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Nitric oxide modulates cerebral blood flow stimulation by acetazolamide in the rat cortex: a laser Doppler scanning study

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Abstract

The involvement of nitric oxide (NO) in cerebral blood flow (CBF) stimulation by acetazolamide was studied in anaesthetised, mechanically ventilated Wistar rats. CBF was monitored by laser Doppler scanning. Acetazolamide induced a long-lasting significant rCBF-increase. Application of N^G-Nitro-L-arginine (L-NNA), an inhibitor of all NO synthetases (NOS), prevented CBF stimulation by acetazolamide. Continuous infusion of the exogenous NO donor SIN-1 (3-morpholinosydnonimine) suppressed L-NNA induced increases of mean arterial blood pressure without effect on rCBF in comparison to baseline. Additional acetazolamide injection then again caused a significant increase of rCBF in spite of NOS-inhibition. We thus conclude that NO is involved in acetazolamide-induced CBF stimulation. The mere continuous presence of NO is sufficient to re-establish the acetazolamide-response in spite of NOS-inhibition. These data suggest that NO acts rather as a modulator than as a mediator of the acetazolamide-induced CBF response.

Keywords: Regional cerebral blood flow; Nitric oxide; Nitro-L-arginine; Scanning laser Doppler flowmetry, Acetazolamide

Acetazolamide is regularly used to test the cerebral reserve capacity in patients with recurrent cerebral ischemic events [16]. Acetazolamide is an inhibitor of carbonic anhydrase, which causes extracellular tissue acidosis in the brain [4,17]. Details of the mechanism of acetazolamide-induced stimulation of cerebral blood flow (CBF) by vasodilatation, however, remain unresolved. Similarities to the CBF stimulation by CO_2 are evident. There is no doubt that nitric oxide (NO) is involved in vasodilation elicited by hypercapnia [5,7,8,15]. The present study was designed to clarify the role of NO in the acetazolamide-induced CBF stimulation, since a recent study claimed acetazolamide induced vasodilatation to be independent of NO [11].

NO is a short-living molecule and in vivo concentration measurements are difficult. So, the current investigation relies on the inhibition of NO-synthetases (NOS) to observe effects on the acetazolamide response with later supplementation of an NO donor. This approach allows to differentiate between mediator and modulator functions of NO. If NO acts as an essential mediator, NOS-inhibition should prevent the acetazolamide-induced CBF stimulation, and the sole reestablishment of the basal NO level by a NO donor after NOS inhibition should not go along with CBF increases after additional acetazolamide injection. In contrast, assuming a modulatory role, the re-establishment of a basal NO level by a NO donor should facilitate an increase of CBF after acetazolamide in spite of NOS-inhibition [1,8,13]. If the stimulation does in fact occur, another trigger signal than NO is involved, and the CBF increase should be comparable in magnitude to the conventional acetazolamide-response.

Male Wistar rats (300 ± 20 g; Charles River, Germany) were used. After premedication with 0.5 mg atropine, animals were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate (36 mg/100 g body weight). During the experiment, artificial ventilation was performed in order to keep p_aCO_2 levels constant. Body temperature was maintained at 37°C. A polyethylene catheter was introduced into the femoral artery and arterial blood pressure was continuously monitored. Arterial blood gases were analyzed before and after acetazolamide, and after nitro-L-arginine (L-NNA) injection (ABL3, Radiometer, Copenhagen, Denmark). The head was fixed in a stereotactic frame and access to the brain surface was gained via a 4 × 5 mm large parietal cranial window, drilled 2 mm lateral and 1 mm caudal to the bregma. Local CBF was assessed by laser Doppler flowme-

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try using a TSI Laser-Flow Blood Perfusion Monitor (Vasamedics, Model BPM 403a, TSI, St. Paul, MN, USA) connected to a needle probe (0.8 mm). In order to estimate regional cortical CBF, a scanning procedure was performed [2,10]. Fifty different locations 100 μm apart from each other were preselected by use of a computer-controlled micromanipulator. The x-, y-, and z-coordinates were stored at the beginning of each experiment to repeatedly re-examine the measuring sites. rCBF changes are reflected by median flow values found in scanning procedures in individual animals and are always expressed in percent baseline.

Control conditions were maintained for 30 min. CBF stimulation was induced by i.p. injection of acetazolamide (Diamox®, Lederle Germany; 100 mg/kg bw), whereas NOS-inhibition was achieved by intraarterial injection of L-NNA (30 mg/kg bw, Sigma, Germany). Endothelial NOS inhibition is followed by increases of arterial pressure which might influence CBF. Therefore, mean arterial blood pressure (MABP) was controlled by a continuous supply of basal NO from the exogenous NO-donor SIN-1 (3-morpholinosydnonimine, Cassela) which was infused after L-NNA injection. The infusion rate was slightly varied in individual experiments to maintain MABP at baseline values; an average intraarterial dosage of 3 mg/kg bw/h was necessary to achieve this goal. CBF-scans were performed at defined time points: two scans during the control phase, four after acetazolamide injection, four after NOS-inhibition and one under SIN-1 infusion. The control phase, the NOS-inhibition phase and the acetazolamide-stimulation phase took 30 min each. Three experimental groups were studied. Group ACZ ($n = 3$) served to demonstrate the time course of CBF changes after acetazolamide-stimulation without NOS-inhibition. The observation time was 40 min after stimulation. In group LNNA-ACZ ($n = 6$) the effect of NOS inhibition before acetazolamide, was tested. L-NNA was intra-arterially infused (over 3 min) 30 min before acetazolamide was given. In Group LNNA-SIN-ACZ ($n = 6$) MABP was kept stable by infusion of the NO donor SIN-1. A time lag of 30 min after L-NNA and 15 min after the beginning of SIN-1 infusion was kept before acetazolamide was finally injected and rCBF studied for 30 min.

For statistical analysis within groups repeated measures analyses of variance (ANOVA) followed by the Student–Newman–Keuls-test (SNK) for multiple comparisons were performed. Differences between the experimental groups were determined by ANOVA followed by SNK. Differences were considered statistically significant at an error probability of $P < 0.05$.

MABP under baseline conditions was between 70 and 80 mmHg (Table 1), i.e. above the autoregulation threshold under chloral hydrate anesthesia [2]. The injection of acetazolamide alone (group ACZ) had no significant influence on MABP. The application of L-NNA in group LNNA-ACZ induced a statistically significant increase of MABP. With SIN-1 infusion in group LNNA-SIN-ACZ the MABP remained stable after L-NNA injection (Table 1).

There were no significant differences in pO_2 between the different experimental groups. Within the groups a statistically non-significant increase of p_aO_2 was seen after acetazolamide injection (Table 1).

Baseline arterial p_aCO_2 of the different experimental groups was without statistical difference and it was kept so by adaptation of the artificial ventilation.

Arterial pH decreased after acetazolamide injection. The inhibition of NOS alone had no effect on pH. If L-NNA was combined with acetazolamide, pH was significantly reduced compared to baseline, but did not vary much from pH seen during acetazolamide exposure alone (Table 1).

In group ACZ, rCBF increased to 158% baseline after acetazolamide ($P < 0.05$, Fig. 1). Additional measurements performed in short intervals revealed that the stimulating effect on rCBF starts as soon as 5 min after injection, and

Table 1
Physiological parameters (means ± standard error of mean)[a]

	baseline	L-NNA	L-NNA + SIN-1	+ acetazolamide
p_aCO_2 (mmHg)				
Group ACZ	44.3 ± 1.4	—-	—-	44.1 ± 2.3
Group LNNA-ACZ	38.9 ± 1.2	38.1 ± 1.1	—-	41.4 ± 3.1
Group LNNA-SIN-ACZ	41.1 ± 2.5	42.1 ± 2.6	42.1 ± 2.4	41.4 ± 2.5
p_aO_2 (mmHg)				
Group ACZ	126.9 ± 7.5	—-	—-	130.4 ± 6.4
Group LNNA-ACZ	108.4 ± 7.9	111.6 ± 5.5	—-	117.9 ± 10.0
Group LNNA-SIN-ACZ	100.8 ± 4.1	99.4 ± 1.7	99.4 ± 3.7	120.2 ± 4.1*
PH				
Group ACZ	7.35 ± 0.02	—-	—-	7.28 ± 0.03*
Group LNNA-ACZ	7.37 ± 0.03	7.32 ± 0.02	—-	7.28 ± 0.03*
Group LNNA-SIN-ACZ	7.34 ± 0.01	7.30 ± 0.01	7.31 ± 0.03	7.25 ± 0.01*
MABP (mmHg)				
Group ACZ	71.7 ± 8.0	—-	—-	75.7 ± 6.0
Group LNNA-ACZ	75.1 ± 4.4	109.3 ± 3.9*	—-	100.0 ± 9.3*
Group LNNA-SIN-ACZ	75.3 ± 2.3	100.0 ± 4.9*	67.7 ± 2.1	73.2 ± 4.2

[a] * significantly different compared to baseline ($P < 0.05$).

even 95 min after a single dose of acetazolamide this stimulatory effect can still be observed (data not shown).

The injection of L-NNA (group LNNA-ACZ) decreased rCBF significantly to 63.1 ± 10.1% from baseline 20 min after application (Fig. 1). The additional injection of acetazolamide after NOS-inhibition failed to stimulate rCBF in comparison to baseline and L-NNA (80.2 ± 6.0% baseline after 30 min).

The continuous infusion of the NO-donor SIN-1 (group LNNA-SIN-ACZ) reversed the L-NNA-induced MABP increase, without significant effect on rCBF as compared to baseline (103.5 ± 19.8% baseline): after L-NNA, rCBF decreased to 79.1 ± 3.6% baseline, and after L-NNA plus NO-donor SIN-1, flow reached baseline-level again (Fig. 1). NOS-inhibition in combination with substitution of basal NO levels by SIN-1 was followed by an impressive recovery of the acetazolamide-induced rCBF stimulation to 190.2 ± 30.8% baseline, a reaction comparable to the typical acetazolamide-response seen in group ACZ.

The activation of NO synthetases catalyzes the conversion of arginine to citrulline with production of NO [5,14]. The NO radical as final mediator activates guanylate cyclase and causes relaxation in smooth muscle induced by increased cyclic GMP levels [14] resulting in marked vasodilatation. Thus, after complete inhibition of all NOSs vasodilatation from any stimulus mediated by NO should no longer occur.

In the cerebral circulation, basal release of NO is involved in regulating resting CBF. The current data show that in group LNNA-ACZ inhibition of NOS by L-NNA had a

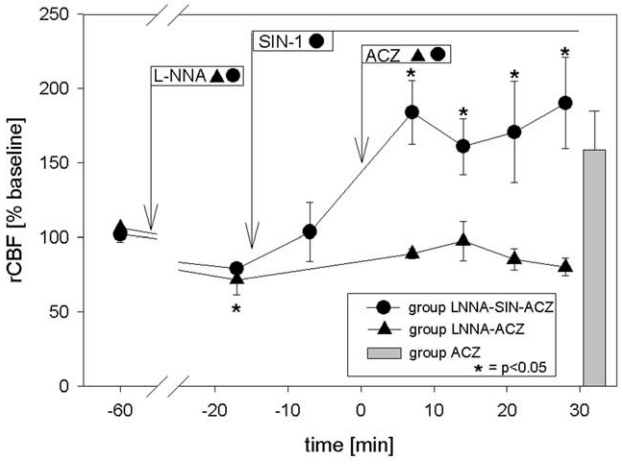

Fig. 1. Effect of acetazolamide on rCBF with and without SIN-1 controlled MABP after L-NNA. In group LNNA-ACZ injection of L-NNA caused a significant reduction of rCBF, which remained below baseline values after application of acetazolamide. In group LNNA-SIN-ACZ rCBF was also reduced by L-NNA though not statistically significant. Under SIN-1 infusion rCBF returned to baseline values without any additional effect on rCBF. Under NOS inhibition but in presence of a basal NO level the injection of acetazolamide caused a significant increase in rCBF, comparable to the effect seen in group ACZ after 35 min (shown as vertical grey bar). Data are means ± standard error of mean.

statistically significant influence on resting CBF which decreased. This is in perfect accordance with a recent publication by Lacza et al. [12], who found that the CBF reduction after NOS inhibition correlates with the basal CBF level. In group LNNA-SIN-ACZ the CBF decrease after L-NNA was statistically non-significant. It cannot be excluded that a more significant decrease would have become apparent if we had measured rCBF later than 30 min after inhibition, since Irikura et al. [9] found a minimal decrease after 30 min, and a more significant reduction 60 min after topical or intravenous application of L-NNA or L-NAME.

Pre-treatment with the NOS inhibitor L-NNA prevented the acetazolamide response. We have to conclude that NO-dependent mechanisms contribute to vasodilatation caused by acetazolamide. Kiss et al. [11] had proposed that NO is not involved in the acetazolamide reaction. In that study the NOS-inhibitor L-NMMA (N^G-monomethyl-L-arginine) was injected in a dosage not affecting MABP. However, the fact that the injection of the NOS-inhibitor did not lead to a significant increase in MABP could indicate that not all NOS had been blocked and therefore could still produce NO.

A vast body of information has accumulated proving that NO is also involved in hypercapnic cerebrovasodilatation [6]. The nearly complete abolishment of the CBF-response in the presence of NOS inhibitors led to the proposal that NO could participate in mechanisms in which extracellular acidosis induces smooth muscle relaxation. There are, however, findings which do not fit the hypothesis of a simple mediator cascade where extracellular acidosis causes NO production in so far undetermined cellular elements which then in turn leads to cGMP generation in smooth muscle cells [14], and hence, relaxation [6]. In the isolated basilar artery, hypercapnia increases vessel diameters but not cGMP, although hypercapnic vasodilatation can be reduced by inhibitors of NOS or cytosolic guanylate cyclase [18]. Furthermore, NOS inhibitors do not completely block the CO_2 response and are ineffective at pCO_2 levels > 100 mmHg [7]. Most importantly, however, a dose-dependent CO_2-reactivity can be restored by the application of a NO donor such as SIN-1 which just provides a stable basal background level of NO [8]. Hence, the degree of the CBF response depends on the carbon dioxide concentration in the tissue, whereas there appears to be no dependency on the NO concentration [7,8].

The current data are in perfect agreement with these observations. Our results demonstrate that indeed pre-treatment with L-NNA attenuated the acetazolamide effect. If however, a basal NO level was provided by a low-dose infusion of SIN-1, a NO donor, which in the concentration used, had no effect on resting rCBF (group LNNA-SIN-ACZ), NOS inhibition could no longer affect the acetazolamide reaction.

A similar modulatory role of NO has been described for the CBF response to elevated extracellular K^+ [1] and

neurovascular coupling in the somatosensory cortex [13]. In all four cases - CO_2, acetazolamide, K^+ and somatosensory stimulation - the sole presence of NO appears sufficient to permit the relaxation of vascular smooth muscle, which itself, however, is mediated by other factors. The nature of these factors remains to be clarified. In the case of acetazolamide local tissue acidosis might influence the open-state of a membrane ion-channel only if NO is present or intracellular cGMP has a given level. Future studies will have to concentrate on the interaction of acidosis with the smooth muscle membrane, with special focus on ion channels which can be modulated by acidosis and cGMP or NO.

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