Regulation of rat pial arteriolar smooth muscle relaxation in vivo through multidrug resistance protein 5-mediated cGMP efflux

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Xu, Hao-Liang, Vitaliy Gavrilyuk, Hailemariam M. Wolde, Verna L. Baughman, and Dale A. Pelligrino. Regulation of rat pial arteriolar smooth muscle relaxation in vivo through multidrug resistance protein 5-mediated cGMP efflux. Am J Physiol Heart Circ Physiol 286: H2020–H2027, 2004. —Multidrug resistance protein 5 (MRP5) has been linked to cGMP cellular export in peripheral vascular smooth muscle cells (VSMCs) and is widely expressed in brain vascular tissue. In the present study, we examined whether knockdown of MRP5 in pial arterioles [via antisense oligodeoxynucleotide (ODN) applications] affected nitric oxide (NO)/cGMP-induced dilatations. The antisense or (as a control) missense ODN was applied to the cortical surface ~24 h before study via closed cranial windows. The efficacy of the antisense vs. missense ODN in eliciting selective reductions in MRP5 expression was confirmed by analysis of MRP5 mRNA in pial tissue. Unexpectedly, in initial studies, a significantly lower maximal pial arteriolar diameter increase in the presence of the NO donor N·nitro-L-arginine. In the NO synthase (NOS)-inhibited rats, the maximal SNAP response was much higher in the antisense ODN-treated rats (35 vs. 48% diameter increase, respectively). It was suspected that this related to a reduced vascular smooth muscle cell sensitivity to cGMP due to prolonged exposure to increased intracellular cGMP levels elevated by overnight restriction of cGMP efflux. That postulate was supported by a finding of a diminished vasodilating response to the cGMP-dependent protein kinase-activating cGMP analog 8-p-chlorophenyl-s-cGMP in antisense vs. missense ODN-treated rats. To prevent desensitization, additional rats were studied in the presence of chronic basal cGMP generation via Nω-nitro-arginine. In the NO synthase (NOS)-inhibited rats, the maximal SNAP response was much higher in the antisense (62% increase) vs. the missense ODN (40% increase) group. A similar result was obtained when monitoring responses to the soluble guanylyl cyclase-activating drugs YC-1 and BAY 41–2272. Moreover, in the presence of NO inhibition, the normal SNAP-induced rise in periarachnoid cerebrospinal fluid cGMP levels, which reflects cGMP efflux, was absent in the antisense ODN-treated rats, a finding consistent with loss of MRP5 function. In conclusion, if one minimizes the confounding effects of basal cGMP production, a clearer picture emerges, one that indicates an important role for MRP5-mediated cGMP efflux in the regulation of NO-induced cerebral arteriolar relaxation.

GUANOSINE 3′,5′-cyclic monophosphate; nitric oxide; antisense oligodeoxynucleotide

GUANOSINE 3′,5′-cyclic monophosphate (cGMP) is an important mediator of cerebral vasodilation. Indeed, one of the enzymes responsible for cGMP biosynthesis, the soluble guanylyl cyclase (sGC), is considered to be the principal receptor for nitric oxide (NO), in its capacity to promote cerebral vascular smooth muscle (VSM) cell (VSMC) relaxation (16). Regulation of VSMC cGMP content and, thus, the level of cerebral VSM relaxation can occur either through modifying cGMP synthesis or cGMP cellular loss. In the latter case, this is likely to involve phosphodiesterase (PDE)-mediated cGMP hydrolysis (2). In addition, based on evidence from noncerebral tissues, VSMC cGMP loss may be regulated through ATP-dependent cGMP efflux (15). Exposure of cerebral arteries in vivo to a purported inhibitor (zaprinast) of the principal cGMP-hydrolyzing enzyme (PDE5) was shown to elicit a dose-dependent vasodilation (19). Ostensibly, this would seem to be support cGMP hydrolysis as an important control site for regulating cerebral vasodilation. However, recent findings indicate that zaprinast, along with several other selective PDE5 blockers, may also inhibit cGMP cellular efflux (12). Furthermore, cGMP rapidly appears in the cerebrospinal fluid (CSF) bathing pial vessels, following stimuli known to be accompanied by increased cGMP production within VSMCs (1, 23). Because cGMP generation only occurs intracellularly, the cGMP present in the extracellular compartment must arise via cellular export pathways. Given the magnitude of CSF cGMP elevations observed in vivo, the possibility exists that cGMP efflux may play an important role in the regulation of cerebral vasodilating function.

Multidrug resistance protein 5 (MRP5) has been linked to ATP-dependent cGMP efflux in peripheral tissues (12). The mRNA for this protein is well expressed in brain and vascular tissues (4, 32). Nevertheless, to date, the role of cGMP efflux in controlling cerebrovascular tone has not been examined. In this study, we examined, in male rats, whether knockdown of MRP5 in pial arterioles [using antisense vs. missense oligodeoxynucleotide (ODN) topical applications, given 24 h before study] affected NO- and cGMP-mediated dilatations. In addition, initial findings indicated a diminished response to NO in antisense ODN-treated rats. This suggested the possibility that elevations in VSMC cGMP levels of several hours or longer, presumably resulting from interfering with efflux of basally produced cGMP, may desensitize cerebral VSMCs to the vasodilating actions accompanying subsequent applications of sGC-stimulating agents. Thus additional rats were evaluated, where basal cGMP generation was restricted via chronic NO synthase (NOS) inhibition in advance of ODN treatment.

METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago. Age-matched male Sprague-Dawley rats (300–400 g) were used (supplied by Charles River; Wilmington, MA). Pial arteriolar reactivities were evaluated using a closed window and intravital microscopy system (29). The windows were placed ~24 h before study. The procedure for “chronic” placement of cranial windows in experiments utilizing topical...
applications of ODNs was described in a previous paper from our laboratory (29). Three hundred microliters of a solution containing either 5 μM MRP5 antisense (5′-AAATCCCTTCCATCCAC-3′) or 5 μM missense (5′-CTCACTTCTCACTTTTC-3′) ODN was injected into the space under the cranial window. Six bases (3 at the 5′-end and 3 at the 3′-end) were phosphorothioated so as to minimize nuclease-mediated ODN breakdown. On the day of study, after anesthesia induction with halothane and paralysis (curare), the rats were tracheotomized and mechanically ventilated. Bilateral femoral arterial and venous catheters, used for arterial blood gas measurement and drug infusion, were inserted under continuous anesthesia with 0.8% halothane-70% N2O-30% O2. After catheterization, the rat was placed in a head holder, and the cranial window inflow, outflow, and intracranial pressure-monitoring cannulas were connected. Halothane was discontinued, and 10 μg/kg fentanyl bolus was given intravenously. Anesthesia was maintained during the study with fentanyl (25 μg·kg⁻¹·h⁻¹ iv) and 70% N2O-30% O2. The space under the window was filled with artificial cerebrospinal fluid (aCSF, pH = 7.35) that was equilibrated with a gas mixture consisting of 20% O2-5% CO2 with a balance of N2. The aCSF solution was suffused at 0.5 ml/min and controlled at 37°C. Body temperature was maintained at 37°C with a servocontrol heating pad, and mean arterial pressure and intracranial pressure were monitored continuously during the experiment. Vascular reactivity was assessed by measuring the diameters of pial arterioles (300 μm). The vessels were viewed by video microscopy and measurements made using a calibrated video microscaler (see Ref. 29). In all experiments, the initial diameter measurements were made >1 h after halothane and after 40 min of drug-free aCSF suffusion. The pial arteriolar response to 3 min of hypercapnia [arterial PCO₂ (Paco₂) = 65–70 mmHg] was then examined. The results were expressed in terms of CO₂ reactivity (percent diameter increase/mmHg Paco₂ change).

In initial experiments, dose-dependent pial arteriolar reactivities to suffusions of an NO donor [5-nitroso-N-acetyl-penicillamine (SNAP) at 0.01, 0.1, 0.3, 1.0, and 10 μM] were assessed. Diameter measurements from three separate pial arterioles were obtained (averaged) after 5-min suffusion at each concentration. In all cases, comparisons were made between the antisense and missense ODN-treated rats. Because initial findings indicated a lower response to SNAP in MRP5 antisense vs. missense ODN-treated rats, we were compelled to include additional experimental groups. We suspected that interfering with cGMP influx under basal conditions, via knockdown of MRP5, led to a prolonged increase in intracellular cGMP levels before study, resulting in desensitizing of the vessels to the effects of subsequent acute increases in cGMP elicited by applications of the NO donor. We examined whether the diminished reactivity to acute endogenous cGMP elevations resulted from reduced cGMP-dependent protein kinase (PKG) function (e.g., Ref. 20), as opposed to cGMP-induced, PKG-independent feedback inhibition of SGC activity (see, for example, Ref. 27). To that end, evaluations of vasodilating responses to the cell-permeable, PDE-resistant, highly selective PKG activator 8-p-chlorophenylthio-cGMP (8-pCPT-cGMP) were performed in antisense and missense ODN-treated animals. After the initial CO₂ response evaluation, 8-pCPT-cGMP was suffused at 1.0, 3.0, 10, 30, and 100 μM concentrations (5 min at each level). One additional experimental manipulation was instituted for the purpose of minimizing basal cGMP generation in advance of ODN treatments. Thus rats chronically treated with Nω-nitro-l-arginine (l-NNA, at 100 mg·kg⁻¹·day⁻¹ iv over 4 days) were studied. We previously found that this treatment regimen produced a virtually complete suppression of brain NOS activity in rats (25). The protocol utilized in the initial experimental series was repeated. That is, SNAP dose-response evaluations were carried out in antisense vs. missense ODN-treated rats. In three animals from each of the ODN-treated groups, periarcachondal CSF was collected from the outflow cannula during SNAP dose-response evaluations and analyzed for cGMP content using a kit (Research Biochemicals). In separate animals, we again examined the dose-related reactivities of pial arterioles to 8-pCPT-cGMP, adhering to the same protocols as given earlier. Finally, we evaluated pial arteriolar reactivities to two SGC activators, YC-1 and BAY 41–2272. Thus, in both antisense and missense ODN-treated rats, suffusions of YC-1 (at 3.0, 10, and 30 μM) or BAY 41–2272 (at 0.01, 0.1, 1.0, and 10 μM) were performed. These evaluations were done to confirm that the changes in vascular reactivity observed were indeed cGMP related. The YC-1 and BAY 41–2272 were prepared in DMSO, and subsequent dilutions were made with aCSF. The final DMSO concentrations were, therefore, 0.03, 0.1, and 0.3%, at the three YC-1 doses, and ranged from 0.0001 to 0.1% for BAY 41–2272. We have established, in preliminary experiments, that within the above concentration range, DMSO does not affect pial arteriolar caliber. However, a full dose-response curve for YC-1 could not be generated because to achieve the higher concentrations of YC-1 required, we would have needed to expose the pial arterioles to the potentially confounding influences from DMSO concentrations of ≥1%.

Four animals each from the antisense and missense ODN-treated groups were analyzed for MRP5 mRNA expression, and (as a control) MRP4 expression, in pial tissue removed from under the cranial windows. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also analyzed to provide an internal reference. The tissue was obtained on the day after ODN application (see Refs. 29, 30) and prepared for real-time quantitative RT-PCR evaluations utilizing procedures similar to those described in recent publications from our laboratory (6, 8). In brief, RNA samples (1 μg) were converted to cDNA using random hexamer primers and amplified in a 20-μl reaction under stringent annealing temperatures in a Corbett Rotor-Gene real-time PCR machine, in the presence of SYBR Green. Relative mRNA concentrations were calculated from the takeoff points of the PCR reactions, using the comparative 2⁻ΔΔCt method, where Ct is the takeoff point (13). Cerebral cortical tissue from an untreated rat was used as a reference sample. Correct product synthesis was confirmed by agarose gel electrophoresis and melting curve analysis. For MRP5, the forward primer sequence was 5′-CCCCCTTCTCTTTGTCAACCTTGTG-3′ and the reverse was 5′-TGGGGAGGAGCCTTGTTG-3′, with an expected product length of 232 bp. For MRP4, the forward primer was 5′-GGACACTGAATCAGCACAACTCTGG-3′, and the reverse was 5′-GTCGCTGCAATGATGCCGTTCA-3′, which yield a 229-bp product. The primers used for GAPDH detection were 5′-GCCAATGATGACATCAAGAG-3′ and 5′-TCCAGGGGTTTCTACTCTTGG-3′, which yield a 264-bp product.

All drugs and reagents were obtained from Sigma (St. Louis, MO) and dissolved in aCSF unless otherwise stated. The 8-pCPT-cGMP was from Biolog (La Jolla, CA), and BAY 41–2272 was obtained from Calbiochem (San Diego, CA). The ODNs were obtained from Sigma Genosys (St. Louis, MO). Values are presented as means ± SE. Comparisons of arteriolar diameter values within groups were made using one-way repeated-measures ANOVA, combined with a post hoc Student-Newman-Keuls analysis. Analyses of diameter changes between groups were made using Student’s t-test. A P value of <0.05 was considered as significant.

RESULTS

In all experiments, the physiological variables were within normal limits. That is, with the exception of a brief period of imposed hypercapnia, Paco₂, pH, and MABP in these groups did not show any significant differences when comparing initial and final values over the course of the experiments. The values measured in animals in the absence and presence of chronic l-NNA treatment are presented in Table 1, along with initial pial arteriolar diameters. Not included in Table 1 are the arterial Po₂ values, which were maintained >100 mmHg in all rats studied. It should be noted that MABP values in the rats subjected to chronic NOS inhibition, not surprisingly, were significantly higher than in their untreated counterparts.
MRP5 message in pial tissue of rats treated with antisense vs. missense ODN. Effective antisense ODN-induced knockdown of specific proteins should not only be associated with reductions in protein expression but also reduced mRNA as well. This is because antisense ODN binding “marks” the target mRNA sequence for destruction by RNase-H (5). The real-time RT-PCR-based mRNA analysis was the only technique used to confirm the effectiveness of the ODN treatment in the present study simply because the inadequacy of currently available MRP5 antibodies (see Ref. 4) makes immunoblot or immunocytochemistry-based protein analyses untenable. In each sample analyzed, the takeoff points Ct were obtained for MRP5, MRP4, and GAPDH. Relative mRNA expression was calculated using the 2-ΔΔCt method (13). Cerebral cortical tissue obtained from an untreated rat was used as a reference sample. No appreciable variations in GAPDH mRNA expression were observed across samples (not shown). On the other hand, a substantial difference in the relative levels of MRP5 mRNA expression was observed when comparing antisense and missense ODN-treated tissues. As summarized in Fig. 1, pial tissue MRP5 mRNA expression was reduced by ~60% in the antisense relative to the missense treatment groups, thereby confirming the effectiveness of the antisense ODN treatment. The selectivity of the antisense ODN treatment was demonstrated by the finding of no changes in the relative expression of MRP4 mRNA (Fig. 1).

Effects of MRP5 knockdown on pial arteriolar responses in rats with normal NOS activity. In the NOS-normal rats, based on nonlinear regression analysis (Prism), a significantly lower pial arteriolar maximal SNAP reactivity was seen in the antisense vs. the missense ODN-treated rats (35 vs. 48%, respectively; Table 2, Fig. 2A). No significant differences in the EC50 values were observed (Table 2). These results suggested the possibility that pial arteriolar VSMCs, in the presence of MRP5 knockdown, may have become desensitized to subsequent NO-stimulated increases in cGMP concentrations. We next examined whether that diminished reactivity occurred via some process acting at the level of sGC or more distally, at the level of PKG or a site downstream from PKG. Significant lower responses to the selective PKG activating agent 8-pCPT-cGMP were observed at all but the lowest dose of 8-pCPT-cGMP in the rats treated with the antisense, compared with missense, ODN (Fig. 3A). The values of the maximal responses, as derived from nonlinear regression analysis (Table 3), were 25.9 ± 2.1% (antisense) and 38.9 ± 1.4% (missense). No significant differences in the EC50 values were observed (Table 3). The similarities in the reductions in vascular reactivities to NO and the PKG activator indicate that the reduced response to NO associated with MRP5 knockdown probably relates to a process occurring at some downstream site in the cGMP signal transduction pathway (perhaps PKG) rather than at the site of cGMP synthesis.

Table 2. SNAP dose-response curve properties

<table>
<thead>
<tr>
<th>Group</th>
<th>EC50, μM</th>
<th>Maximum Dilation, %Pial Arteriolar Diameter Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS-normal</td>
<td></td>
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</tr>
<tr>
<td>Missense</td>
<td>0.41 ± 0.06</td>
<td>47.6 ± 0.5</td>
</tr>
<tr>
<td>Antisense</td>
<td>0.27 ± 0.05</td>
<td>35.0 ± 3.9*</td>
</tr>
<tr>
<td>Chronic l-NNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missense</td>
<td>0.29 ± 0.04</td>
<td>39.7 ± 3.3</td>
</tr>
<tr>
<td>Antisense</td>
<td>0.30 ± 0.05</td>
<td>61.6 ± 7.4*</td>
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</tbody>
</table>

All values are means ± SE. EC50 and maximum dilation values derived from nonlinear regression analysis. SNAP, S-nitroso-N-acetylpenicillamine. *P < 0.05 vs. missense.
Effects of MRP5 knockdown on pial arteriolar responses in chronically NOS-inhibited rats. Opposite to the results seen in the rats with normal NOS activity, the maximal SNAP response in the NOS-inhibited rats (Table 2, Fig. 2B) was much higher (P < 0.05) in the antisense (62% diameter increase) compared with the missense group (40% increase). On the other hand, no significant difference in the EC50 values of the antisense- vs. missense-treated rats was observed (Table 2). Knockdown of MRP5 affected pial arteriolar dilating responses to the sGC-activating drugs YC-1 and BAY 41–2272 in a manner similar to that observed when assessing SNAP responses in NOS-inhibited rats. That is, when comparing the pial arteriolar dilations to YC-1 in the antisense- vs. missense-treated rats, a greater response was observed in the antisense group at all three doses (Fig. 4A). Measurements of pial arteriolar reactivity to suffusions of the purportedly more potent NO-independent sGC activator BAY 41–2272 (22) also revealed a more pronounced response in the antisense ODN group, at all but the lowest dose applied (Fig. 4B).

Table 3. 8-pCPT-cGMP dose-response curve properties

<table>
<thead>
<tr>
<th>Group</th>
<th>EC50, μM</th>
<th>Maximum Dilation, %Pial Arteriolar Diameter Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS normal</td>
<td>9.2 ± 2.3</td>
<td>38.9 ± 1.4</td>
</tr>
<tr>
<td>Missense</td>
<td>12.5 ± 3.6</td>
<td>25.9 ± 2.1*</td>
</tr>
<tr>
<td>Antisense</td>
<td>11.1 ± 2.8</td>
<td>29.2 ± 0.1</td>
</tr>
<tr>
<td>Chronic L-NNA</td>
<td>8.0 ± 1.5</td>
<td>41.1 ± 2.3*</td>
</tr>
</tbody>
</table>

All values are means ± SE. 8-pCPT-cGMP, 8-p-chlorophenylthio-cGMP. EC50 and maximum dilation values derived from nonlinear regression analysis. * P < 0.05 vs. missense.
the results with SNAP, the pial arteriolar response to the PKG activator 8-pCPT-cGMP was found to be enhanced in the antisense- vs. missense-treated groups of chronically NOS-inhibited rats (Fig. 3B). That is, a more pronounced vasodilation was seen at the two highest doses applied, with the maximal responses (as derived from nonlinear regression analysis) being 41% and 29% in the antisense vs. missense groups, respectively. No differences in EC50 values were noted (Table 3).

Periarachnoid CSF cGMP changes. In several rats from each of the chronically NOS-inhibited ODN treatment groups, periarachnoid CSF was collected from the outflow cannulas before and during SNAP dose-response evaluations and analyzed for cGMP content. The results of that analysis are summarized in Fig. 5. Under control conditions (missense ODN treatment), increases in CSF cGMP content were observed starting at 0.1 μM SNAP, leveling off at ~2 times the baseline level (~330 pM cGMP) at 1.0–10 μM SNAP. On the other hand, in the antisense ODN-treated group, no increases from the baseline level of CSF cGMP (~340 pM) were seen.

CO2 reactivity. In the presence of normal NOS activity, pial arteriolar dilations during exposure to hypercapnia were significantly diminished in rats treated with the MRP5 antisense ODN compared with their missense-treated counterparts. This was reflected in a ~40% lower CO2 reactivity (Fig. 6). In rats chronically treated with L-NNA, CO2 reactivity was ~20% higher in the antisense vs. missense ODN group, although that difference was not statistically significant. Similar to the findings with SNAP and the PKG-activating drug 8-pCPT-cGMP, the former results also seem to imply a diminished sensitivity to cGMP-elevating and PKG-activating stimuli (in this case, hypercapnia; see Ref. 16), arising from MRP5 knockdown in the presence of normal levels of resting NO and cGMP generation. It merits some mention that in the missense-treated group, the statistically significant reduction (40%) in CO2 reactivity in chronically NOS-inhibited vs. control rats was somewhat lower than that previously observed in rats after acute NOS inhibition. However, this is consistent with the results of previous studies showing that prolonged loss of NO generating function is associated with a compensatory increase...
in the contributions from non-NO factors in promoting hypercapnia-induced dilation of cerebral vessels (11, 24).

**DISCUSSION**

The present investigation involved the use of antisense ODN methodologies to examine the role of MRP5 in the regulation of cerebral vasodilating function. Evidence derived from these experiments suggested that, presumably via its capacity to regulate cGMP efflux from VSMCs, MRP5 plays an important role in NO/cGMP-induced pial arteriolar relaxation. Thus cGMP efflux can be added to the list of mechanisms that may participate in the regulation of cGMP-mediated vasodilation. Others include contributions from sGC, PDEs (especially PDE5), and PKG (16).

One potential limitation associated with the protocols employed in this investigation relates to the use of the antisense ODN approach. When designing the MRP5 antisense ODN, care was taken to avoid four contiguous G residues, which may impart a secondary structure that can prevent hybridization. Additionally, the ODN was modified with end-phosphorothioate (i.e., 3 bases at the 5' end and 3 bases at the 3' end), which protects the oligonucleotide from rapid nuclease-mediated destruction, while preserving its ability to promote RNase-H-mediated cleavage of the target mRNA (9). A BLAST analysis was also performed to confirm the specificity of the sequence. We then established the effectiveness of the antisense ODN by performing mRNA analyses of pial tissue from antisense and missense ODN-treated rats using a quantitative real-time PCR technique. The lack of suitable MRP5 antibodies precluded any protein expression analyses. The PCR analysis revealed, for the optimized ODN sequences used in these experiments, a 60% lower MRP5 mRNA expression in pial tissue harvested from antisense vs. missense ODN-treated rats. This is generally consistent with results from previous studies in our laboratory, where the cranial window approach was used to target other proteins for ODN-mediated knockdown in pial vessels (29, 30). Furthermore, the selectivity of antisense over missense ODN treatment in the current study was suggested by additional results showing an absence of any differences in MRP4 message.

Another limitation in this investigation relates to the fact that our experimental model does not permit any direct analysis of changes in cGMP efflux from pial VSM. In the present study, we compared cGMP levels measured in samples of cranial window CSF effluent (periaqueductal CSF) in antisense vs. missense ODN rats. To simplify this comparison, we only measured SNAP-induced cGMP changes in chronically NOS-inhibited animals. Although there is little question that the cGMP appearing in the CSF originates intracellularly and is extruded to the extracellular compartment (presumably via the MRP5-related system), the specific cellular source cannot be readily established. Indeed, MRP5 transcripts have been identified in cultures of neurons and astrocytes (10), as well as in cerebral microvessels (32) and pial tissue (current study). Because our CSF samples are collected over fairly short time intervals (minutes), it is quite likely that only sGC-containing cells that are in contact with, or in close proximity to, the periaqueductal CSF are capable of appreciably contributing to changes in CSF cGMP content. The sGC-expressing cells in closest contact to the periaqueductal CSF are pial VSM and astrocytes (via the glia limitans), followed by vascular endothelium and, finally, neurons. As we reported in an earlier study (23), acute increases in NO result in a rapid rise in periaqueductal CSF cGMP levels. This was also shown in the present study. The rapid response, coupled with the fairly high sGC content of VSMCs and astrocytes (e.g., Ref. 21), makes those cells the most likely sources of the cGMP appearing in the cortical subarachnoid CSF. The one notable result of this analysis was that the cGMP increases normally measured during SNAP exposure were blocked in the antisense group. Viewed another way, the NOS-inhibited, antisense ODN-treated group displayed the greater vasodilating response to SNAP, even though the CSF cGMP changes were clearly more restricted. Because CSF flow and collection rates were constant, the only factors that could account for a diminished CSF cGMP elevation in the presence of a sGC-activating agent like SNAP would be enhanced PDE activity, reduced sGC activity, or a diminished cGMP efflux. Yet, there are no plausible physiological circumstances we can envisage where increased cGMP hydrolysis or reduced synthesis could occur along with an enhancement of the vasodilating response, unless VSMC sensitivity to cGMP was substantially augmented. At first glance, the results showing an enhanced reactivity to the PKG-activating drug 8-pCPT-cGMP in chronically NOS-inhibited rats might be taken as evidence of a heightened cGMP sensitivity. As will be discussed later, VSMC sensitivity to cGMP may, in fact, be affected by MRP5 knockdown; but this relates to reductions in VSMC sensitivity to cGMP. However, because cGMP analogs have been reported to compete with cGMP for cellular export sites (3), it is conceivable that the enhanced pial arteriolar response to 8-pCPT-cGMP in the NOS-inhibited MRP5-knockdown (vs. control) group relates to a reduced analog extrusion from VSMCs. Thus, although we cannot completely discount alternative explanations, it seems more likely that the disappearance of the SNAP-induced cGMP increase in the CSF was due to suppression of cellular cGMP extrusion.

Although the present results suggest that cGMP efflux may be an important site for regulating VSM relaxation in response to increases in NO/cGMP in cerebral arterioles, recent findings in noncerebral, nonvascular cell systems indicated a modest to minimal role for MRP5 (and MRP4) in regulating intracellular cGMP levels (26). In contrast to the low cGMP affinity transport system suggested by Wielinga et al. (26), Jedlitschky et al. (12), using another cellular preparation, reported that MRP5 mediates a high-affinity cGMP export. These disparate findings seem to suggest that the relative role played by cGMP efflux in controlling intracellular cGMP levels may vary according to tissue and cell type. Based on the apparently appreciable sensitivity of pial arterioles to MRP5 knockdown, we would speculate that high-affinity cGMP export system is present in cerebral arteriolar VSM.

Additional data obtained in the present study demonstrated that MRP5 knockdown, in the face of normal basal NO and cGMP generation, reduced arteriolar reactivity to the sGC-independent agent 8-pCPT-cGMP at least as much, if not more, than the response to SNAP. This finding bears some similarity to results reported for endothelial NOS (eNOS)-overexpressing transgenic mice (31), where the reactivities of aorta to the vasorelaxant actions of the NO donor SNAP and the cGMP analog 8-bromo-cGMP were found to be diminished. More-
over, as we found, that repressed vascular reactivity could be prevented by chronic NOS inhibition. Thus prolonged elevations in basal NO and cGMP levels can result in diminished reactivities of arteries/arterioles to subsequent elevations in cGMP, a change that can be reversed by limiting resting cGMP generation. Because both sGC-dependent and -independent PKG-activating stimuli (i.e., SNAP, hypercapnia, and 8-pCPT-cGMP) were affected in our study, the mechanism responsible for the repressed vascular reactivity cannot be confined to cGMP-mediated inhibition of sGC activity (see, for example, Refs. 7, 27) and must include actions at or distal to PKG (e.g., Ref. 18). Furthermore, the reduced reactivity to NO/cGMP cannot be attributed to cGMP-mediated potentiation of PDE5 activity (14). If that were the case, then vasodilating responses to the PDE5-insensitive cGMP analogs should be affected to a lesser degree, or not at all, compared with NO donors.

In eNOS transgenic mice (31), both a decrease in PKG expression and a reduced sGC activity were noted. Others have also reported reductions in VSM PKG expression after prolonged exposure to exogenous NO (20). However, it is not clear whether cGMP elevations contributed to that effect (18). Nevertheless, whatever the mechanism involved, it would appear that prolonged elevations in cGMP, in cerebral VSMCs, are just as likely to interfere with cGMP actions as with cGMP synthesis. Finally, even though intracellular cGMP concentrations in pial VSM could not be measured, increased intracellular cGMP levels under baseline conditions, in the antisense-treated and NOS-normal rats of the current study, can be inferred based on the similarity of our results to the transgene mouse data, where a diminished vascular reactivity to NO/ cGMP, which could be reversed by chronic NOS inhibition, was seen.

Present findings indicated that only when the confounding effects of basal cGMP generation were minimized (via chronic NOS inhibition) could the role of MRPS in the regulation of cerebral vasodilation be revealed. Thus MRPS knockdown was associated with enhanced pial arteriolar reactivities to sGC activation by NO, as well as sGC activation via agents that can act independently from NO, i.e., YC-1 and BAY 41–2272. However, there is evidence that the principal effect of YC-1 is to sensitize sGC to the actions of NO, more so than directly stimulating sGC (17). On the other hand, BAY 41–2272 has been shown to possess a substantial sGC stimulatory capacity in the absence of NO (22). Nevertheless, despite the likelihood that SNAP, YC-1, and BAY 41–2272 activate sGC via somewhat different mechanisms, they do exhibit a shared action, i.e., to increase cGMP production and intracellular cGMP levels. Moreover, that common action does not include blocking cGMP hydrolysis, because BAY 41–2272 is devoid of PDE5 inhibitory effects (22). As a cautionary note, the above evaluations were performed in animals with chronically elevated arterial pressures. Thus one cannot completely ignore the possibility of a hypertension-related modulatory influence on the results obtained. Nevertheless, because the vasodilating responses to all three agents were specifically enhanced in the presence of MRPS antisense vs. missense ODN exposure, those findings remain, at the least, strongly suggestive of the importance of MRPS-related cGMP efflux in the control of pial arteriolar tone in vivo.

In conclusion, present findings indicate that MRPS-linked cGMP efflux can be added to the list of mechanisms controlling NO-induced relaxation in cerebral VSMCs. Furthermore, we did not achieve a complete knockdown in MRPS transcrip- tion. Whether this translates to an equivalent incomplete knockdown in MRPS protein expression and function is not known. Assuming that this might be the case, it is quite possible that the results of this study actually underestimate the relative role of cGMP efflux in controlling cerebral VSMC relaxation.

GRANTS

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