British Journal of Pharmacology (2005) 144, 1051–1058
www.nature.com/bjp

Pharmacological neutropenia prevents endothelial dysfunction but not smooth muscle functions impairment induced by middle cerebral artery occlusion

[1]Olivier Pétrault, [1]Thavarak Ouk, [1]Sophie Gautier, [1]Maud Laprais, [1]Patrick Gelé, [1,2]Michèle Bastide & *,[1]Régis Bordet

[1]EA 1046-Laboratoire de Pharmacologie, Institut de Médecine Prédictive et de Recherche Thérapeutique, Université de Lille 2 et Centre Hospitalier Universitaire de Lille, 1 place de Verdun 59045, Lille Cedex, France and [2]IUT A, Université Sciences et Techniques de Lille, Villeneuve d'Ascq, France

1 The polymorphonuclear neutrophils (PMN) activation and mobilization observed in acute cerebral infarction contribute to the brain tissue damage, but PMN could also be involved in postischemic functional injury of ischemied blood vessel.

2 This study was undertaken to investigate whether pharmacological neutropenia could modify the postischemic endothelial dysfunction in comparison to smooth muscle whose impairment is likely more related to reperfusion and oxidative stress.

3 A cerebral ischemia–reperfusion by endoluminal occlusion of right middle cerebral artery (MCA) was performed 4 days after intravenous administration of vinblastine or 12 h after RP-3 anti-rat neutrophils monoclonal antibody (mAb RP-3) injection into the peritoneal cavity, on male Wistar rats with 1-h ischemia then followed by 24-h reperfusion period. Brain infarct volume was measured by histomorphometric analysis and vascular endothelial and smooth muscle reactivity of MCA was analysed using Halpern myograph.

4 Neutropenia induced a neuroprotective effect as demonstrated by a significant decrease of brain infarct size. In parallel to neuroprotection, neutropenia prevented postischemic impairment of endothelium-dependent relaxing response to acetylcholine. In contrast, smooth muscle functional alterations were not prevented by neutropenia. Ischemia–reperfusion-induced myogenic tone impairment remained unchanged in vinblastine and mAb RP-3-treated rats. Postischemic Kir2.x-dependent relaxation impairment was not prevented in neutropenic conditions. The fully relaxation of smooth muscle response to sodium nitroprusside was similar in all groups.

5 Our results evidenced the dissociate prevention of pharmacologically induced neutropenia on postischemic vascular endothelial and smooth muscle impairment. The selective endothelial protection by neutropenia is parallel to a neuroprotective effect suggesting a possible relationship between the two phenomena.

British Journal of Pharmacology (2005) **144,** 1051–1058. doi:10.1038/sj.bjp.0706124
Published online 7 February 2005

Keywords: Cerebral ischemia; polymorphonuclear neutrophils; endothelium; smooth muscle; potassium channels

Abbreviations: ACh, acetylcholine; 5-HT, serotonin (5-hydroxytryptamine); ICAM, intercellular cell adhesion molecules; I/R, ischemia/reperfusion; Kir, inward rectifier potassium channel; mAb RP-3, anti-rat neutrophils monoclonal antibody; MABP, mean arterial blood pressure; MCA, middle cerebral artery; MMP, matrix metalloproteinase; NO, nitic oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxinitrite; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; SNP, sodium nitroprusside; TMP, transmural pressure; Vb, vinblastine; Veh, vehicle

Introduction

After focal cerebral ischemia, the function of cerebral arteries is critical to maintain cerebral perfusion and preserve neuronal integrity. The duration and level of the blood flow deficit are associated with the severity of brain damage. Two different regions can be distinguished: the core where neurons are definitively death; the penumbra that can progress to infarction if maintenance of a partial blood supply is not sufficient. The level of cerebral blood flow in penumbra is partly dependent of vascular functions, which are impaired in course of ischemia and reperfusion. The protection of vascular bed during ischemia/reperfusion (I/R) represents a potent pharmacological target to limit infarct extent, explaining the necessity to understand the physiopathology of I/R-induced functional alterations of blood vessel. Several studies reported impairments of many functional vascular bed including endothelium relaxing response (Cipolla *et al*., 1997), myogenic activity (Cipolla & Curry, 2002) and smooth muscle relaxation consecutive to inward rectifier potassium channels (Kir2.x) activation (Marrelli *et al*., 1998; Bastide *et al*., 1999). Ischemia and reperfusion affect differently endothelium and smooth muscle functions suggesting that several mechanisms could be

*Author for correspondence; E-mail: bordet@univ-lille2.fr
published online 7 February 2005

involved in the different compartments. Indeed, ischemia alone induces an alteration of endothelial relaxing response, which is worsen by reperfusion, while reperfusion is necessary to induce smooth muscle functional abnormalities in the absence of effect induced by ischemia alone (Cipolla *et al*., 1997; Pétrault *et al*., 2004).

It is now established that maintenance of a partial or complete blood flow is essential to preserve cerebral tissue whereas reperfusion paradoxically induces excessive generation of reactive oxygen species (ROS) known to impair cell membranes (lipid peroxidation and protein oxidation), ion channels and electrical properties of vascular smooth muscle (Kourie, 1998). Reperfusion-induced ROS contribute to a decrease of the NO availability responsible for the post-ischemic endothelial dysfunction (Drexler, 1999; Cai & Harrison, 2000). This decrease of NO bioavaibility is not explained by NO synthase (NOS) alteration, since endothelial NOS expression increased in the course of cerebral ischemia (Leker *et al*., 2001). Numerous reports have shown that oxidative stress modulation early in the course of reperfusion could reduce cerebrovascular oxidative damages. We have previously demonstrated that pharmacological modulation of oxidative stress prevents postischemic Kir2.x impairment (Bastide *et al*., 2003, Pétrault *et al*., 2004). In contrast, the oxidative stress modulation during the reperfusion by an antioxidant agent did not prevent the endothelial dysfunction (Pétrault *et al*., 2004), suggesting the involvement of other mechanisms.

Polymorphonuclear neutrophils (PMN) activation are involved in vascular reactivity modulation in different pathological circumstances. The prevention of PMN adhesion contributes to the protective effect of preconditioning against cardiac ischemia and reperfusion-induced endothelial injury (Beauchamp *et al*., 1999; Laude *et al*., 2002). PMN activation by injection of phorbol ester impaired the endothelium-dependent relaxing response to acetylcholine (Akopov *et al*., 1994). In subarachnoid hemorrhage model, vascular dysfunction-induced cerebral vasospasm has been related to inflammatory cytokine cascade released by leukocytes and could be improved by endothelial NOS increase (Mori *et al*., 2001; Takizawa *et al*., 2001; McGirt *et al*., 2002). Involvement of PMN in functional vascular injury occurring in course of cerebral ischemia has not been yet investigated but is suspected in regard to their role in cerebral ischemia physiopathology.

The leukocytes involvement in physiopathology of I/R has been previously demonstrated using induction of neutropenia by vinblastine and participated to infarction and oxidative stress in the cortex but not in the striatum (Beray-Berthat *et al*., 2003). In addition, PMN accumulation in acute cerebral infarction was correlated with brain tissue damage (Akopov *et al*., 1996a, b). Neutrophils have the capacity to generate ROS like superoxide anion, hydroxyl radical, protagonists known to be deleterious in the course of ischemia and reperfusion phenomena (Matsuo *et al*., 1995; Love, 1999). Owing to its chemical interaction with superoxide anion and its transformation in the highly harmful peroxinitrite ($ONOO^-$), indirect oxidative stress decreases the bioavailability of NO (Forman *et al*., 1998). On the other hand, several studies have suggested that PMN involvement in matrix metalloproteinase 9 (MMP-9) proform releasing and the blood-barrier breakdown, participated to aggravate infarction by their consecutive hemorrhage complications (Gautier *et al*., 2003; Justicia *et al*., 2003).

All these data suggest that I/R-induced PMN activation could contribute to the mechanisms of vascular bed reactivity impairment in particular endothelium dysfunction. To test this hypothesis, we investigated, in the present study, whether in pharmacologically induced neutropenia conditions the post-ischemic endothelial dysfunction is modified in comparison to smooth muscle, whose impairment is more related to reperfusion and oxidative stress.

Methods

All experiments were performed in strict accordance with the guidelines of the National Institutes of Health and French Department of Agriculture, on male Wistar rats (IFFA Credo, France) weighing 280–320 g according to rat middle cerebral artery (MCA) occlusion model.

Animals and drugs administration

MCA occlusion or sham surgery was consequently performed 4 days after intravenous administration of vinblastine ($0.5\,mg\,kg^{-1}$) or vehicle (saline 0.9%) to male Wistar rats. Two injections of anti-rat neutrophils monoclonal antibodies (mAb RP-3; Sekiya *et al*., 1989) in peritoneal cavity were carried out 12 h before MCA occlusion (1 ml mAb RP-3 diluted solution) and during the surgery (2 ml mAb RP-3 diluted solution). The numbers of white blood cells were counted, and hemograms of 100 white blood cells were examinated by Giemsa staining (Hemoquick). Four groups of rats have been defined: (i) sham-operated rats (Sham); (ii) vehicle-treated ischemic/reperfused (Veh + I/R); (iii) vinblastine-treated I/R (Vb + I/R); (iv) mAb RP-3-injected rats (mAb RP-3 + I/R).

MCA occlusion model

Anesthesia was induced by chloral hydrate administered i.p. at a dose of $300\,mg\,kg^{-1}$. A rectal probe was inserted and body temperature maintained by the use of a heating lamp at $37 \pm 0.5^{\circ}C$. The caudal artery was exposed and cannulated with a 24G polyethylene catheter and connected to a blood pressure monitor. Mean arterial blood pressure (MABP, mmHg) was monitored throughout the experiment and blood samples were taken before, during and after ischemia to measure blood pH, arterial PaO_2 (mmHg) and arterial $PaCO_2$ (mmHg).

The ostium of the right MCA was occluded intraluminally as previously described (Bastide *et al*., 1999). The right carotid arteries were exposed through a midline cervical incision and the common carotid and external carotid arteries were ligated with a silk suture. The pterygopalatine artery was exposed by developing a plane alongside the internal carotid artery, and was ligated at its origin with a fine silk. Aneurysm clip was placed across internal carotid artery and an arteriotomy was made in the common carotid artery stump allowing the introduction of a 4/0 monofilament nylon suture with its tip rounded by flame heating. This was secured in place and the aneurysm clip on the internal carotid artery was removed. The suture was gently advanced into the internal carotid artery

and passed into the intracranial circulation to lodge in the narrower lumen of the origin of the MCA. Mild resistance to this advancement indicated that the intraluminal occluder had entered the anterior cerebral artery. After 60 min, the suture was carefully removed, until its tip was blocked by ligature placed on common carotid artery, to permit reperfusion. The caudal artery catheter was removed and the artery was ligated to prevent bleeding. The animals were placed in cage to recover from anesthesia at room temperature and were allowed to eat and drink freely. The sham operation occurs with the same manipulation without introduction of the monofilament.

Infarct volume measurement

At 24 h after reperfusion the rats were killed with an overdose of pentobarbital (200 mg kg^{-1}) injected i.p. and brains were rapidly removed in an ice-cold saline solution gassed with 5% CO_2 and 95% O_2. MCAs were carefully dissected to the vascular smooth muscle cells preparation or for vasoreactivity analysis and thereafter brains were frozen and coronally dissected into 50-μm-thick slices on a cryostat at 12 levels separated by 1-mm intervals, according to stereotaxic sections maps (Paxinos & Watson, 1986). Sections were stained by cresyl fast violet. The unstained area of the brain was defined as infarcted. Cortical and subcortical infarcted areas and total hemispheric areas were calculated separately for each coronal slice by an image analysis software (Color Image 1.32, NIMH, Bethesda, MD, U.S.A.) after digitization by scanner process. Total infarct volumes as well as hemispheric volumes (in mm^3) were calculated by the use of numerical integration of the respective areas for all the sections per animals and the distance between them. A corrected total infarct volume was calculated to compensate for the effect of brain edema (Lin *et al*., 1993). The corrected volume was calculated using the following equation: corrected infarct volume = total infarct volume × (left hemisphere volume/right hemisphere volume).

Preparation of arterial segment and pressurized arteriograph system

Segment of dissected right MCA was mounted in a small vessel arteriograph (Living Systems Instrumentation, Burlington, VT, U.S.A.) on two glass cannulas perfused with saline solution. The artery was secured on the proximal and distal cannulas with nylon ties. The distal cannula was closed in order to work in no flow condition. The arteriograph chamber was continuously supplied with saline solution equilibrated with 5% CO_2/95% O_2 and maintained at 37°C and pH 7.4. The proximal cannula was connected to a pressure transducer, a miniature peristaltic pump and a servo controller that continually measured and adjusted transmural pressure (TMP). The entire arteriograph system was positioned on the stage of an inverted microscope equipped with a video camera and a monitor. The lumen diameter was measured by image analysis with a video dimension analyser. The output of the video dimension, which is proportional to diameter, was continually sent to a digital tape recorder (DTR-1205).

Experimental protocol

All mounted and pressurized arteries were equilibrated at a TMP of 25 mmHg for 1 h to stabilize before experiments were conducted. The spontaneous contracting response of cerebral arteries to pressure contributes to autoregulation of cerebral blood flow and characterises the myogenic tone. To evaluate myogenic reactivity, the TMP was increased step by step in increments of 25 mmHg from 25 to 100 mmHg and arterial diameter was recorded at each TMP once stable (5 min). At the end of each experiment and after another 25 mmHg TMP equilibration, TMP was once again increased by 25 mmHg increment steps from 25 to 100 mmHg in addition with papaverine (10 μM), a phosphodiesterase inhibitor, to record passive fully relaxed diameter. Then, the TMP was adjusted at 75 mmHg for the remainder of the experiment. Acetylcholine (ACh) relaxing dose–response was determined by cumulative addition of ACh (0.001–30 μM) on MCA beforehand constricted by serotonine (1 μM-induced 90% of the maximum constriction). After washout with fresh saline solution allowing the return to basal diameter, a 15 mM KCl-containing saline solution was superfused to the vessel and induced a smooth-muscle dependent vasodilation, which is carried by activation of Kir2.x channels, most probably Kir2.1 (Bradley *et al*., 1999), and inhibited by administration of $BaCl_2$ (75 μM) a Kir2.x blocker. To test the NO-mediated smooth muscle relaxation, a single concentration of sodium nitroprusside (SNP 10 μM) was added in the bath after preconstriction with 5-HT (1 μM).

Drugs and solutions

The ionic composition of the physiologic saline solution used for all vasoreactivity experiments was as follows (mM): 119 NaCl, 24 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4 7H_2O$, 10 glucose, 1.6 $CaCl_2$, pH 7.4. All vasoactive drugs (ACh, 5-HT, SNP and KCl) were purchased from Sigma Chemical Co. ACh, 5-HT and SNP were made fresh daily as stock solutions of 0.1 M and stored at 4°C for all experimental time. RP-3 anti-rat neutrophils monoclonal antibodies were supplied from BD Pharmingen, and vinblastine from Laboratoire EuroGenerics.

Data calculation and statistical analysis

Spontaneous arterial tone was evaluated as a percent of decrease in diameter from the fully relaxed diameter in papaverine and was plotted at each TMP. The relaxing responses to ACh and SNP were respectively expressed as the percent of increase of the luminal diameter and the percent of relaxation related to basal tone after preconstriction. The 15 mM KCl-induced vasorelaxation is estimated as the % of increase of the basal tone diameter.

All values were expressed as mean ± s.e. Continuous variables (infarct volumes, myogenic tone, MCA diameter changes) were compared with a one-way ANOVA in four treatment groups followed, when ANOVA was significant, by a *post hoc* protected least significant difference (PLSD) Fisher test. A value of $P<0.05$ was considered as significant.

Results

Effect of vinblastine and RP-3 anti-rat neutrophils antibody on leukocytes

Ischemia followed by reperfusion (Veh + I/R) induced a significant ($P<0.05$) increase of PMN number in comparison

Table 1 Variation percent of neutrophil blood counts (PMN), red bood cells (RBC) and white blood cells (WBC) numbers from rats treated by administration of vehicle (saline 0.9%), vinblastine (0.5 mg kg^{-1}) or RP-3 anti-rat neutrophils antibody (mAb RP-3) in middle cerebral artery occlusion conditions (I/R) or not (Sham)

	Sham	*Ischemia/reperfusion (I/R)*		
	Vehicle (n = 4)	*Vehicle* (n = 8)	*Vinblastine* (n = 8)	*mAb RP-3* (n = 4)
PMN (% variation)	—	+50.1%	−99.8%	−39.1%
RBC ($\times 10^6$/mm^3)	10.3 ± 1.0	10.2 ± 0.6	7.2 ± 0.5	9.5 ± 1.2
WBC (/mm^3)	4533 ± 677	4380 ± 540	700 ± 220	4605 ± 530

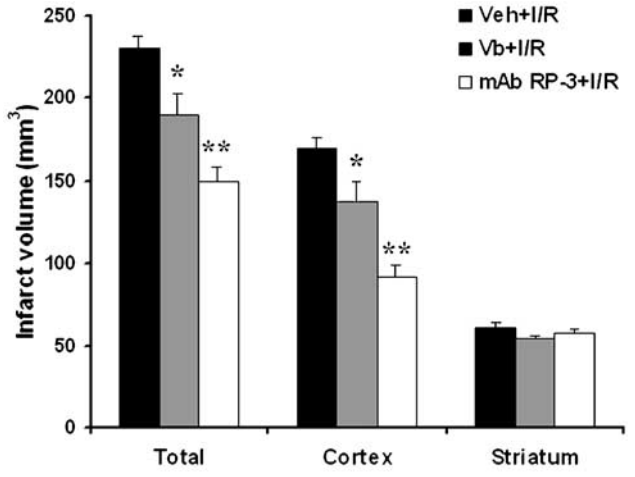

Figure 1 Effect of administration (i.v.) of vehicle (Veh: saline 0.9%, $n = 10$) or vinblastine (Vb: 0.5 mg kg^{-1}, $n = 10$) and injections (i.p.) of RP-3 anti-rat neutrophils antibody (mAb RP-3, $n = 4$) on total, cortical and striatal infarct volume (corrected for edema) induced by 1 h focal cerebral ischemia and followed by 24 h reperfusion period. Values are mean ± s.e.m. *$P < 0.05$ as compared with vehicle-treated animals.

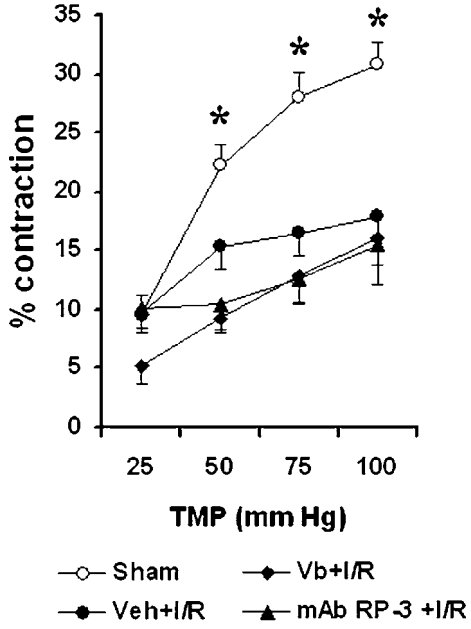

Figure 2 Effect of administration (i.v.) of vehicle (saline 0.9%) or vinblastine (0.5 mg kg^{-1}) and RP-3 anti-rat neutrophils antibody (mAb RP-3) on response of middle cerebral arteries (MCA) to pressure. Basal tone present in each MCA at different transmural pressures (TMP) was evaluated as a percent of spontaneous decrease in diameter from the fully relaxed diameter in response to papaverine (10 μM). MCA were originated from sham-operated animals (Sham), or ischemia (1 h) following by reperfusion (24 h)-operated rats (I/R). Values are mean ± s.e.m. *$P < 0.05$ as compared to I/R groups.

to sham-operated animals (Sham) as shown in Table 1. After I/R, treatment by vinblastine and mAb RP-3 reduced in a significant manner ($P < 0.05$) the number of PMN. Vinblastine administration decreased all white blood cells number to 80% while mAb RP-3 had a specific action on PMN (Table 1).

Effect of neutropenia on cerebral infarct volume

In rats treated with vinblastine 4 days before I/R at the dose of 0.5 mg kg^{-1}, there was a significant ($P < 0.05$) decrease in total infarct volume with edema correction (190.5 ± 12.5 mm^3) as compared to infarct volume in vehicle-treated animals (231.1 ± 7.2 mm^3). Administration of mAb RP-3 reduced brain infarct volume (149.7 ± 8.6 mm^3). These neuroprotective effects of vinblastine and mAb RP-3-induced neutropenia were significant ($P < 0.05$) in the cortical part of infarcted area (Veh + I/R: 169.9 ± 6.8 mm^3, Vb + I/R: 136.5 ± 13.6 mm^3 and mAb RP-3 + I/R: 92.0 ± 7.5 mm^3) but not in striatal part as shown in Figure 1.

Effect of neutropenia on myogenic activity

As shown in Figure 2, ischemia and reperfusion induced a significant lost of pressure-induced contracting response of MCA (Veh + I/R) as compared to sham-operated MCA (Sham) with respectively 15.8 ± 2.5 *versus* 28.1 ± 1.9% decrease of diameter. Vinblastine- and mAb RP-3-induced neutropenia did not correct the lost of MCA contractility in the course of I/R as illustrated in Figure 2 (Vb + I/R: 16.5 ± 1.9% and mAb RP-3 + I/R: 12.6 ± 2.1%).

Effect of neutropenia on 15 mM KCl-induced smooth muscle relaxation

Smooth muscle cell-dependent relaxation of MCA was evaluated by application of 15 mM KCl in the different groups of rats. In Veh + I/R animals, the relaxation of the occluded MCA (4.6 ± 1.4%) was significantly reduced in comparison to sham rats (26.0 ± 2.4%) and was not influenced by vinblastine treatment (Vb + I/R: 8.5 ± 3.5%) and mAb RP-3 administrations (mAb RP-3 + I/R: 7.7 ± 1.2%; Figure 3).

Effect of neutropenia on endothelium reactivity

No difference in 5-HT-induced maximum contracting response was observed between the four groups (Table 2). The ACh-induced endothelium-dependent relaxing response was impaired in vehicle-treated ischemic animals (Veh + I/R) as proven by decrease in maximal relaxation (12.1 ± 2.8%) in comparison to sham-operated group (24.9 ± 3.0%; $P < 0.05$). This impairment was prevented in vinblastine-treated animals, as shown by the lack of significant difference between Vb + I/R *versus* Sham and by the significant difference between Vb + I/R *versus* Veh + I/R (Figure 4, Table 2). Administrations of RP-3 anti-rat neutrophils monoclonal antibodies prevented endothelial dysfunction as illustrated by dose–response curve in Figure 4 and the significant difference between the maximal relaxing response of mAb RP-3 + I/R MCAs as compared to Veh + I/R MCAs (Table 2). Endothelium-independent relaxing responses to SNP (10 μM) were similar in all four groups (Table 2).

Discussion

In the present study, we demonstrated that the postischemic endothelial dysfunction was totally prevented by pharmacologically induced neutropenia in I/R conditions. In contrast, smooth muscle compartment alterations were not influenced by pharmacological neutropenia as proved by the persistence of impairment of myogenic tone and Kir2.x activation-induced relaxation. Ischemia and reperfusion induced an increase of PMN number. Vinblastine treatment drastically reduced number of neutrophils and white blood cells while mAb RP-3 treatment specifically diminished PMN. The beneficial effects on endothelial function are parallel to a protective effect against brain infarct, as demonstrated by a significant decrease in infarct size, in particular in the cortical part of ischemia.

Our data concerning neutropenia-induced decrease of infarct size are in accordance with previous studies showing that pharmacologically-induced neutropenia is responsible for a neuroprotection. This neuroprotective effect points out the potential role of infiltration of infarct area by PMN. This deleterious role has been demonstrated in most studies, whereas it remains controversial because of the lack of infiltration in some studies (Hayward *et al*., 1996; Beray-

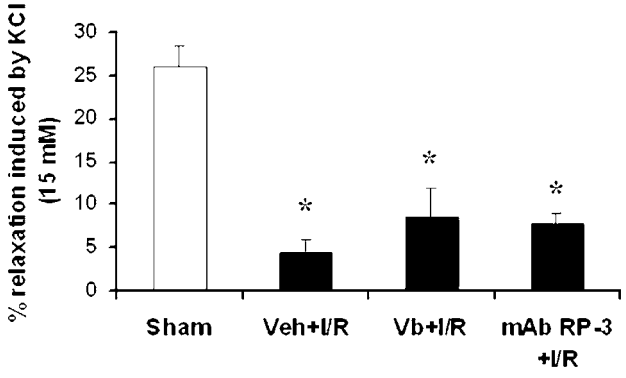

Figure 3 Percent change in diameter in response to 15 mM KCl of middle cerebral arteries originated from sham-operated animals (Sham), or ischemia (1 h) following by reperfusion (24 h)-operated rats (I/R) treated by vinblastine (Vb: 0.5 mg kg^{-1}) or not (Veh: saline vehicle 0.9%) and RP-3 anti-rat neutrophils antibody (mAb RP-3). Values are mean ± s.e.m. *$P < 0.05$ as compared to Sham.

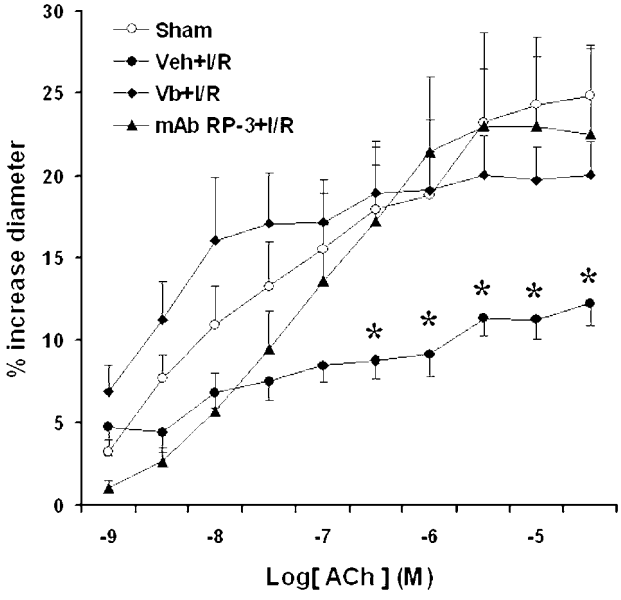

Figure 4 Dose–response curves to ACh for vehicle (saline 0.9%), vinblastine (0.5 mg kg^{-1}) or RP-3 anti-rat neutrophils antibody (mAb RP-3) treated animals in ischemia/reperfusion (I/R) or sham operations. Relaxation was expressed as the percent change in diameter of 5-HT (1 μM) preconstricted middle cerebral artery in response to cumulative concentration of ACh (0.001–30 μM). Values are mean ± s.e.m. *$P < 0.05$ as compared to Sham.

Table 2 Acetylcholine (ACh), potassium chloride (KCl), serotonin (5-HT) and sodium nitroprusside (SNP) vasoreactive effects on middle cerebral artery (MCA)

	Sham	*Ischemia/reperfusion (I/R)*		
	Vehicle (n = 7)	*Vehicle* (n = 7)	*Vinblastine* (n = 8)	*mAb RP-3* (n = 4)
ACh 10 μM (% relaxation)	24.3 ± 2.9	11.3 ± 1.2*	19.8 ± 2.0$^{\#}$	23.0 ± 5.4$^{\#}$
KCl 15 mM (% relaxation)	26.0 ± 2.4	4.6 ± 1.3*	6.1 ± 2.9*	7.0 ± 1.1*
SNP 10 μM (% relaxation)	58.4 ± 5.2	52.5 ± 4.9	50.4 ± 6.0	55.7 ± 12.0
5-HT 1 μM (% constriction)	29.6 ± 2.0	29.8 ± 2.0	27.5 ± 4.5	33.3 ± 5.6

MCA were dissected from rats in sham-operated conditions (Sham) or after 1 h ischemia followed by 24 h reperfusion period (I/R) and treated by vehicle (Veh), vinblastine or RP-3 anti-rat neutrophils monoclonal antibody (mAb RP-3). Values are mean ± s.e.m. *$P < 0.05$ as compared to Sham, $^{\#}P < 0.05$ as comparison to vehicle-treated I/R group.

Berthat *et al*., 2003) or because of the lack of relationship between infarct severity and time course of this infiltration (Fassbender *et al*., 2002). Other available strategies (antibodies against adhesion molecules, chemical compounds that prevent PMN adhesion or mice deficient in adhesion molecules) have also given controversial results. Here, we demonstrate for the first time with two different methods directed either against all white blood cells (vinblastine), either specifically against PMN (mAb RP-3) that neutrophils would participate to brain infarct development. Nevertheless, it could exist a differential involvement of PMN between cortex and striatum since we confirmed, as previously demonstrated, that the vinblastine- or mAb RP-3-induced neutropenia only decreases cortical infarct volume but not striatal one. This dissociate effect could be related to a more pronounced reduction of blood flow in striatum than in cortex after MCA occlusion, as suggested by the lack of cortical protection induced by neutropenia in model of cerebral ischemia with a drastic drop in cortical blood flow (Abe *et al*., 1988; Memezawa *et al*., 1992). These data support the hypothesis of a possible link between vascular wall and polymorphonuclear leukocytes, in particular in penumbra, where cerebral blood flow is decreased but not nearly null as in core.

Several works have demonstrated that PMN modulation could modify endothelial function of blood vessel. Indeed, in a canine experimental I/R model, adenosine, which is known to diminish PMN recruitment during extended reperfusion, preserved endothelial function (Budde *et al*., 2004). In isolated bovine mesenteric arteries, perfusion of PMN decreased endothelial relaxing response to ACh. This effect was cancelled by superoxide dismutase supplementation suggesting involvement of ROS (De Kimpe *et al*., 1993). In addition, PMN activation by injection of phorbol ester impaired ACh-mediated endothelium relaxation without affecting the relaxation due to endothelium-independent mechanisms. This phenomenon might favour cerebral vasoconstriction, particularly resulting in augmented vessel responses to aggregating platelets (Akopov *et al*., 1994a). One of mechanisms to explain cerebral vasospasm previously described in subarachnoid haemorrhage models, is the impairment of endothelium-dependent relaxation of cerebral arteries associated with enhanced expression in the basilar artery of ICAM-1, which promotes adhesion and infiltration of leukocytes (Handa *et al*., 1991; 1995; Akopov *et al*., 1996a).

We demonstrated here with two different methods that pharmacologically induced neutropenia prevents postischemic endothelial dysfunction, suggesting that activation of leukocytes and their adhesion to endothelium could contribute to occurrence of endothelial dysfunction. In contrast, induction of a neutropenia has no significant effect on both Kir2.x-dependent smooth muscle relaxation and endothelium-independent relaxing response. This dissociate effect supports our hypothesis that mechanisms underlying endothelial dysfunction and Kir2.x-dependent smooth muscle alteration are different. This differential effect could explain that in previous works, we found that an antioxidant agent prevented Kir2.x current impairment but not endothelial dysfunction, while in a model of ischemic tolerance with pleiotropic effect there was a protection of both smooth muscle and endothelial dysfunctions (Bastide *et al*., 2003; Pétrault *et al*., 2004). This dissociate effect points out that endothelial dysfunction is likely not directly related to ROS such as superoxide anion, contrasting with the probable direct effect of these free radicals on ion channels. Indeed, oxidative damages of cell membranes (lipid peroxidation and protein oxidation) have been shown to impair ion channels and electrical properties of vascular smooth muscle (Kourie, 1998).

The molecular mechanisms of endothelial dysfunction remain to be discussed. The decrease of endothelial response to ACh could not be explained by an alteration of constitutive NO synthase since it has been reported that endothelium NOS expression was increased during cerebral I/R (Leker *et al*., 2001). PMN are able to produce oxidative stress by membrane-associated NADPH oxidase, which participates to the production of superoxide anion, and by myeloperoxidase activation, which releases hypochloric acid, another powerful oxidant (Babior, 2000; Witko-Sarsat *et al*., 2000). It has been shown that the cerebral oxidative stress, which can be evaluated by glutathione levels measurement, was concomitant with the presence of PMN in brain parenchyma (Beray-Berthat *et al*., 2003). Therefore, PMN could constitute one of mechanisms of endothelial alteration by their oxidative interactions with NO, resulting in a decrease of its bioavaibility consecutive to peroxinitrite formation. Otherwise, active MMPs, proteases released by activated leukocytes, increased degradation of extracellular matrix and altered endothelial barrier permeability and the blood vessel integrity. The loss of microvessel extracellular matrix is also associated with the loss of endothelial reactivity during focal ischemia (Del Zoppo & Mabuchi, 2003). Recent studies have shown that PMN were responsible for release of pro-MMP-9 but not of pro-MMP-2 during cerebral ischemia in the rodent, which contributed to worsen brain infarct lesion and to occur hemorrhagic transformations (Heo *et al*., 1999; Asahi *et al*., 2000; 2001a, b; Justicia *et al*., 2003). The relationship between pharmacologically mediated neutropenia and endothelial function prevention supports hypothesis that PMN play a crucial role in occurrence of endothelium injury beyond its pathophysiological involvement in extent of neuronal lesions.

The parallel occurrence of endothelial protection and decrease of infarct volume raise also the question of the link between these two phenomena. Our data could suggest that pharmacological protection of endothelium is involved in neuroprotection associated with neutropenia. Protection of endothelium could contribute to increase neuroprotection whereas it is only not the determinant factor. Thus, while RP3- and vinblastine-induced neutropenia were associated with a similar endothelial protection, there was a difference in level of neuroprotection between the two strategies. In contrast, we demonstrated recently that a pure antioxidant was able to limit the extent of ischemia despite lack of endothelial protection but in parallel with prevention of Kir2.x-dependent mechanisms impairment. In a model of ischemic tolerance by a low dose of lipopolysaccharide, we found a prevention of both endothelial dysfunction and Kir2.x-dependent mechanisms impairment because of its pleiotropic effects (Bastide *et al*., 2003). Nevertheless, the mAb RP-3-induced neutropenia, which induced a comparable level of neuroprotection to LPS, does not prevent Kir2.x-dependent relaxation impairment. Our results point out that involvement of the different compartments of vascular wall is complex. Further experiments with other pharmacological agents will be necessary to prove the relationship between vasculoprotection and neuroprotection.

In conclusion, we demonstrate for the first time that PMN participate to endothelial dysfunction occurrence in course of cerebral ischemia, suggesting that a phamacological modulation of their activation could contribute to neuroprotection.

This work was supported by Société Française de Pharmacologie (O. Pétrault), of Conseil Régional du Nord-Pas de Calais and by the Délégation à la Recherche du C.H.&U. (P. Gelé). This work was granted by Génopole Lille Nord-Pas de Calais (#01360124) and Fondation pour la Recherche Médicale.

References

ABE, K., ARAKI, T. & KOGURE, K. (1988). Recovery from edema and of protein synthesis differs between the cortex and caudate following transient focal cerebral ischemia in rats. *J. Neurochem.,* **51,** 1470–1476.

AKOPOV, S.E., SERCOMBE, R. & SEYLAZ, J. (1994). Endothelial dysfunction in cerebral vessels following carotid artery infusion of phorbol ester in rabbits: the role of polymorphonuclear leukocytes. *J. Cereb. Blood Flow Metab.,* **14,** 1078–1087.

AKOPOV, S., SERCOMBE, R. & SEYLAZ, J. (1996a). Cerebrovascular reactivity: role of endothelium/platelet/leucocyte interactions. *Cerebrovasc. Brain Metab. Rev.,* **8,** 11–94.

AKOPOV, S.E., SIMONIAN, N.A. & GRIGORIAN, G.S. (1996b). Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. *Stroke,* **27,** 1739–1743.

ASAHI, M., ASAHI, K., JUNG, J.C., DEL ZOPPO, G.J., FINI, M.E. & LO, E.H. (2000). Role of matrix metalloproteinase-9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. *J. Cereb. Blood Flow Metab.,* **20,** 1681–1689.

ASAHI, M., SUMII, T., FINI, M., ITOHARA, S. & LO, E. (2001a). Matrix metalloproteinase 2 gene knockout has no effect on acute brain injury after focal ischemia. *Neuroreport,* **12,** 3003–3007.

ASAHI, M., WANG, X., MORI, T., SUMII, T., JUNG, J., MOSKOWITZ, M., FINI, M. & LO, E. (2001b). Effects of matrix metalloproteinase-9 gene knockout on the proteolysis of blood–brain barrier and white matter components after cerebral ischemia. *J. Neurosci.,* **21,** 7724–7732.

BASTIDE, M., BORDET, R., PU, Q., ROBIN, E., PUISIEUX, F. & DUPUIS, B. (1999). Relationship between inward rectifier potassium current impairment and brain injury after cerebral ischemia/reperfusion. *J. Cereb. Blood Flow Metab.,* **19,** 1309–1315.

BASTIDE, M., GELE, P., PETRAULT, O., PU, Q., CALIEZ, A., ROBIN, E., DEPLANQUE, D., DURIEZ, P. & BORDET, R. (2003). Delayed cerebrovascular protective effect of lipopolysaccharide in parallel to brain ischemic tolerance. *J. Cereb. Blood Flow Metab.,* **23,** 399–405.

BEAUCHAMP, P., RICHARD, V., TAMION, F., LALLEMAND, F., LEBRETON, J.P., VAUDRY, H., DAVEAU, M. & THUILLEZ, C. (1999). Protective effects of preconditioning in cultured rat endothelial cells: effects on neutrophil adhesion and expression of ICAM-1 after anoxia and reoxygenation. *Circulation,* **100,** 541–546.

BERAY-BERTHAT, V., CROCI, N., PLOTKINE, M. & MARGAILL, I. (2003). Polymorphonuclear neutrophils contritube to infarction and oxidative stress in the cortex but not in the striatum after ischemia–reperfusion in rats. *Brain Res.,* **987,** 32–38.

BABIOR, B.M. (2000). Phagocytes and oxidative stress. *Am. J. Med.,* **109,** 33–44.

BRADLEY, K.K., JAGGAR, J.H., BONEV, A.D., HEPPNER, T.J., FLYNN, E.R.M., NELSON, M. & HOROWITZ, B. (1999). Kir2.1 encodes the inward rectifier potassium channel in rat arterial smooth muscle cells. *J. Physiol.,* **515,** 639–651.

BUDDE, J., MORRIS, C., VELEZ, D., MURAKI, S., NING-PING, W., GUYTON, R. & ZHI-QING, Z. (2004). Reduction of infarct size and preservation of endothelial function by multidose intravenous adenosine during extended reperfusion. *J. Surg. Res.,* **116,** 104–115.

CAI, H. & HARRISON, D.G. (2000). Endothelial dysfunction in cardiovascular diseases. The role of oxidant stress. *Circ. Res.,* **87,** 840–844.

CIPOLLA, M.J. & CURRY, A.B. (2002). Middle cerebral artery function after stroke: the threshold duration of reperfusion for myogenic activity. *Stroke,* **33,** 2094–2099.

CIPOLLA, M.J., MCCALL, A.L., LESSOV, N. & PORTER, J.M. (1997). Reperfusion decreases myogenic reactivity and alters middle cerebral artery function after focal cerebral ischemia in rats. *Stroke,* **28,** 176–180.

DEL ZOPPO, G.J. & MABUCHI, T. (2003). Cerebral microvessel responses to focal ischemia. *J. Cereb. Blood Flow Metab.,* **23,** 879–894.

DE KIMPE, S.J., VAN HEUVEN-NOLSEN, D. & NIJKAMP, F.P. (1993). Acetylcholine-induced relaxation in bovine isolated mesenteric arteries is suppressed by polymorphonuclear leukocytes. *Br. J. Pharmacol.,* **109,** 8–13.

DREXLER, H. (1999). Nitric oxide and coronary endothelial dysfunction in humans. *Cardiovasc. Res.,* **43,** 572–579.

FASSBENDER, K., ROGOSCHKE, A., KUHL, S., SZABO, K., FATAR, M., BACK, W., BERTSCH, T., KREISEL, S. & HENNERICI, M. (2002). Inflammatory leukocyte infiltration in focal cerebral ischemia: unrelated to infarct size. *Cerebrovasc. Dis.,* **13,** 198–203.

FORMAN, L.J., LIU, P., NAGELE, R.G., YIN, K. & WONG, P.Y. (1998). Augmentation of nitric oxide, superoxide and peroxynitrite production during cerebral ischemia and reperfusion in the rat. *Neurochem. Res.,* **23,** 141–148.

GAUTIER, S., PETRAULT, O., GELE, P., LAPRAIS, M., BASTIDE, M., BAUTERS, A., DEPLANQUE, D., JUDE, B., CARON, J. & BORDET, R. (2003). Involvement of thrombolysis in recombinant tissue plasminogen activator-induced cerebral hemorrhages and effect on infarct volume and postischemic endothelial function. *Stroke,* **34,** 2975–2979.

HANDA, Y., KABUTO, M., KOBAYASHI, H., KAWANO, H., TAKEUCHI, H. & HAYASHI, M. (1991). The correlation between immunological reaction in the arterial wall and the time course of the development of cerebral vasospasm in a primate model. *Neurosurgery,* **28,** 542–549.

HANDA, Y., KUBOTA, T., TSUCHIDA, A., KOBAYASHI, H. & KAWANO, H. (1995). Expression of intercellular adhesion molecule 1 (ICAM-1) on the cerebral artery following subarachnoid haemorrhage in rats. *Acta Neurochir.,* **132,** 92–97.

HEO, J.H., LUCERO, J., ABUMIYA, T., KOZIOL, J.A., COPELAND, B.R. & DEL ZOPPO, G.J. (1999). Matrix metalloproteinases increase very early during experimental focal cerebral ischemia. *J. Cereb. Blood Flow Metab.,* **19,** 624–633.

HAYWARD, N.J., ELLIOTT, P.J., SAWYER, S.D., BRONSON, R.T. & BARTUS, R.T. (1996). Lack of evidence for neutrophil participation during infarct formation following focal cerebral ischemia in the rat. *Exp. Neurol.,* **139,** 188–202.

JUSTICIA, C., PANES, J., SOLE, S., CERVERA, A., DEULOFEU, R., CHAMORRO, A. & PLANAS, A.M. (2003). Neutrophil infiltration increases matrix metalloproteinase-9 in the ischemic brain after occlusion/reperfusion of the middle cerebral artery in rats. *J. Cereb. Blood Flow Metab.,* **23,** 1430–1440.

KOURIE, J.I. (1998). Interaction of reactive oxygen species with ion transport mechanisms. *Am. J. Physiol.,* **275,** C1–C24.

LAUDE, K., BEAUCHAMP, P., THUILLEZ, C. & RICHARD, V. (2002). Endothelial protective effects of preconditioning. *Cardiovasc. Res.,* **55,** 466–473.

LEKER, R.R., TEICHNER, A., OVADIA, H., KESHET, E., REINHERZ, E. & BENHUR, T. (2001). Expression of endothelial nitric oxide synthase in the ischemic penumbra: relationship to expression of neuronal nitric oxide synthase and vascular endothelial growth factor. *Brain Res.,* **909,** 1–7.

LIN, T.N., HE, Y.Y., WU, G., KHAN, M. & HSU, C.Y. (1993). Effects of brain edema on infarct volume in a focal cerebral ischemia model in rats. *Stroke,* **24,** 117–121.

LOVE, S. (1999). Oxidative stress in brain ischemia. *Brain Pathol.,* **9,** 119–131.

MARRELLI, S.P., JOHNSON, T.D., KHOROVETS, A., CHILDRES, W.F. & BRYAN, R.M. (1998). Altered function of inward rectifier potassium channels in cerebrovascular smooth muscle after ischemia/reperfusion. *Stroke,* **29,** 1469–1474.

MATSUO, Y., KIHARA, T., IKEDA, M., NINOMIYA, M., ONODERA, H. & KOGURE, K. (1995). Role of neutrophils on radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion on extracellular ascorbyl radical formation. *J. Cereb. Blood Flow Metab.,* **15,** 941–947.

MCGIRT, M.J., LYNCH, J.R., PARRA, A., SHENG, H., PEARLSTEIN, R.D., LASKOWITZ, D.T., PELLIGRINO, D.A. & WARNER, D.S. (2002). Simvastatin increases endothelial nitric oxide synthase and ameliorates cerebral vasospasm resulting from subarachnoid hemorrhage. *Stroke,* **33,** 2950–2956.

MEMEZAWA, H., MINAMISAWA, H., SMITH, M.L. & SIESJÖ, B.K. (1992). Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat. *Exp. Brain Res.,* **89,** 67–78.

MORI, T., NAGATA, K., TOWN, T., TAN, J., MATSUI, T. & ASANO, T. (2001). Intracisternal increase of superoxide anion production in a canine subarachnoid hemorrhage model. *Stroke,* **32,** 636–642.

PAXINOS, G. & WATSON, C. (1986). *The Rat Brain Stereotaxic Coordinates*. Academic Press: London.

PÉTRAULT, O., BASTIDE, M., COTELLE, N., GELE, P., GAUTIER, S., LAPRAIS, M., VAMECQ, J., DURIEZ, P. & BORDET, R. (2004). The neuroprotective effect of the antioxidant flavonoid derivate di-tert-butylchalcone is parallel to preventive effect on post-ischemic Kir2.x impairment but not to post-ischemic endothelial dysfunction. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **370,** 395–403.

SEKIYA, S., GOTOH, S., YAMASHITA, T., WATANABE, T., SAITOH, S. & SENDO, F. (1989). Selective depletion of rat neutrophils by *in vivo* administration of a monoclonal antibody. *J. Leukoc. Biol.,* **46,** 96–102.

TAKIZAWA, T., TADA, T., KITAZAWA, K., TANAKA, Y., HONGO, K., KAMEKO, M. & UEMURA, K.I. (2001). Inflammatory cytokine cascade released by leukocytes in cerebrospinal fluid after subarachnoid hemorrhage. *Neurol. Res.,* **23,** 724–730.

WITKO-SARSAT, V., RIEU, P., DESCAMPS-LATSCHA, B., LESAVRE, P. & HALBWASCHS-MECARELLI, L. (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.,* **80,** 617–653.

(Received August 2, 2004
Revised October 15, 2004
Accepted December 1, 2004)