Agonist-specific differences in mechanisms mediating eNOS-dependent pial arteriolar dilation in rats


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Xu, H.-L., D. L. Feinstein, R. A. Santizo, H. M. Koenig, and D. A. Pelligrino. Agonist-specific differences in mechanisms mediating eNOS-dependent pial arteriolar dilation in rats. Am J Physiol Heart Circ Physiol 282: H237–H243, 2002.—Nitric oxide (NO), derived from the endothelial isoform of NO synthase (eNOS), is a vital mediator of cerebral vasodilation. In the present study, we addressed the issue of whether the mechanisms responsible for agonist-induced eNOS activation differ according to the specific receptor being stimulated. Thus we examined whether heat shock protein 90 (HSP90), phosphatidylinositol-3-kinase (PI3K), and tyrosine kinase participate in ACh- versus ADP-induced eNOS activation in cerebral arterioles in vivo. Pial arteriolar diameter changes in anesthetized male rats were measured during sequential applications of ACh and ADP in the absence and presence of the nonselective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME), the neuronal NOS (nNOS)-selective inhibitor ARR-17477, the HSP90 blocker 17-(allylamino)-17-demethoxygeldanamycin (AAG), the PI3K inhibitor wortmannin (Wort), or the tyrosine kinase blocker tyrphostin 47 (T-47). Only NOS inhibition with L-NAME (not ARR-17477) reduced ACh and ADP responses (by 65–75%), which suggests that all of the NO dependence in the vasodilating actions of those agonists derived from eNOS. Suffusions of AAG, Wort, and T-47 were accompanied by substantial reductions in ACh-induced dilations but no changes in the responses to ADP. These findings suggest that muscarinic (ACh) and purinergic (ADP) receptor-mediated eNOS activation in cerebral arterioles involve distinctly different signal transduction pathways.

heat shock protein 90; muscarinic; endothelial nitric oxide synthase; phosphatidylinositol-3-kinase; purinergic; tyrosine kinase

The important role of the endothelial isoform of nitric oxide synthase (eNOS or NOS III) in the regulation of cerebral vascular tone has long been recognized. For many years, it was thought that Ca$^{2+}$-induced binding of calmodulin (Cam) to eNOS was the major (if not the only) trigger for eNOS activation. Accumulating evidence indicates that eNOS activity is regulated by mechanisms that extend well beyond a simple relationship with the intracellular Ca$^{2+}$ content ([Ca$^{2+}]_{i}$) of endothelial cells. Indeed, eNOS activity can be modulated through changes in Ca$^{2+}$ sensitivity and avidity of Cam binding. A key element in Cam interactions with eNOS is the plasma membrane structural protein caveolin-1 (Cav-1), which is a 22- to 24-kDa protein that binds to and blocks eNOS activity (10, 25, 32). Upon Ca$^{2+}$-induced Cam binding to eNOS, Cav-1 is displaced and the enzyme is activated. Recent findings point to the existence of a variety of factors regulating the interactions between eNOS, Cav-1, and Cam to the extent that the specific mechanisms responsible for eNOS activation have taken on an added level of complexity and may differ according to the stimulus applied (6, 11).

The potentiation of eNOS activity is sometimes classified on the basis of whether the stimulus results in an increase in [Ca$^{2+}]_{i}$ in endothelial cells. A number of vasodilating stimuli have been reported to lead to increased eNOS activity in the absence of Ca$^{2+}$ changes or to an enhanced sensitivity of eNOS to Ca$^{2+}$-Cam. Although multiple mechanisms have been proposed, experimental findings have identified several principal candidates. These include association of eNOS with heat shock protein 90 (HSP90) (9, 33); increased activity of pathways regulating the phosphorylation of specific serine/threonine sites on the eNOS homodimer [e.g., those mediated by Akt subsequent to its phosphatidylinositol-3-kinase (PI3K)-related phosphorylation (20, 26)]; and tyrosine phosphorylation of eNOS-regulating proteins (5, 15). There is little available information regarding the potential contributions from the aforementioned factors to agonist-induced eNOS activation in cerebral vessels. Nevertheless, findings by Kitazono and coworkers (17, 18) suggest an important role for PI3K and tyrosine kinase in ACh-mediated dilation of rat large cerebral arteries in vivo. On the other hand, the role of HSP90 in eNOS-mediated dilation of large cerebral arteries remains unclear with data favoring HSP90 participation in basal but not agonist-stimulated eNOS-mediated relaxation (16). However, nothing is known about the influence of these factors in the eNOS activation and dilation of cerebral arterioles elicited by receptor agonists.

In the present study, we examined whether HSP90, PI3K, and tyrosine kinase participate in agonist-induced eNOS activation in cerebral arterioles in vivo.
Using an established intravital microscopy model for monitoring pial arteriolar diameter changes, experiments were designed to address two principal questions. First, does inhibition of HSP90, PI3K, or tyrosine kinase affect the pial arteriolar diameter increases elicited by agonists known to activate eNOS in these vessels? Second, is participation of HSP90, PI3K, and tyrosine kinase a general feature of agonist-induced eNOS stimulation, or do the contributions from these proteins depend on the specific receptor being activated? To that end, we compared the vasodilations elicited by muscarinic (ACh) and purinergic (ADP) receptor agonists before and after exposure to 1) the NOS inhibitor Nω-nitro- L-arginine methyl ester (L-NAME), 2) the HSP90 blocker 17-(allylamino)-17-de-methoxygeldanamycin (AAG), the tyrosine kinase blocker tyrphostin 47 (T-47), or the PI3K inhibitor wortmannin (Wort). AAG and ADP were chosen because, in addition to activating distinctly different receptor populations, they represent the two agonists displaying the greatest endothelium-derived NO dependency in adult rodent pial arterioles (23, 28, 29, 40).

METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (body wt 250–350 g) were used. Animals were anesthetized with halothane, and paralysis was induced with curare (1 mg/kg body wt). Tracheotomy was performed and animals were ventilated on a rodent respirator. Bilateral femoral arterial and venous catheters were then inserted during administration of 0.8% halothane-70% N2O-O2 balance O2 anesthesia. The rat was placed in a prone position and the head was immobilized. A craniotomy (~10 mm in diameter) was performed over the midline of the skull, and the dura was carefully removed while the sagittal sinus was kept intact. An acrylic cranial window (11 mm in diameter and 1 mm in thickness) with inflow, outflow, and intracranial pressure (ICP) monitoring ports was fixed to the skull with cyanoacrylate gel. The halothane was discontinued and a loading cannula was inserted into the aCSF (see Ref. 28). The aCSF was perfused at 0.5 ml/min and was maintained at 37°C with a partial pressure of O2 (P O2) of 40–45 mmHg, a partial pressure of O2 P O2 of 50–60 mmHg, and pH 7.35. The ICP was controlled at 5–10 mmHg by adjusting the height of the outflow cannula. The reaction of 25- to 50-μm-diameter pial arterioles on the exposed cortical surface was assessed via measurement of diameter changes. A microscope (Nikon) and color, charge-couple device camera (Sony) arrangement was equipped with an epi-illumination dark-field system (Fryer; Carpentersville, IL). Images were displayed on a video monitor. Measurements of vessel diameters were made using a calibrated video microscaler (Optech).

The rats were divided into six experimental groups: 1) nonselectively NOS inhibitor (L-NAME (n = 4)); 2) neuronal NOS (nNOS) inhibited with 0.3 mM ARR-17477 (n = 4); 3) HSP90 inhibited using 10 μM AAG (n = 4); 4) tyrosine kinase inhibited via 1.0 μM T-47 (n = 4); 5) PI3K inhibited with 0.01 μM Wort (n = 4); and 6) vehicle/time control (n = 3). The concentrations applied for the NOS inhibitors (30), AAG (16, 41), T-47 (18), and Wort (17) were chosen based on information from the literature and lie within the respective selectivity ranges. With the exception of the drug (vehicle) treatments, the experimental protocols were identical in each group (e.g., inhibiting agents were all applied topically). Initial diameter measurements were made after a 30- to 40-min period of cortical suffusion with drug-free aCSF (initiated at 1 h post halothane). Hypercapnia (PaCO2 ≈65 mmHg) was then imposed for 3 min, and the CO2 “reactivity” of the arterioles was calculated (as percent diameter increase per mmHg of CO2 change). At 10 min after the return to normocapnia, a suffusion of the NO donor S-nitroso-N-acetylpenicillamine (SNAP), was initiated at concentrations of 0.1 and then 1.0 μM (for 5 min at each level). Baseline diameters were restored via 10–15 min of suffusion with drug-free aCSF. This was followed by suffusion with ADP (10 then 100 μM; 5 min at each level) before return to baseline for 15 min and subsequent suffusion with ACh (10 then 100 μM; 10 min at each level). Fifteen minutes after the return to baseline, inhibitor suffusions were initiated and the sequence of vasodilator exposures was subsequently repeated. The duration of the NOS inhibitor suffusions (L-NAME and ARR-17477) before beginning the repeat exposure to the series of vasodilating stimuli was 1 h. The rest of the drugs were suffused for ~20 min before initiation of the repeat sequence and they remained in the aCSF thereafter. L-NAME and ARR-17477 were prepared in aCSF, and the other drugs were prepared in equal parts of ethanol and DMSO. Dilutions were subsequently made in aCSF. The stock drug solution was prepared so that the final aCSF contained at most one part per thousand of ethanol-DMSO, which is the concentration of vehicle used in the time-control evaluations. In all experiments, mean arterial blood pressure (MABP) was continuously monitored, and arterial blood samples were taken at 30-min intervals for measurement of P O2, P CO2, and pH using a Radiometer Copenhagen (model ABL 520) blood gas/pH analyzer. The rats were euthanized with a halothane overdose at the termination of the experiment.

Statistical comparisons of pial arteriole diameter changes between groups in the NOS inhibitor treatment experiments were made using a two-way ANOVA combined with a post hoc Tukey analysis. For comparisons of diameter values within a given experiment, a repeated-measures, two-way ANOVA design and post hoc Tukey analysis were used. A level of P < 0.05 was considered significant in all statistical tests. Values are presented as means ± SE. Wort, ADP, L-NAME, and T-47 were purchased from Sigma (St. Louis, MO). AAG was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). ARR-17477 was a gift from Astra Arcus (Worcester, MA).

RESULTS

In rats from all study groups with the exception of the periods of imposed hypercapnia, the arteriolar P CO2, pH, and MABP did not show any significant variations over the length of the experiment. Thus the overall mean (± SE) initial and final P CO2, and MABP values were 7.41 ± 0.01 and 7.40 ± 0.01, 38.5 ± 1.0 and 37.0 ± 1.2 mmHg; and 123 ± 2 and 121 ± 3 mmHg, respectively. During all experiments, P O2 was maintained ~100 mmHg.

In vehicle/time-control experiments, no differences in the pial arteriolar responses to hypercapnia, SNAP,
ADP, or ACh were seen when the diameter increases before and after initiation of vehicle suffusion were compared (data not shown).

**NOS inhibition.** The initial diameter values in the L-NAME- and ARR-17477-treated groups were 38.7 ± 1.9 and 36.3 ± 1.8 μm, respectively (means ± SE). Before NOS inhibitor administration, no significant variations in baseline diameters (i.e., values measured during normocapnia and between periods of vasodilator applications) were observed. However, 1-h suffusions of L-NAME and ARR-17477 were accompanied by significant 14.8 ± 2.7% and 10.7 ± 3.1% reductions in arteriolar diameters, respectively. For the remainder of these experiments, no subsequent variations in those “new” baseline diameter values in the intervals between vasodilator exposures were noted. Topically applied L-NAME and ARR-17477 (Fig. 1) produced significant and similar decreases in the vasodilating responses to hypercapnia (50–70% reduction in CO2 reactivity). This is in accordance with the established role of nNOS but not eNOS in hypercapnic cerebral vasodilation in rats (e.g., Refs. 30 and 39). On the other hand, only L-NAME affected ACh- and ADP-induced responses (65–75% reductions; see Fig. 2). No changes were seen in the presence of ARR-17477 (Fig. 2). The response to the NO donor SNAP was not altered by either NOS inhibitor (see Fig. 1).

**Heat shock protein 90.** A modest but statistically insignificant 8% increase in baseline diameter was observed when initial (36.5 ± 1.1 μm) and final (39.5 ± 1.7 μm) values were compared. Twenty minutes of suffusion with AAG was not associated with any diameter change (data not shown). The pial arteriolar responses to hypercapnia and SNAP were unaffected by AAG (data not shown), whereas the ACh response completely disappeared in the presence of AAG (Fig. 3). A modest but statistically insignificant 10–20% reduction in the level of ADP-induced vasodilation was observed after AAG administration (Fig. 3).

**Tyrosine kinase.** Initial (33.4 ± 1.7 μm) and final (34.5 ± 0.8 μm) baseline diameter values for this group of experiments were similar. Suffusion of T-47 for 20 min elicited no change in pial arteriolar diameter (data not shown), nor was it associated with any significant alteration in the diameter increases produced by hypercapnia or SNAP (data not shown). The ADP response was similarly unaffected in the presence of the tyrosine kinase inhibitor (Fig. 4). However, suffusion of T-47 was accompanied by a nearly complete (~90%) suppression of ACh-induced vasodilation (Fig. 4).

**Phosphatidylinositol-3-kinase.** No significant differences were found when initial (35.6 ± 2.1 μm) and final (36.1 ± 2.6 μm) baseline diameter values were compared. Wort suffusion (20 min) was not associated with any significant alteration in arteriolar diameter (data not shown). Similar to the results obtained with the HSP90 and tyrosine kinase inhibitors, PI3K blockade with Wort did not affect pial arteriolar relaxation during hypercapnia or suffusion of SNAP (data not shown) or ADP (Fig. 5). The response to ACh, however, was significantly diminished in the presence of Wort at both the 10 μM (~80% reduction from the initial response) and 100 μM (~60% reduction from the initial response) concentrations of ACh (Fig. 5).

**DISCUSSION**

There were a number of key findings in this study. First, a significant portion of the pial arteriolar vasodilation responses to both ACh and ADP in male rats are eNOS dependent with no apparent contributions from nNOS. Second, ACh-induced cerebral arteriolar relaxations were markedly diminished in the presence of inhibitors of HSP90, PI3K, or tyrosine kinase, whereas ADP reactivity was unaffected. It has been clearly established that ACh-induced activation of eNOS in pial arterioles is mediated via muscarinic receptors (e.g., Ref. 24). On the other hand, ADP is
most likely to act via endothelial purinergic P2Y1 receptors (see Ref. 2) in stimulating eNOS. These results therefore indicate that the signal transduction pathways mediating muscarinic and purinergic receptor-induced eNOS activation are quite different. Furthermore, current findings imply that serine/threonine and tyrosine phosphorylations of proteins as well as the presence of HSP90 are vital to the process linking the interaction of ACh with its receptor to the resulting increase in eNOS activity in cerebral arterioles.

A number of studies have placed PI3K as an important upstream effector in fluid shear stress-induced eNOS activation (e.g., Ref. 4). Although it is given less attention in the literature, PI3K also appears to play an important role in the eNOS activation elicited by some receptor agonists. The ability of PI3K blockers to diminish the NO generation and vasodilation actions of ACh and other G protein-coupled receptor agonists linked to eNOS activation has been demonstrated in peripheral (13) and cerebral (17) vascular tissues. Current evidence strongly suggests that PI3K acts through the serine/threonine kinase Akt (protein kinase B) to increase eNOS activity (for example, see Refs. 20 and 26). Specifically, PI3K activation increases the levels of phosphatidylinositol-3-phosphate (PIP₃). It has been proposed that PIP₃ in turn directly binds to Akt (at the pleckstrin homology domain) and renders the Akt molecule “receptive” to phosphorylation by phosphatidylinositol-3-kinase-dependent kinase (4, 36). On activation, Akt...
phosphorylates eNOS, the major site in rats being serine-1177 (6, 11). Phosphorylation of eNOS at serine-1177 is thought to enhance the Ca^{2+}-Cam sensitivity of eNOS, which permits greater levels of NO generation in the presence of lesser (or no) increases in [Ca^{2+}]). The apparently [Ca^{2+}]-increase-independent activation of eNOS ascribed to fluid shear stress and pulsatile flow in multiple studies appears to rely heavily on the PI3K-Akt-eNOS phosphorylation sequence (6, 11).

The present findings would indicate that this pathway plays a role in agonist-induced eNOS activation and cerebral arteriolar dilation as well, but in an agonist (receptor)-selective manner. That is, ACh-induced pial arteriolar responses were substantially diminished in the presence of PI3K blockade, but ADP responses were completely unaffected.

A second indication in this study that differences exist in the mechanisms mediating muscarinic and purinergic receptor-linked eNOS activation is in the effects of HSP90 inhibition. Thus ACh-induced dilations were completely blocked, whereas ADP-induced diameter increases were not altered. The antibiotic ansamycins geldanamycin and its less-toxic derivative AAG potently and selectively block HSP90 via interaction with an amino-terminal ATP binding site (21). Like the findings in this study, in peripheral vessels, geldanamycin administration is accompanied by substantial reductions in ACh-induced dilations (9, 33). Data regarding purinergic receptors are lacking in the literature; however, recent findings in canine basilar artery rings (16) indicate that HSP90 blockade has no effect on the eNOS-dependent relaxations elicited by the receptor agonists bradykinin and substance P. Thus one might consider the possibility that HSP90 dependence of agonist-induced eNOS activation in cerebral vessels is unique to ACh.

Exactly how HSP90 facilitates ACh-mediated stimulation of eNOS in cerebral arterioles is not known. Some possibilities are suggested by results of studies on peripheral vascular tissue. This includes HSP90 binding to eNOS, perhaps acting as an allosteric modulator, and maintaining eNOS in a stable and optimal conformation (see Ref. 9). Another possibility is binding of HSP90 to Akt, thereby preserving the active state of Akt by preventing its dephosphorylation (31). Wherever this binding occurs, it has been proposed that HSP90 increases the ability for Cam to displace Cav-1 from its binding site on eNOS (12). Whether these or other possibilities are valid for ACh-induced eNOS activation in cerebral vessels remains to be established.

The third principal difference we observed in the mechanisms mediating ACh- versus ADP-induced cerebral arteriolar dilations relates to the effects of tyrosine kinase inhibition. The ability of tyrosine kinase inhibitors to block ACh-induced dilations in cerebral vessels in vivo was previously reported for rat basilar artery (17, 18). Most (e.g., Refs. 1 and 7) but not all (37) studies on peripheral vessels indicate a tyrosine kinase role in ACh-induced dilations. The site or sites of tyrosine phosphorylation that are important to muscarinic receptor-mediated eNOS activation are not precisely known. Some suggested sites include PI3K, HSP90, and HSP90-activated protein kinase, HSP90. Although reports would seem to favor phosphorylation on an eNOS-associated protein rather than eNOS (see Refs. 5, 15, 38), present findings also revealed that the pial arteriolar diameter increases elicited by ADP did not involve any influence from tyrosine kinase. Because there is a virtual absence of any information in the literature regarding the tyrosine kinase dependence of purinergic receptor-mediated vasodilations, no further discussion is warranted.

Studies in peripheral and cerebral vascular tissue have shown that ACh administration is accompanied by increases in endothelial [Ca^{2+}]_{i} (e.g., Refs. 1, 7, 17, 37). However, the question remains as to whether those [Ca^{2+}]_{i} changes alone are sufficient in all cases to activate eNOS. In cerebral endothelial cells (17), ACh does indeed elicit increases in endothelial [Ca^{2+}]_{i}. However, Kitayama and colleagues (17) reported that even though the PI3K inhibitor Wort blocked ACh-induced basilar artery dilations, it did not reduce the endothelial [Ca^{2+}]_{i} increase elicited by ACh. One might speculate therefore that such results are indicative of a scenario where two pathways are activated when ACh interacts with its receptor: one linked to a [Ca^{2+}]_{i} increase and the other linked to Ca^{2+}-Cam sensitization of eNOS. Moreover, both pathways must be operative for eNOS activation to occur. Based on current findings, the Ca^{2+}-sensitization pathway would involve PI3K-Akt phosphorylation of eNOS coupled with HSP90 binding (at some step) and tyrosine phosphorylation.

The absence of any effects of PI3K, HSP90, or tyrosine kinase inhibition on the pial arteriolar response to ADP certainly indicates that this pathway is not necessary for ADP/purinergic receptor-mediated eNOS activation. One might therefore consider the possibility that the absence of any need for increased Ca^{2+}-Cam sensitization in ADP-elicited activation of eNOS (as opposed to ACh) is due to a much greater [Ca^{2+}]_{i} increase in endothelial cells when purinergic (versus muscarinic) receptors are stimulated. There is clear evidence that both P2Y1 and P2Y2 receptor activation can elicit substantial increases in [Ca^{2+}]_{i} in the endothelium of large cerebral arteries in rats (22). Similar increases in [Ca^{2+}]_{i}, with P2Y1 activation have also been shown in cerebral capillaries (35). There are a number of examples in the literature to indicate that the endothelial [Ca^{2+}]_{i} increases that accompany purinergic receptor agonist applications greatly exceed those associated with muscarinic agonists. Paeemeire et al. (27) reported that ATP produced substantial increases in [Ca^{2+}]_{i} in cultured rat brain endothelial cells, whereas the changes associated with carbachol administration were undetectable. Although that study involved ATP rather than ADP, the rather robust increases in cerebrovascular endothelial [Ca^{2+}]_{i} elicited by P2Y2 and P2Y1 agonists (22) suggest that a
similar relationship (relative to ACh) may have been observed had ADP been used instead of ATP. However, in cerebral arteries and arterioles, direct comparisons of endothelial [Ca\textsuperscript{2+}] changes along with measurements of diameter increases in the presence of ACh and ADP must be performed to establish whether eNOS Ca\textsuperscript{2+} sensitivity is indeed greater when muscarinic or purinergic agonists are used to trigger the response.

In conclusion, current findings indicate that although ACh and ADP dilate pial arterioles mainly via increasing eNOS-derived NO generation, the pathways utilized to achieve that effect are quite different. Thus the ACh response is strongly dependent upon PI3K, HS90, and tyrosine kinase, whereas ADP-induced dilations are unaffected by inhibition of those proteins. A PI3K-HS90-tyrosine kinase dependence in eNOS activation has been linked to Ca\textsuperscript{2+}-Cam sensitization. We therefore speculate that the [Ca\textsuperscript{2+}], increases that accompany muscarinic, but not purinergic receptor stimulation, are below the threshold required for eNOS activation, which thereby necessitates the operation of a parallel Ca\textsuperscript{2+}-sensitizing pathway.

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