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Modulation of cerebral vascular tone by activated glia: involvement of nitric oxide

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Abstract

The ability of activated glia to affect cerebral vascular tone has been evaluated using an *in vitro* experimental system in which basilar arteries were incubated with glial cultures activated by treatment with lipopolysaccharide (LPS). Vascular tone was measured with an isometric myograph. Contraction in response to high KCl and serotonin was reduced in arteries co-incubated for 24 h with LPS-activated glia, whereas the response to acetylcholine was not modified. The reduced contraction was prevented when the nitric oxide synthase (NOS) inhibitor L-*N*-nitro-arginine (L-NNA) was added throughout the whole incubation time (activation of glial cells with LPS + co-incubation of glial cells with cerebral arteries). Under these conditions, nitrite levels were drastically reduced. A reduced contraction to KCl was also observed after treatment of the cerebral vessel with sodium nitroprusside. In contrast, L-NNA added to the vessel did not modify the response to contracting stimuli and the expression of endothelial NOS was not modified in cerebral arteries pre-incubated with activated glia. These results suggest that activated glia, which finds an *in vivo* correlate in several neuropathological conditions, can contribute to changes of vascular tone by modifying the levels of nitric oxide (NO) to which the vessel is exposed.

Keywords: astrocyte, contraction, cytokines, gliosis, microglia.

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Glia plays a fundamental role in the regulation of neuronal functions and neurone–glia interaction is a complex event that implies the existence of reciprocal exchanges among different cell types (Haydon 2001). This interaction is realized not only by glia supporting survival and maturation of developing as well as of mature neurones (Villegas *et al*. 2003; Lemke 2001) and intervening in the host defense mechanisms (Kreutzberg 1996), but also by glia responding to synaptic transmitters released by neurones (Carmignoto 2000).

Glial cells also contribute to the formation of the blood–brain barrier, thus operating an indirect homeostatic regulation of brain environment. Astrocytes and other glial cells release, in fact, soluble factors that are able to modulate endothelial permeability (Abbott 2000, 2002) and, interestingly, endothelial cells can affect astrocyte differentiation (Mi *et al*. 2001). In addition, glial cells can also control cerebral blood flow by regulating vascular tone (Anderson and Nedergaard 2003). Recent evidence suggests that the increased blood flow occurring under physiological conditions in areas of intense neuronal activity, namely functional hyperemia, recognizes a fundamental role for astrocytes (Harder *et al*. 1998; Zonta *et al*. 2003).

The chemical nature of the glia-derived signals in the glia-vascular communication is currently unclear, but several candidate molecules have been proposed. Among these are the eicosanoids, including prostaglandins and thromboxane and a variety of lipoxygenase, epoxygenase and cyclooxygenase products (Murphy *et al*. 1994; Harder *et al*. 1998; Zonta *et al*. 2003). These factors can exert direct effects on smooth muscle cells but their action also influences endothelial cells by modulation of the synthesis and release of several vasoactive products (Faraci 1992). More recently, astrocytic calcium signalling has also been implicated in the

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Abbreviations used: ACh, acetylcholine; eNOS, endothelial nitric oxide synthase; 5-HT, 5-hydroxytryptamine; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-NNA, L-*N*-nitro-arginine; LPS, lipopolysaccharide; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; TNF-α, tumour necrosis factor-α.

communication at the gliovascular interface (Simard *et al*. 2003).

In pathological conditions such as neurodegenerative diseases, ischemia, stroke and other cerebral injuries, glial cells change their morphology and releasing capacity (Chen and Swanson 2003). During this condition, known as 'reactive gliosis', both astrocytes and microglia provide a rich source of cytokines and growth factors, which may influence nearby neurones and probably brain vessels (Stoll *et al*. 1998; Streit *et al*. 1999; Abbott 2000; Chen and Swanson 2003).

Although much is known on the interplay between glia cells and endothelium under physiological and pathological conditions, as well as on the physiological regulation of vascular tone by astrocytes, little attention has been paid to the control of vascular contractility by reactive glia. To ascertain whether during neuropathological conditions implying glial reactivity, changes of cerebral vascular tone occur, we have used an experimental paradigm in which rat basilar arteries were incubated with mixed glial cells activated by exposure to lypopolysaccaride (LPS). Thus, in the present study, attention has been focused on activated glia to identify its selective contribution to the control of vascular contractility. The ability of cerebral vessels to respond to depolarizing, contracting and dilating agents has then been evaluated with an isometric myograph.

Materials and methods

All animal experimental procedures were carried out in accordance with the Italian and EU regulations on animal welfare and were authorized by the Italian Ministry of Health.

Glial cell culture

Glial cells were prepared from 1–3-day-old Sprague-Dawley rats. Cortices were deprived of meninges and dissociated with a 0.05% trypsin solution. Cells were added to 75-mm^2 flasks and maintained in Dulbecco's modified Eagle's medium (Invitrogen srl, Milan, Italy) supplemented with 10% fetal bovine serum, penicillin (100 U/mL)/streptomycin (100 μg/mL) and glutamine (2 mM), all from Invitrogen srl, in an incubator at 37°C and 5% CO_2. Confluent cultures at 14 days *in vitro* were detached with a 0.05% trypsin solution, collected and re-plated onto 35-mm dishes (Corning, Acton, MA, USA) until use. These cultures have been thoroughly characterized (Saneto and de Vellis 1987) and consist of a confluent layer of flat, large cells identified as astrocytes and sparse, smaller, round cells that stain positively for oligodendrocyte specific markers. On top of the astrocyte layer, large, flat cells are clearly distinguishable and represent the microglial population. Microglia was obtained by vigorous shaking of flasks for 4 h at 37°C. Pure astrocytes were derived by prolongation of the shaking procedure for an additional 4 h to remove oligodendrocyte contamination.

Cerebral artery isolation and incubation with glial cells

The basilar artery of Sprague-Dawley male rats (260–300 g) was transferred to a buffer solution (NaCl 118 mM; KCl 4.6 mM; $NaHCO_3$ 25 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; glucose 10 mM; EDTA 0.025 mM; pH 7.4 at 37°C), dissected, deprived of meninges and cut into four segments (1.5–2 mm long). Each segment was incubated for 24 h with glial cells, either untreated or pre-exposed for 16–20 h to LPS (2 μg/mL; Sigma St Louis, MO, USA), at 37°C in a humidified atmosphere of 5% CO_2. A schematic diagram of the paradigm used is illustrated in Fig. 1.

Measurement of contractile tension in isolated basilar artery

Arterial segments (1.5–2 mm long) were threaded onto 40-μm stainless steel wires and mounted in an isometric myograph (610 M, Danish Myo Technology, Aarhus, Denmark). After mounting, each preparation was equilibrated unstretched, for 30 min, in physiological solution, maintained at 37°C and aerated with a gas mixture of 95% O_2−5% CO_2. The normalized passive resting force and the corresponding diameter were determined for each preparation from its own length–pressure curve, according to Mulvany and Halpern (1977). Each preparation was maintained, until the end of experiment, at a passive relaxation corresponding to 90% of tension exerted by a pressure of 100 mmHg on the vessel wall. Contraction and relaxation force was registered by an acquisition and registration program (Myodaq e Myodata, Danish Myo Technology). After normalization and 30 min of equilibration in physiological solution, preparations were stimulated with 100 mM KCl (in a solution in which NaCl had been largely replaced by KCl; final concentrations were NaCl 22.6 mM; KCl 98.8 mM; $NaHCO_3$ 25 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; glucose 10 mM; EDTA 0.025 mM). These supramaximal KCl concentrations are largely reported in the literature (Salomone *et al*. 1997; Takizawa *et al*. 1997) for measurement of maximal contractile response. After wash-out and 30 min recovery, they were exposed to cumulative concentrations of 5-hydroxytryptamine (5-HT, 1 nM-10 μM), to induce contraction, and acetylcholine (ACh, 1 nM-10 μM), to induce relaxation.

Western blot analysis

For western blot analysis, after co-incubation with glial cells, the rat basilar artery was homogenized with the aid of an automatic pellet pestle in a small volume of lysis buffer containing a cocktail of protease inhibitors including bestatin, aprotinin, leupeptin and

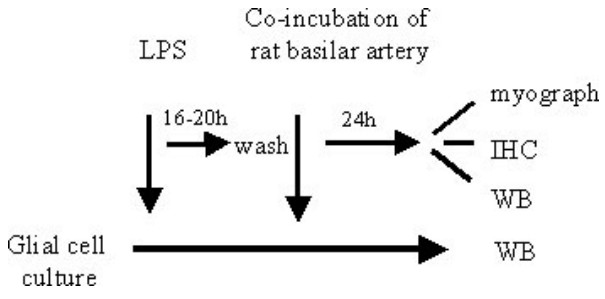

Fig. 1 Schematic diagram of the experimental paradigm utilized throughout the study. Confluent glia cultures were exposed to lipopolysaccharide (LPS, 2 μg/mL) for 16–20 h prior to washing and co-incubation with a rat basilar artery for additional 24 h. When indicated, drugs were added during this incubation time. Arterial segments were then used for measurement of contractile tension or processed for immunohistochemistry (IHC) or western blot (WB). When indicated, glial cells were used for western blot analysis.

sodium EDTA. The same procedure was used to extract proteins from the glia culture, except for homogenization that was not necessary. After sonication, an aliquot of the sample was processed for protein concentrations according to the method of Bradford. Samples were concentrated and boiled for 5 min. Electrophoresis was performed in 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (30 mA/h) using 60 μg of cell proteins per lane. Proteins were then transferred onto a nitrocellulose membrane (Hybond ECL, Amersham, Little Chalfont, UK) for 2 h at 23°C using a transblot semi-dry transfer cell. After blocking, the membranes were incubated with polyclonal rabbit anti-endothelial nitric oxide synthase (eNOS) (1 : 500), anti-inducible NOS (iNOS) (1 : 200) and anti-neuronal NOS (nNOS) (1 : 200) antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, then repeatedly washed, and exposed to horseradish peroxidase-conjugated anti-rabbit IgG (1 : 5000; Amersham) for 1 h at room temperature. Proteins were visualized with a chemiluminescent detection system. The same membranes were stripped and re-exposed with mouse anti-actin (1 : 250; Sigma) to check for protein loading.

Immunohistochemistry

After co-incubation with glial cells, rat basilar arteries were maintained in 30% sucrose prior to inclusion in albumin : glyceraldehyde (15 : 1). Then, 40-μm-thick sections of rat basilar arteries were cut on a microtome (Leitz, Wetzlar, Germany) and maintained in a cryoprotective solution at 4°C until processing for immunostaining. Slices were incubated for 30 min with Tris-buffered saline solution containing 4% normal goat serum and 0.2% Tryton X-100. Sections were then incubated overnight at 4°C with polyclonal rabbit anti-eNOS (1 : 500, Santa Cruz Biotechnology), repeatedly washed and incubated with anti-rabbit secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA), for 1 h at room temperature. After reaction with avidin–biotin–horseradish peroxidase (Vectastain ABC Elite kit, Vector Laboratories) for 1 h at room temperature, staining was developed by exposure to 0.05% diaminobenzidine/0.01% H_2O_2 for 5–8 min.

Measurement of nitric oxide

Nitric oxide (NO) production in the glial cell cultures was assessed by the Griess reaction. Briefly, 100 μL aliquots of culture supernatants were incubated with 100 μL Griess reagent [1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride and 5% phosphoric acid] at room temperature for 10 min. Optical density at 540 nm was then determined using a microplate reader.

Statistical analysis

Data were analysed by *t*-test or whenever requested by ANOVA followed by Neuman–Keuls *t*-test for significance. $p < 0.05$ denotes statistical significance.

Results

Co-incubation of cerebral artery segments with mixed glial cultures activated by pre-exposure to LPS affected the response of the vessel to depolarizing concentrations (100 mM) of KCl. The ability of the vessel to contract was in fact significantly reduced ($p < 0.05$) following exposure to LPS-activated glia (Fig. 2a). Glia *per se* was not able to modify vascular tone, as shown by the similarity of the response to KCl in arteries pre-incubated with or without glia (not shown). A reduced contractile response was observed also when cerebral arteries co-incubated with LPS-treated glia were exposed to cumulative concentrations of 5-HT (1 nM–10 μM). Although the ability to respond to low concentrations of 5-HT was similar in vessels pre-incubated with control and LPS-activated glia, contraction to higher concentrations was significantly impaired only in the latter treatment group (Fig. 2b). The response to increasing concentrations of ACh (1 nM–10 μM) was instead similar in the two experimental groups when absolute values were considered (Fig. 2c). The reduced ability of vessels to

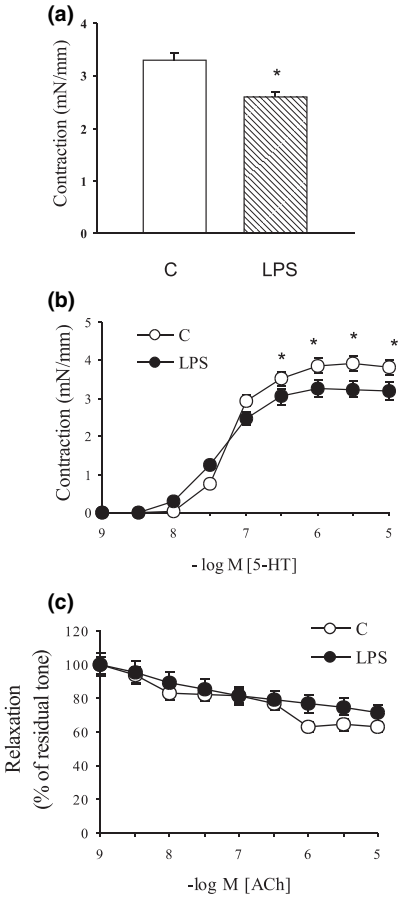

Fig. 2 LPS-treated glia modifies the contractility of rat basilar arteries. The response to depolarizing concentrations (100 mM) of KCl was significantly ($p < 0.05$) reduced in basilar arteries incubated with LPS-pre-treated glia cells (a). The reduced ability to contract was maintained when the same arteries were challenged with increasing concentrations of 5-hydroxytryptamine (5-HT) (b; $p < 0.05$). In contrast, there was no significant difference in the response to acetylcholine (ACh) (c). Data are mean ± SE of 7–9 independent determinations. C, control.

respond to contracting stimuli was related to the reactivity of glia, as it was observed also in cerebral arteries co-incubated with pure astrocyte cultures made reactive by prolonged serum starvation (72 h in the absence of serum). A reduced contraction was again observed when arterials segments were challenged with KCl (Fig. 3a) and increasing concentrations of 5-HT (Fig. 3b), whereas the concentration response curves to ACh were superimposable (Fig. 3c). In contrast, when basilar arteries were incubated with pure microglia cultures pre-treated with LPS, the contractility of the vessel in response to KCl did not differ from the control (3.15 and 3.10 mN/mm for arteries co-incubated with control and LPS-activated glia, respectively).

To ascertain whether soluble factors released by activated glial cells were responsible for the modified response to depolarizing concentrations of KCl, the experiment reported in Fig. 2 was repeated in the presence of 10 μM of the Golgi inhibitor brefeldin-A. Under these conditions in which vesicular release from glial cells was inhibited, basilar arteries co-incubated with LPS-pre-treated glia, reacquired their ability to fully respond to KCl (Fig. 4a). The concentration response curve to increasing concentrations of 5-HT maintained a slight shift to the right in vessels exposed to LPS-pre-treated glia in the presence of brefeldin A (pD2 of 7.26 ± 0.05 and 6.69 ± 0.13, respectively; Fig. 4b; $p < 0.005$), whereas the dilating action of increasing concentrations of ACh was not modified by the drug (Fig. 4c). A similar pattern of response was observed when glial cells were pre-treated with dexamethasone (1 μM) for the 24 h preceding and throughout LPS exposure, as well as

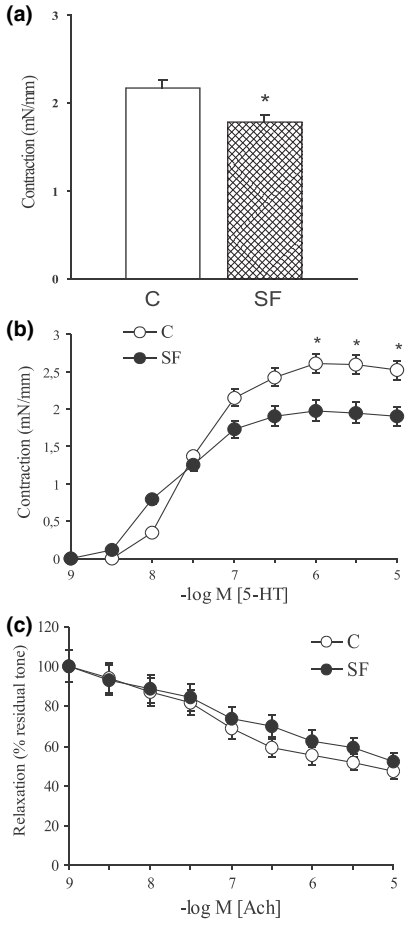

Fig. 3 Astrocytes activated by serum deprivation modify the contractility of rat basilar arteries. Basilar arteries co-incubated with serum-deprived astrocytes (SF) responded with a lower contractility ($p < 0.05$) to 100 mM KCl (a) and cumulative concentrations of 5-hydroxytryptamine (5-HT) (b). Dilation induced by increasing concentrations of acetylcholine (ACh) (c) was similar in the two experimental groups. Values represent mean ± SE of five to seven determinations. C, control.

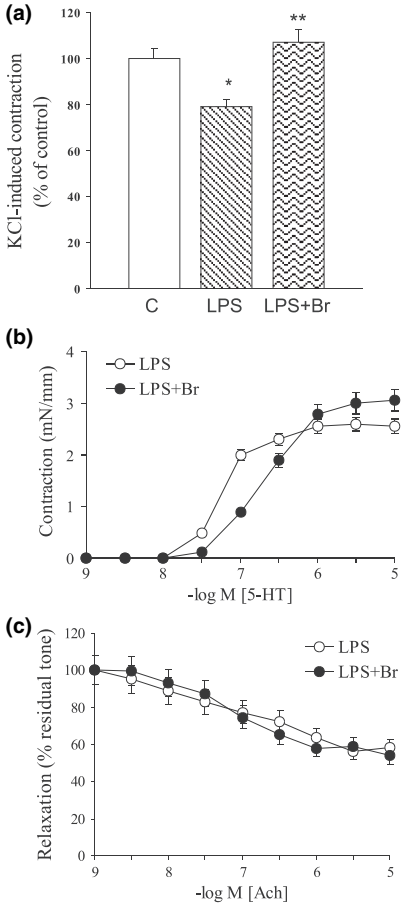

Fig. 4 Brefeldin A prevents the reduction of KCl-stimulated contractility induced by co-incubation with lipopolysaccharide (LPS)-treated glial cultures. Pre-treatment with brefeldin A (Br; 10 μg/mL) in the 24 h following LPS removal completely reverted the reduced response to 100 mM KCl (a) and to 5-hydroxytryptamine (5-HT) (b). The relaxing activity of acetylcholine (ACh) was similar in arteries incubated with LPS- and LPS/brefeldin A-treated glia (c). Data are mean ± SE of six arterial segments. *$p < 0.05$ vs. control; **$p < 0.05$ vs. LPS alone. C, control.

during co-incubation with the vessel. Under these conditions the response of the vessel to KCl and 5-HT was restored (not shown). As factors released by activated glia include pro-inflammatory cytokines, cerebral arteries were incubated for a period of 20–24 h preceding the contractile tension measurement with selected cytokines. As shown in Table 1, treatment of vessels with interleukin-1β (IL-1β; 1 ng/mL) or tumour necrosis factor-α (TNF-α; 20 ng/mL) significantly reduced the subsequent ability of the arterial segment to respond to KCl, whereas interferon-γ (IFN-γ; 10 ng/mL) was inactive.

To establish whether endothelial NO was involved in the modified response to KCl, the expression of the endothelial isoform of NO synthesizing enzyme (eNOS) was evaluated by western blot and immunohistochemical analyses. As shown in Figs 5(a) and (c) exposure of the vessel to activated glia did not modify the expression of eNOS as measured by detection of a 145 kDa specific band (Fig. 5a) or specific staining of the vascular endothelium (Fig. 5c). Accordingly, addition of the NOS inhibitor L-*N*-nitro-arginine (L-NNA; 100 μM) directly into the incubation chamber where the vessel was mounted did not produce a significant effect on the response to KCl of arterial segments pre-incubated with either control or LPS-treated glia (Fig. 5b), while inducing *per se* an increased vascular tone (not shown). In contrast, the reduced contraction to KCl was partially prevented when the NOS inhibitor L-NNA was added to glia cultures during the LPS exposure and also, subsequently, during the co-incubation with the vessel (Fig. 6a). This condition was characterized by maintenance of lower NO levels throughout the study (Table 2). This effect was instead not detectable when L-NNA was added only during the exposure of glia to LPS and removed thereafter, concomitantly with the addition of the vessel to the glia culture (Fig. 6a). In contrast to the conditions described above, this situation was characterized by concentrations of NO that were maintained low by the presence of L-NNA, but returned to control levels rapidly after removal of the drug (Table 2). As for the source of NO, increased expression of iNOS and no change of nNOS was observed by western blot in glial cells exposed to LPS (Fig. 6b). It is noteworthy that treatment of glial cells with brefeldin-A, during the co-incubation with the vessel, produced a significant reduction of NO levels (Table 3), suggesting that autocrine or paracrine events were responsible for the increased production of glial NO. Finally, a role for exogenous NO in vessel contractility was supported by the reduced contraction in response to KCl observed in cerebral arteries incubated with 1 μM sodium nitroprusside for 24 h preceding measurement of contractile tension (Fig. 7).

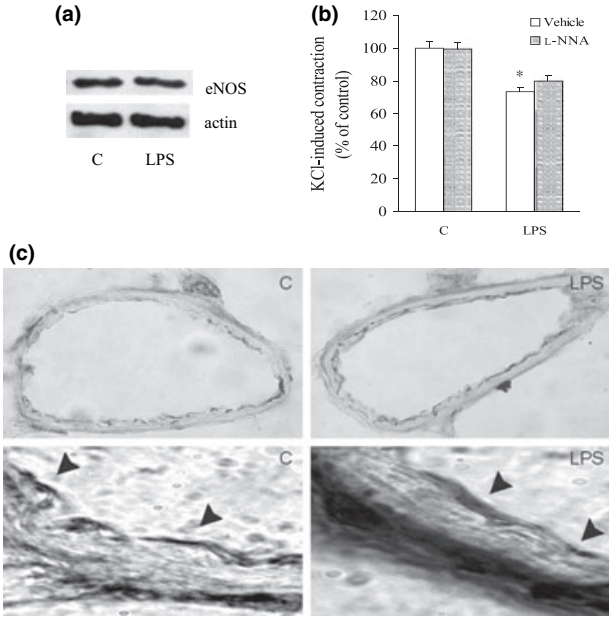

Fig. 5 Vascular endothelial nitric oxide synthase (eNOS) is not involved in the reduction of the contractile response of cerebral arteries exposed to lipopolysaccharide (LPS)-activated glia. Expression of eNOS was analysed by western blot (a) and immunohistochemistry (c) in vascular extracts or in whole arterial segments incubated for 24 h with LPS-stimulated glia (LPS). (b) The response to KCl (100 mM) in vessels pre-exposed to control (C) and activated glia (LPS) and treated with L-*N*-nitro-arginine (L-NNA) (100 μM) directly in the incubation chamber prior to measurement of contractile tension. *$p < 0.05$ vs. control.

Table 1 Effects of exogenously added cytokines on the contractility of rat basilar arteries

	Basilar artery contraction mN/min (% of control)
Control	100.0 + 2.6
IL-1β	84.5 + 3.2*
TNF-α	65.6 + 5.8
IFN-γ	94.2 + 3.3*

Rat basilar arteries were exposed to interleukin-1β (IL-1β; 1 ng/mL); tumour necrosis factor α (TNF-α; 20 ng/mL) and interferon-γ (IFN-γ; 10 ng/mL) for 24 h prior to stimulation with KCl and assessment of vascular contractility. Values are mean + SE of four determinations and are reported as percentage of respective control. Control values ranged between 1.94 and 2.85 mN/min.
*$p < 0.05$ vs. control by Student's *t*-test.

Discussion

Neuropathological conditions including acute neuronal injury or neurodegeneration involve morphological and functional changes of glial cells (Chen and Swanson 2003). In our experimental model, astrogliosis occurring during neuronal damage was mimicked *in vitro* by exposure of

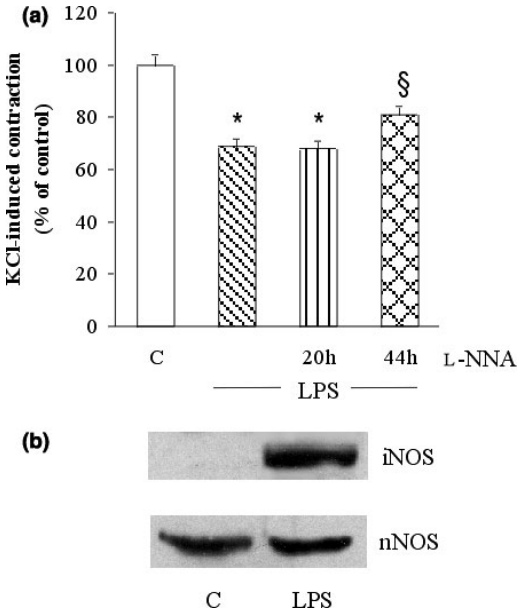

Fig. 6 Sustained inhibition of nitric oxide synthase (NOS) is necessary to reduce the impaired contractility of arteries incubated with lipopolysaccharide (LPS)-activated glial cells. The NOS inhibitor L-*N*-nitro-arginine (L-NNA) (100 μM) was added during the 20-h LPS treatment (20 h) or during the all incubation period (20-h LPS treatment + 24-h wash-out; 44 h). Values are mean ± SE of six determinations. *$p < 0.05$ vs. control; §$p < 0.05$ vs. LPS. (b) inducible NOS (iNOS) and neuronal NOS (nNOS) expression in glia cultures under basal conditions or following a 20-h treatment with LPS.

primary glial cultures to LPS. These cultures are characterized by changes in astrocyte and microglia morphology, enhanced glial fibrillary acidic protein expression, and

Table 2 Time-dependency of nitric oxide synthase inhibition by L-*N*-nitro-arginine

	Nitrite/nitrate (% of control)
Control	100 ± 6.7
LPS (20 h)	341 ± 12.1*
LPS (20 h) + L-NNA (20 h)	200 ± 9.9*
LPS (20 h) + L-NNA (20 h) + 24 h wash-out	350 ± 17.6*
LPS (20 h) + L-NNA (44 h)	163 ± 8.0*†

Mixed glial cultures were incubated with lipopolysaccharide (LPS, 2 μg/mL) for 20 h, with and without 100 μM L-*N*-nitro-arginine (L-NNA). Incubation with the nitric oxide synthase (NOS) inhibitor was carried out during the treatment with LPS (20 h) or in the presence of LPS (20 h) and also during the following 24 h (44 h). Nitrite and nitrate were measured by the Griess assay at the end of the incubation. Basal values in the present assay conditions were approximately 25 μM. Values are reported as percentage of control and represent mean ± SE of four determinations.

*$p < 0.05$ vs. control.

†$p < 0.05$ vs. LPS + L-NNA + wash-out.

Table 3 Soluble factors released by glial cells contribute to the increased production of nitric oxide

	Nitrite/nitrate (% of control)
Control	100 ± 4.8
LPS	249 ± 8.1*
LPS + brefeldin A	199 ± 8.4*†

Mixed glial cultures were incubated with lipopolysaccharide (LPS, 2 μg/mL) for 20 h, washed and exposed to the Golgi inhibitor brefeldin A (10 μg/mL) for additional 24 h. Values are reported as percentage of control and represent mean ± SE of three determinations each run in triplicate.

*$p < 0.05$ vs. control.

†$p < 0.05$ vs. LPS alone.

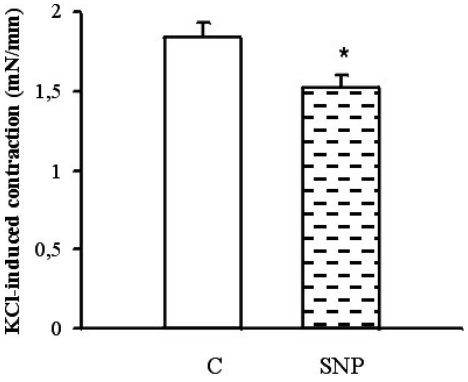

Fig. 7 Exogenous nitric oxide reduces the contractility of cerebral basilar arteries. Vessels were incubated with 1 μM sodium nitroprusside (SNP) for the 24 h preceding measurement of contractile tension. Values are mean ± SE of three independent determinations. *$p < 0.05$ vs. control (C).

increased release of several cytokines (Ridet *et al*. 1997; Wu and Schwartz 1998; Nakamura *et al*. 1999; Vitkovic *et al*. 2000). The choice of basilar arteries has been mainly dictated by the fact that the reactivity of this artery can be easily studied by conventional isometric tension recording. In addition, it reflects most of the characteristics of cerebral vessels, including expression of various receptors and response to environmental conditions. Thus the experimental model used has allowed analysis of the interplay between glia and cerebral vessels that may occur during glia reactivity, resembling a situation in which, in principle, a closer interaction, such as that of smaller vessels and astrocytic endfeet, is realized.

Cerebral vessels incubated with LPS-stimulated mixed glia cultures exhibited changes of vascular contractility in response to KCl and high 5-HT concentrations. The significantly modified response to depolarizing concentrations of KCl was somehow unexpected as KCl was used to induce an

aspecific contractile response to be used as an index of arterial contractility, as from an established experimental protocol (Salomone *et al*. 1997).

Interestingly, the modification of vascular reactivity was observed only in vessels incubated with mixed glia cultures in which astrocytes, oligodendrocytes and microglia were present, but not in pure astroglial cultures, suggesting that the presence of microglia is critical for the response to LPS, as also from previous observations (Zielasek *et al*. 1992). In contrast, the lack of effect of pure microglia indicates the primary role of astrocytes in this effect, also giving support to the reported ability of astrocytes to enhance the responses of microglial cells to activating stimuli (Solà *et al*. 2002). Furthermore, the reduced contraction of arterial segments to KCl and 5-HT was present also when pure astrocytes were activated by prolonged serum deprivation, another condition that mimics reactive gliosis *in vitro* (Wu and Schwartz 1998).

Prevention of the reduced contractile response of basilar cerebral artery to KCl by treatment with the Golgi inhibitor brefeldin-A suggests that a releasable factor produced by glial cells is responsible for the observed effect. Whether brefeldin-A exerted its action on microglia cells rather than astrocytes cannot be established from the present data. Certainly soluble factors released by microglia contribute to the activation of astrocytes, but cytokines produced by astrocytes seem to be involved primarily in the observed response, as suggested by the lack of effect in cerebral vessels incubated with pure microglia and the similarity of the effect observed in vessels exposed to LPS-activated glia and serum-deprived astrocytes. Furthermore, a reduced contraction to KCl was present in cerebral arteries exposed to TNF-α or IL-1β. A role for these cytokines in the modulation of vascular tone has already been demonstrated. In particular, IL-1β is known to inhibit phenylephrine-induced contraction in rat aorta (Takizawa *et al*. 1997) and vasodilation induced by IL-1β has been included among the pathogenetic mechanisms of septic shock (Takizawa *et al*. 1997). More controversial is the action of TNF-α on vascular contractility, as changes of both contraction (Gunnett *et al*. 2000) and relaxation (Greenberg *et al*. 1993) of vessels exposed to the cytokine have been reported. However, enhanced production of vasodilatatory prostaglandins has been observed in endothelial cells from brain microvessels treated with TNF-α (Kilbourn *et al*. 1990; Mark *et al*. 2001).

Paracrine effects in the interplay between astrocytes and endothelial cells have already been invoked as causative events in the disruption of blood–brain barrier integrity occurring during CNS inflammation (Didier *et al*. 2003). The effect produced by exogenously added cytokines in our system are comparable or even greater than that induced by co-incubation with activated glia, but the concentrations used to obtain this effect are higher than the levels of cytokines achieved in the glia culture and no effect was observed when IL-1β and TNF-α were used in the range of concentrations of pg/mL (not shown). Thus, although it is plausible that released cytokines intervene in the modulatory action of astrocytes on vascular contractility, a paracrine or autocrine effect of cytokines released by microglia or astrocytes on the same glial cells cannot be ruled out. Accordingly, a significant reduction of NO production is observed following treatment of astrocyte/microglia cultures with brefeldin-A. Thus, it seems that soluble factors released by glial cells stimulate astrocytes to produce and release NO and the NO-enriched environment in which the vessel is incubated for the 24 h preceding measurement of contractile tension modifies the response to subsequently applied stimuli. In support of a primary role for glia-derived NO, inhibition of NO production during both LPS activation of glia and co-incubation of the vessel with glial cells, partially prevented the reduced contractile response to depolarizing concentrations of KCl. In contrast, a similar effect was not present when inhibition of NO was carried out either during LPS stimulation or directly in vessels previously incubated with LPS-stimulated glia. In the latter conditions, the increased basal contraction measured upon L-NNA addition was completely blunted in the presence of KCl. This was not surprising, as lack of effect of L-NNA on the contractile response induced by maximal stimulation of 5-HT receptors has been reported in bovine pial vessel (Elhusseiny and Hamel 2001). The primary action of NO in changes of vascular contractility is also strongly supported by the reduced response to KCl observed following exposure of the vessel to sodium nitroprusside. Thus, activation of glia *in vitro* does not modify vascular production of NO, as also suggested by lack of changes in the expression of endothelial NOS, but contributes to an altered vascular contraction by exposing the vessel to increased concentrations of NO. Exogenous NO has been shown to inhibit NO production by cerebral endothelial cells in culture (Borgerding and Murphy 1995). In our system, eNOS expression was not modified in cerebral arteries pre-incubated with activated glia and, under the same conditions, we have not been able to detect any iNOS (data not shown). Accordingly, the ability of arteries to dilate in response to ACh is fully preserved, further strengthening the concept that endothelium-produced NO is not involved in the observed effect.

Whether NO originates from astrocytes or microglia cannot be established from the present data due to the experimental model used. iNOS seems responsible for its production as indicated by the marked induction of this isotype in the mixed glia culture and no change in the levels of eNOS. Although microglia in a mixed glia culture is considered at a too low concentration to contribute significantly to the production of NO (Simmons and Murphy 1992), the presence of astrocytes in a mixed culture is known to increase NO production by microglia (Solà *et al*. 2002). This question is, however, still controversial, as both cell types (Kong *et al*. 1996), or

exclusively microglia (Vincent *et al*. 1996, 1997; Yang *et al*. 1998), have been reported to be responsible for NO production. However, the similarity of the effects observed in cerebral arteries incubated with LPS-stimulated mixed glia cultures and serum-deprived astrocytes confirm a primary role for astrocytes, as also supported by the high levels of NO detected in pure astrocyte cultures activated by exposure to inflammatory cytokines (Falsig *et al*. 2004). This may achieve particular significance *in vivo* as it suggests that reactive astrocytes *per se*, even in the absence of a massive microglia infiltration, can affect vascular tone.

In conclusion, the present study demonstrates that reactive glial cells induce changes of cerebral vascular tone. This may be ascribed to cytokines released by activated glia that directly affect the ability of the vessels to contract, but mainly to soluble factors produced by glial cells that, in a paracrine or autocrine manner, cause enhanced production of NO. Overall these results suggest that the state of reactive gliosis that accompanies several neuropathological conditions takes active part to tissue homeostasis by reducing the response to contracting stimuli thus modifying the trophic support to affected areas.

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