons, EPO prevents NMDA-receptor mediated glutamate toxicity (Morishita *et al*., 1997), which is an important factor in triggering the initial processes leading to ischaemic neuronal damage (Lipton, 1999). Glutamate toxicity is in part mediated by NO, and studies in cultured hippocampal cells showing that EPO protects against neuronal death induced by NO-generating agents suggested that EPO upregulates the expression of antioxidant enzymes (Sakanaka *et al*., 1998). However, EPO may as well interact with NOS-induced formation of NO on a transcriptional level, as EPO in vascular smooth muscle cells inhibited cytokine-induced increases in iNOS-mRNA, iNOS-protein expression, and NO production (Akimoto *et al*., 1999; Kusano *et al*., 1999). Similar to other haematopoietic factors, EPO has been shown to provide a trophic effect on neurons (Konishi *et al*., 1993; Siren *et al*., 2001) and on brain capillary endothelial cells (Yamaji *et al*., 1996). Interestingly, EPO receptor mRNA is upregulated in the ischaemic penumbra zone after middle cerebral artery occlusion (Sadamoto *et al*., 1998), and the enhanced expression of EPO receptors in neurons, astrocytes and endothelial cells precedes the upregulation of EPO synthesis after focal ischaemia (Bernaudin *et al*., 1999). In line with this, analyses of EPO receptor transcripts in brain indicate that the neuroprotective effects of EPO and its receptor in susceptible brain areas depend upon both a hypoxia-induced stimulation of EPO formation and an increase in the expression of EPO receptors resulting in increased sensitivity to EPO (Chin *et al*., 2000).

It still remains unknown, however, to what extent the neuroprotective effects of circulating recombinant EPO

Table 2 Lower limits of MABP and plateaus of CBF in the four experimental groups

	Sham operated		*SAH*	
	Vehicle (Group A)	*EPO* (Group B)	*Vehicle* (Group C)	*EPO* (Group D)
Lower limit (mm Hg)	91±3	98±3	–	93±4
CBF plateau (% of baseline)	111	105	–	104

Data are presented as means±s.d. $n=8$ in all groups. Differences between lower limits in groups A, B and D are non-significant.

depend upon its passage into the brain. Strong evidence now indicates that dysfunction of cerebrovascular endothelium is a significant contributor to ischaemic brain damage after SAH (Faraci & Heistad, 1998; Sobey & Faraci, 1998). In view of the anti-inflammatory effect of EPO in vascular tissue (Akimoto *et al*., 1999; Kusano *et al*., 1999; Squadrito *et al*., 1999), it is conceivable that circulating EPO by activation of endothelial EPO receptors may oppose inflammatory pathways in cerebral arteries induced by SAH, independent of any entrance of EPO into the brain. Impairment of endothelial NO activity after SAH can result in enhanced adherence and infiltration of leukocytes in cerebral arteries (Faraci & Heistad, 1998). In a rodent model of blunt head injury, systemic administration of recombinant EPO reduced the infiltration of mononuclear inflammatory cells (Brines *et al*., 2000).

The quantity of EPO administered in this study is not far from that now used in clinical therapy. In humans, similar doses have been tested in clinical trials without the demonstration of adverse effects (Cheung *et al*., 1998) and some patients may even receive higher doses. Arterial hypertension may develop during prolonged treatment with recombinant EPO but in uremic patients this is not considered to outweigh the advantages of the therapy (Luft, 2000). Thromboembolic complications due to an increase in blood viscosity can be prevented by haemodilution. In addition, single-dose administration of EPO is less effective in producing a reticulocyte response than is repeated administrations (Cheung *et al*., 1998). Obviously, subcutaneous administration of recombinant EPO, as used in the present study, has clear advantages over intraperitoneal and intrathecal injection. Greater feasibility combined with a minimal infection risk may in clinical settings facilitate an early onset of therapy. In view of recent studies indicating the existence of an endogenous EPO/EPO-receptor system that appears to be upregulated in ischaemic brain areas (Sadamoto *et al*., 1998; Yamamoto *et al*., 2000), amplification of this system by systemic administration of recombinant EPO may represent a potential therapeutic strategy in the treatment of secondary neuronal damage after SAH. In our study, beneficial effects of EPO on cerebral autoregulation was observed 48 h after the drug had been administered immediately after the induction of SAH. Previous studies have indicated that EPO has no acute neuroprotective effects and that beneficial effects in models of focal brain ischaemia depend on an administration of EPO within 6 h after the injury (Brines *et al*., 2000). The presence of this window of protection suggests that the effect of EPO is secondary to induction of protective genes. Further studies are needed to clarify if the effects of recombinant EPO on CBF autoregulation after SAH can also be demonstrated after more extended administration. Furthermore, the dose-response relation has to be examined.

In conclusion, the present study demonstrates that a single subcutaneous bolus of recombinant EPO administered immediately after the induction of SAH protects against SAH-induced loss of CBF autoregulatory function. Our results may reflect that early activation of endothelial EPO receptors after SAH has a beneficial effect in initial cerebrovascular inflammatory processes leading to impaired CBF autoregulation.

This study was supported by grants donated by the Danish Medical Research Council, the H:S Foundation, the Daloon Foundation, the P.A. Messerschmidt Foundation, and the Michaelsen Foundation, Denmark. Recombinant erythropoietin (Eprex™) was kindly donated by Janssen-Cilag, Denmark.

References

AKIMOTO, T., KUSANO, E., MUTO, S., FUJITA, N., OKADA, K., SAITO, T., KOMATSU, N., ONO, S., EBATA, S., ANDO, Y., HOMMA, S. & ASANO, Y. (1999). The effect of erythropoietin on interleukin-1beta mediated increase in nitric oxide synthesis in vascular smooth muscle cells. *J. Hypertens.,* **17,** 1249 – 1256.

ALAFACI, C., SALPIETRO, F., GRASSO, G., SFACTERIA, A., PASSALACQUA, M., MORABITO, A., TRIPODO, E., CALAPAI, G., BUEMI, M. & TOMASELLO, F. (2000). Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage. *Eur. J. Pharmacol.,* **406,** 219 – 225.

BERNAUDIN, M., BELLAIL, A., MARTI, H.H., YVON, A., VIVIEN, D., DUCHATELLE, I., MACKENZIE, E.T. & PETIT, E. (2000). Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia,* **30,** 271 – 278.

BERNAUDIN, M., MARTI, H.H., ROUSSEL, S., DIVOUX, D., NOUVELOT, A., MACKENZIE, E.T. & PETIT, E. (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.,* **19,** 643 – 651.

BRINES, M.L., GHEZZI, P., KEENAN, S., AGNELLO, D., DE LANEROLLE, N.C., CERAMI, C., ITRI, L.M. & CERAMI, A. (2000). Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 10526 – 10531.

BUEMI, M., GRASSO, G., CORICA, F., CALAPAI, G., SALPIETRO, F.M., CASUSCELLI, T., SFACTERIA, A., ALOISI, C., ALAFACI, C., STURIALE, A., FRISINA, N. & TOMASELLO, F. (2000). In vivo evidence that erythropoietin has a neuroprotective effect during subarachnoid hemorrhage. *Eur. J. Pharmacol.,* **392,** 31 – 34.

CALAPAI, G., MARCIANO, M.C., CORICA, F., ALLEGRA, A., PARISI, A., FRISINA, N., CAPUTI, A.P. & BUEMI, M. (2000). Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *Eur. J. Pharmacol.,* **401,** 349 – 356.

CESARINI, K.G., HARDEMARK, H.G. & PERSSON, L. (1999). Improved survival after aneurysmal subarachnoid hemorrhage: review of case management during a 12-year period. *J. Neurosurg.,* **90,** 664 – 672.

CHEUNG, W.K., GOON, B.L., GUILFOYLE, M.C. & WACHOLTZ, M.C. (1998). Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin after single and multiple subcutaneous doses to healthy subjects. *Clin. Pharmacol. Ther.,* **64,** 412 – 423.

CHIN, K., YU, X., BELESLIN-COKIC, B., LIU, C., SHEN, K., MOHRENWEISER, H.W. & NOGUCHI, C.T. (2000). Production and processing of erythropoietin receptor transcripts in brain. *Brain Res. Mol. Brain Res.,* **81,** 29 – 42.

DELGADO, T.J., BRISMAR, J. & SVENDGAARD, N.A. (1985). Subarachnoid haemorrhage in the rat: angiography and fluorescence microscopy of the major cerebral arteries. *Stroke,* **16,** 595 – 602.

FARACI, F.M. & HEISTAD, D.D. (1998). Regulation of the cerebral circulation: Role of endothelium and potassium channels. *Physiol. Rev.,* **78,** 53 – 97.

HANSEN, T.D., WARNER, D.S., TODD, M.M. & VUST, L.J. (1989). Effects of nitrous oxide and volatile anaesthetics on cerebral blood flow. *Br. J. Anaesth.,* **63,** 290 – 295.

HAUERBERG, J. & JUHLER, M. (1994). Cerebral blood flow autoregulation in acute intracranial hypertension. *J. Cereb. Blood Flow Metab.,* **14,** 519 – 525.

HERTZ, M.M., BARRY, D.I., HEMMINGSEN, R. & BOLWIG, T.G. (1984). The intracarotid 133xenon injection method for measurement of cerebral blood flow (CBF) in rats: evaluation of the effect of bolus volume. *Acta Physiol Scand.,* **122,** 397 – 400.

HIRASHIMA, Y., NAKAMURA, S., ENDO, S., KUWAYAMA, N., NARUSE, Y. & TAKAKU, A. (1997). Elevation of platelet activating factor, inflammatory cytokines, and coagulation factors in the jugular vein of patients with subarachnoid hemorrhage. *Neurochem. Res.,* **22,** 1249 – 1255.

JUUL, S.E., ANDERSON, D.K., LI, Y. & CHRISTENSEN, R.D. (1998). Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr. Res.,* **43,** 40 – 49.

KENDALL, M.G. & STUART, A., (eds) (1958). *The advanced theory of statistic*. pp. 141 – 165 New York, NY: Hafner.

KIKUCHI, T., OKUDA, Y., KAITO, N. & ABE, T. (1995). Cytokin production in cerebrospinal fluid after subarachnoid haemorrhage. *Neurol. Res.,* **17,** 106 – 108.

KONISHI, Y., CHUI, D.H., HIROSE, H., KUNISHITA, T. & TABIRA, T. (1993). Trophic effect of erythropoietin and other hematopoietic factors on central cholinergic neurons in vitro and in vivo. *Brain Res.,* **609,** 29 – 35.

KUSANO, E., AKIMOTO, T., INOUE, M., MASUNAGA, Y. UMINO, T., ONO, S., ANDO, Y., HOMMA, S., MUTO, S., KOMATSU, N. & ASANO, Y. (1999). Human recombinant erythropoietin inhibits interleukin-1beta-stimulated nitric oxide and cyclic guanosine monophosphate production in cultured rat vascular smooth-muscle cells. *Nephrol. Dial. Transplant.,* **14,** 597 – 603.

LASSEN, N.A. (1968). Brain extracellular pH: the main factor controlling cerebral blood flow. *Scand. J. Clin. Invest.,* **22,** 247 – 251.

LIPTON, P. (1999). Ischemic cell death in brain neurons. *Physiol. Rev.,* **79,** 1431 – 1568.

LUFT, F.C. (2000). Erythropoietin and arterial hypertension. *Clin. Nephrol.,* **53,** S61 – S64.

MA, X., WILLUMSEN, L., HAUERBERG, J., PEDERSEN, D.B. & JUHLER, M. (2000). Effects of graded hyperventilation on cerebral blood flow autoregulation in experimental subarachnoid hemorrhage. *J. Cereb. Blood Flow Metab.,* **20,** 718 – 725.

MARTI, H.H., WENGER, R.H., RIVAS, L.A., STRAUMANN, U., DIGICAYLIOGLU, M., HENN, V., YONEKAWA, Y., BAUER, C. & GASSMANN, M. (1996). Erythropoietin gene expression in human, monkey and murine brain. *Eur. J. Neurosci.,* **8,** 666 – 676.

MASUDA, S., OKANO, M., YAMAGISHI, K., NAGAO, M., UEDA, M. & SASAKI, R. (1994). A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. *J. Biol. Chem.,* **269,** 19488 – 19493.

MATHIESEN, T., EDNER, G., ULFARSSON, E. & ANDERSSON, B. (1997). Cerebrospinal fluid interleukin-1 receptor antagonist and tumor necrosis factor-alpha following subarachnoid hemorrage. *J. Neurosurg.,* **87,** 215 – 220.

MENDELOW, A.D. (1988). Pathophysiology of delayed ischaemic dysfunction after subarachnoid haemorrhage: experimental and clinical data. *Acta Neurochir.,* **45** (Suppl): 7 – 10.

MORISHITA, E., MASUDA, S., NAGAO, M., YASUDA, Y. & SASAKI, R. (1997). Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience,* **76,** 105 – 116.

OLESEW, J. (1972). The effect of intra caroid epinephrine, norepinephrine, and angiotensin on the regional cerebral blood flow in man. *Neurology,* **22,** 978 – 987.

OLESEN, J., PAULSON, O.B. & LASSEN, N.A. (1971). Regional cerebral blood flow in man determined by the initial slope of the clearance of intra-arterially injected 133Xe. *Stroke,* **2,** 519 – 540.

PAULSON, O.B., STRANDGAARD, S. & EDVINSSON, L. (1990). Cerebral autoregulation. *Cerebrovasc. Brain Metab. Rev.,* **2,** 161 – 192.

RASMUSSEN, G., HAUERBERG, J., WALDEMAR, G., GJERRIS, F. & JUHLER, M. (1992). Cerebral blood flow autoregulation in experimental subarachnoid haemorrhage in rat. *Acta Neurochir. (Wien.),* **119,** 128 – 133.

SADAMOTO, Y., IGASE, K., SAKANAKA, M., SATO, K., OTSUKA, H., SAKAKI, S., MASUDA, S. & SASAKI, R. (1998). Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. *Biochem. Biophys. Res. Commun.,* **253,** 26 – 32.

SAKANAKA, M., WEN, T.C., MATSUDA, S., MASUDA, S., MORISHITA, E., NAGAO, M. & SASAKI, R. (1998). In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 4635 – 4640.

SAYAMA, T., SUZUKI, S. & FUKUI, M. (1998). Expression of inducible nitric oxide synthase in rats following subarachnoid hemorrhage. *Neurol. Res.,* **20,** 79 – 84.

SAYAMA, T., SUZUKI, S. & FUKUI, M. (1999). Role of inducible nitric oxide synthase in the cerebral vasospasm after subarachnoid hemorrhage in rats. *Neurol. Res.,* **21,** 293 – 298.

SCHMIDT, J.F., WALDEMAR, G., VORSTRUP, S., ANDERSEN, A.R., GJERRIS, F. & PAULSON, O.B. (1990). Computerized analysis of cerebral blood flow autoregulation in humans: validation of a method for pharmacologic studies. *J. Cardiovasc. Pharmacol.,* **15,** 983 – 988.

SCHWARTZ, D., MENDONCA, M., SCHWARTZ, I., XIA, Y., SATRIANO, J., WILSON, C.B. & BLANTZ, R.C. (1997). Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. *J. Clin. Invest.,* **100,** 439 – 448.

SCOTT, J.A. & MCGORMACK, D.G. (1999). Selective in vivo inhibition of inducible nitric oxide synthase in a rat model of sepsis. *J. Appl. Physiol.,* **86,** 1739 – 1744.

SHEEHY, A.M., BURSON, M.A. & BLACK, S.M. (1998). Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. *Am. J. Physiol.,* **274,** L833 – L841.

SIREN, A.L., FRATELLI, M., BRINES, M., GOEMANS, C., CASAGRANDE, S., LEWCZUK, P., KEENAN, S., GLEITER, C., PASQUALI, C., CAPOBIANCO, A., MENNINI, T., HEUMANN, R., CERAMI, A., EHRENREICH, H. & GHEZZI, P. (2001). Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 4044 – 4049.

SOBEY, C.G. & FARACI, F.M. (1998). Subarachnoid haemorrhage: what happens to the cerebral arteries? *Clin. Exp. Pharmacol. Physiol.,* **25,** 867 – 876.

SQUADRITO, F., ALTAVILLA, D., SQUADRITO, G., CAMPO, G.M., ARLOTTA, M., QUARTARONE, C., SAITTA, A. & CAPUTI, A.P. (1999). Recombinant human erythropoietin inhibits iNOS activity and reverts vascular dysfunction in splanchnic artery occlusion shock. *Br. J. Pharmacol.,* **127,** 482 – 488.

TUOR, U.I., EDVINSSON, L. & MCCULLOCH, J. (1986). Catecholamines and the relationship between blood flow and glucose use. *Am. J. Physiol.,* **251,** H824 – H833.

VOLDBY, B., ENEVOLDSEN, E.M. & JENSEN, F.T. (1985). Cerebrovascular reactivity in patients with ruptured intracranial aneurysms. *J. Neurosurg.,* **62,** 59 – 67.

WELCH, B.L. (1947). The generalization of 'student's' problem when several different population variances are involved. *Biometrica,* **34,** 28 – 35.

WIDENKA, D.C., MEDELE, R.J., STUMMER, W., BISE, K. & STEIGER, H.J. (1999). Inducible nitric oxide synthase: a possible key factor in the pathogenesis of chronic vasospasm after experimental subarachnoid hemorrhage. *J. Neurosurg.,* **90,** 1098 – 1104.

YAMAJI, R., OKADA, T., MORIYA, M., NAITO, M., TSURUO, T., MIYATAKE, K. & NAKANO, Y. (1996). Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.,* **239,** 494 – 500.

YAMAMOTO, M., KOSHIMURA, K., KAWAGUCHI, M., SOHMIYA, M., MURAKAMI, Y. & KATO, Y. (2000). Stimulating effect of erythropoietin on the release of dopamine and acetylcholine from the rat brain slice. *Neurosci. Lett.,* **292,** 131 – 133.

YAMAMOTO, S., NISHIZAWA, S., TSUKADA, H., KAKIUCHI, T., YOKOYAMA, T., RYU, H. & UEMURA, K. (1998). Cerebral blood flow autoregulation following subarachnoid hemorrhage in rats: chronic vasospasm shifts the upper and lower limits of the autoregulatory range toward higher blood pressures. *Brain Res.,* **782,** 194 – 201.

(Received August 30, 2001
Revised November 20, 2001
Accepted November 22, 2001)