CALL FOR PAPERS | Small Vessels–Big Problems: Novel Insights into Microvascular Mechanisms of Diseases

Purinergic glio-endothelial coupling during neuronal activity: role of P2Y<sub>1</sub> receptors and eNOS in functional hyperemia in the mouse somatosensory cortex

Peter Toth,<sup>1,2,3</sup> Stefano Tarantini,<sup>1,4</sup> Antonio Davila,<sup>5</sup> M. Noa Valcarcel-Ares,<sup>1</sup> Zsuzsanna Tucsek,<sup>1,3</sup> Behzad Varamini,<sup>6</sup> Praveen Ballabh,<sup>7,8</sup> William E. Sonntag,<sup>1,9</sup> Joseph A. Baur,<sup>5</sup> Anna Csiszar,<sup>1,3,4,9</sup> and Zoltan Ungvari<sup>1,3,4,9</sup>

<sup>1</sup>Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; <sup>2</sup>Department of Neurosurgery, University of Pecs, Pecs, Hungary; <sup>3</sup>Szentagothai Research Center, Medical School, University of Pecs, Pecs, Hungary; <sup>4</sup>Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; <sup>5</sup>Institute for Diabetes, Obesity, and Metabolism and Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; <sup>6</sup>Biological Sciences Department, Biola University, La Mirada, California; <sup>7</sup>Department of Cell Biology and Anatomy, New York Medical College, Valhalla, New York; <sup>8</sup>Department of Pediatrics, Regional Neonatal Center, Maria Fareri Children’s Hospital at Westchester Medical Center, New York Medical College, Valhalla, New York; <sup>9</sup>The Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Submitted 15 June 2015; accepted in final form 25 September 2015

Toth P, Tarantini S, Davila A, Valcarcel-Ares MN, Tucsek Z, Varamini B, Ballabh P, Sonntag WE, Baur JA, Csiszar A, Ungvari Z. Purinergic glio-endothelial coupling during neuronal activity: role of P2Y<sub>1</sub> receptors and eNOS in functional hyperemia in the mouse somatosensory cortex. Am J Physiol Heart Circ Physiol 309: H1837–H1845, 2015. First published October 9, 2015; doi:10.1152/ajpheart.00463.2015.—Impairment of moment-to-moment adjustment of cerebral blood flow (CBF) via neurovascular coupling is thought to play a critical role in the genesis of cognitive impairment associated with aging and pathological conditions associated with accelerated cerebrovascular aging (e.g., hypertension, obesity). Although previous studies demonstrate that endothelial dysfunction plays a critical role in neurovascular uncoupling in these conditions, the role of endothelial NO mediation in neurovascular coupling responses is not well understood. To establish the link between endothelial function and functional hyperemia, neurovascular coupling responses were studied in mutant mice overexpressing or deficient in endothelial NO synthase (eNOS), and the role of P2Y<sub>1</sub> receptors in purinergic glio-endothelial coupling was assessed. We found that genetic depletion of eNOS (eNOS<sup>−/−</sup>) and pharmacological inhibition of NO synthesis significantly decreased the CBF responses in the somatosensory cortex evoked by whisker stimulation and by administration of ATP. Overexpression of eNOS enhanced NO mediation of functional hyperemia. In control mice, the selective and potent P2Y<sub>1</sub> receptor antagonist MRS2179 attenuated both whisker stimulation-induced and ATP-mediated CBF responses, whereas, in eNOS<sup>−/−</sup> mice, the inhibitory effects of MRS2179 were blunted. Collectively, our findings provide additional evidence for purinergic glio-endothelial coupling during neuronal activity, highlighting the role of ATP-mediated activation of eNOS via P2Y<sub>1</sub> receptors in functional hyperemia.

NEW & NOTEWORTHY

The findings of this study provide additional evidence for purinergic glio-endothelial coupling during neuronal activity, highlighting the role of ATP-mediated activation of endothelial nitric oxide synthase via P2Y<sub>1</sub> receptors in functional hyperemia. Impairment of this pathway is predicted to play a critical role in the genesis of age-related cognitive impairment.

There is increasing evidence that age-related functional and structural alterations of the cerebral microvascular endothelial cells significantly contribute to the pathogenesis of both vascular cognitive impairment and Alzheimer’s disease (30, 39, 50, 56, 65, 75, 87). Neurovascular coupling is a central homeostatic mechanism adjusting regional cerebral blood flow (CBF) to the increased needs of activated neuronal tissue and thus plays a critical role in the preservation of normal neuronal function, including cognition (30, 34). Importantly, endothelial dysfunction, attributable to impaired NO bioavailability, was shown to associate with impaired neurovascular coupling and cognitive decline in aging and in pathophysiological conditions associated with accelerated microvascular aging including hypertension and obesity (22, 40, 55, 56, 79, 83). Despite these advances, the mechanistic role of endothelial NO production in neurovascular coupling is not completely understood.

Previous studies using pharmacological inhibitors of NO synthesis provided solid evidence that production of NO contributes significantly to functional hyperemia upon neuronal activation; however, the relative contribution of neuronal NO synthase (nNOS or NOS1) and endothelial NO synthase (eNOS or NOS3) is a subject of ongoing debate (4, 9, 70). The
signaling mechanisms that may be involved in triggering endothelial NO production in the cerebral microcirculation during neuronal activation are also not well understood.

There is growing evidence that astrocytes are intimately involved in neurovascular coupling (21, 44, 47, 52, 72). Following synaptic activation, astrocytes release a number of vasoactive mediators in a glutamate-dependent manner, including arachidonic acid metabolites (e.g., epoxyeicosatrienoic acids and prostaglandins), which act directly on the smooth muscle cells of precapillary arterioles (31, 32, 42, 47) eliciting vaso dilatation (termed gliovascular coupling) (59). Activated astrocytes also release ATP, which plays an important role in synchronized activation of neighboring astrocytes and is known to cause dilatation of cerebral arterioles (2, 54, 89, 93).

ATP and adenosine (formed in the extracellular space through hydrolysis of ATP) can bind to purinergic receptors on the smooth muscle cells, exerting direct vasoactive effects (66). Importantly, purinergic receptors, including P2Y1 receptors, are also abundantly expressed on cerebrovascular endothelial cells (90). Previous studies in cultured endothelial cells (17) and in isolated cerebral arterioles (95) demonstrate that ATP and its degradation products activate eNOS via P2Y1 receptors, triggering endothelial production of NO. Despite these advances, the role of P2Y1 receptors in gliovascular coupling remains elusive (17, 94).

The present study was designed to test the hypothesis that functional eNOS and activation of P2Y1 receptors are needed for normal neurovascular coupling response. To achieve these goals, neurovascular coupling was tested in eNOS+/− mice and in a novel mouse model of eNOS overexpression. ATP-induced cerebrovascular responses were also assessed, and the effects of pharmacological inhibition of P2Y1 receptors on neurovascular coupling and purinergic vaso dilatation were determined.

METHODS

Experimental animals. All procedures were approved by the Institutional Animal Use and Care Committees of the participating institutions and in accordance with the ARRIVE guidelines. eNOS-deficient mice (B6.129P2-Nos3tm1Unc/J, developed on a C57BL/6J background; 3 mo old; n = 20) and age-matched wild-type control mice (n = 30) were purchased from the Jackson Laboratories (Bar Harbor, ME).

Mice overexpressing eNOS were developed by the Baur laboratory as follows: a constitutively expressing eNOS construct was generated in eNOS−/− mice and in a novel mouse model of eNOS overexpression. ATP-induced cerebrovascular responses were also assessed, and the effects of pharmacological inhibition of P2Y1 receptors on neurovascular coupling and purinergic vaso dilatation were determined.

METHODS

Experimental animals. All procedures were approved by the Institutional Animal Use and Care Committees of the participating institutions and in accordance with the ARRIVE guidelines. eNOS-deficient mice (B6.129P2-Nos3tm1Unc/J, developed on a C57BL/6J background; 3 mo old; n = 20) and age-matched wild-type control mice (n = 30) were purchased from the Jackson Laboratories (Bar Harbor, ME).

Mice overexpressing eNOS were developed by the Baur laboratory as follows: a constitutively expressing eNOS construct was generated by cloning the murine Nos3 cDNA at the Not1 site between the CAGGS promoter and rabbit globin polyA terminator sequences on the pCAGEN vector backbone (Takahiko Matsuda; Addgene, Cambridge, MA). This was then linearized using Psil and AccI to remove the ampicillin resistance gene. The linearized construct, named pCAGEN-meNOS, was purified from agarose gel with the QiaQuick Gel Extraction Kit (Qiagen, Valencia, CA) and further purified by ethanol precipitation. Linearized pCAGEN-meNOS was injected into fertilized eggs and implanted into pseudopregnant C57BL6/J female mice by the Transgenic and Chimeric Mouse Core at the University of Pennsylvania Perelman School of Medicine. Resultant weanlings were genotyped for expression of the cDNA construct using the primers F: ATTC TCAGGCGACGCTAATCCTC; R: TTCCCA GCTGCTGGCTGA. A female founding pup was confirmed and subsequently backcrossed and maintained on a C57BL/6 Taconic line. Systolic blood pressure was measured by the tail cuff method (CODA Non-Invasive Blood Pressure System; Kent Scientific, Torrington, CT), as previously reported (81). Genetic depletion of eNOS resulted in a mild but significant increase in systolic blood pressure compared with control mice (control: 103 ± 2 mmHg, eNOS+/−: 112 ± 1 mmHg, P < 0.05). Overexpression of eNOS did not significantly affect systolic blood pressure as measured by the tail cuff method.

Endothelium-dependent dilation of isolated cerebral arterioles. To characterize the effect of overexpression of eNOS on endothelium-dependent vasodilatation, responses of isolated cerebral vessels to ATP and acetylcholine (ACH) were obtained, as previously described. In brief, mice were decapitated, the brains were removed, and branches of the middle cerebral artery were isolated using microsurgical technique (67, 68, 79–81). Vascular segments were mounted onto two glass micropipettes in an organ chamber and pressurized to 60 mmHg. The hydrodynamic resistance of the micropipettes was matched. Inflow and outflow pressures were controlled and measured by a pressure servo-control system (Living Systems Instrumentation, Burlington, VT). Inner vascular diameter was measured with a custom-built videomicroscope system and continuously recorded using a computerized data-acquisition system, as reported (77). All vessels were allowed to stabilize for 60 min in oxygenated (5% CO2, balanced with air) Krebs buffer (at 37°C). After a stable, spontaneous pressure-induced myogenic tone developed, changes in vascular diameter were measured in response to administration of ATP (10−7 mol/l) and ACh (10−7 mol/l) (Sigma Aldrich, St. Louis, MO). At the end of each experiment, the passive, maximal diameter was obtained at 60 mmHg in the presence of Ca2+-free Krebs buffer containing the L-type calcium channel inhibitor nifedipine (10−5 mol/l). Dilation is expressed as a percentage and calculated as: [(d – d0)/(pd – d0)]×100, where d is the actual diameter, d0 is baseline diameter, and pd is the passive diameter.

Immunoblotting. Cerebral tissues of control, eNOS transgene (TG), and eNOS−/− were lysed with the Qiagen TissueLyzer II system (20 Hz for 2 min at 4°C) in lysis buffer (25 mM Tris, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% Na-deoxycholate; 1% Nonidet P-40; 0.5% SDS; 1 mM EDTA) supplemented with complete protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). SDS-PAGE was performed on 4–15% Tris-HCl gels (Bio-Rad, Hercules, CA) using 50 μg of cleared lysate boiled for 5 min in 1× Laemmli buffer. Gels were transferred to PVDF membranes (Millipore, Billerica, MA) and blocked with 5% fatty acid-free BSA (Roche Applied Science). Primary antibody for eNOS (NOS3, c-20, sc-654; Santa Cruz Biotechnology, Dallas, TX) was diluted 1:500 in TBS-Tween 20 according to the manufacturer’s instructions. The primary antibody was then removed using a stripping buffer (62.5 mM Tris, pH 6.8; 2% SDS; 100 mM β-mercaptoethanol) for 15 min at 50°C, thoroughly washed, and blocked in 5% BSA. The blot was reprobed with β-actin-horseradish peroxidase (ab49900; Abcam, Cambridge, MA) for the protein loading control. Antibody binding was detected using chemiluminescent horseradish peroxidase substrate (PerkinElmer Life Sciences, Billerica, MA).

Surgical procedures. Mice were anesthetized (α-chloralose, 50 mg/kg plus urethane, 750 mg/kg ip), endotracheally intubated, and ventilated (MousVent G500; Kent Scientific). Rectal temperature was maintained at 37°C using a thermostatic heating pad (Kent Scientific). End-tidal CO2 (with dead space) was kept between 3.2% and 3.7% to maintain blood gas values within the physiological range, as reported (79–81). Animals were immobilized and placed on a stereotaxic frame (Leica Microsystems, Buffalo Grove, IL). The scalp and periosteum were pulled aside, and a craniotomy was made with a dental drill over the left somatosensory cortex corresponding to the barrel field. The dura was gently removed, and the open cranial window was continuously superfused with artificial cerebrospinal fluid (composition: 119.0 mM NaCl, 26.2 mM NaHCO3, 2.5 mM KCl, 1.0 mM NaH2PO4, 1.3 mM MgCl2, 10.0 mM glucose, 2.5 mM CaCl2; pH 7.3, 37°C). The right femoral artery was cannulated for arterial blood pressure measurement (Living Systems Instrumentation) (79–81) to ensure that the blood pressure was within the physiological range throughout the experiments (90–100 mmHg).
CBF responses to whisker stimulation and pharmacological studies. To assess neurovascular coupling, a laser Doppler probe (Transonic Systems, Ithaca, NY) was positioned above the barrel cortex (1.0–1.5 mm posterior and 3.0–3.5 mm lateral to bregma), and the contralateral whiskers were stimulated for 1 min at 5 Hz from side to side. Changes in CBF (n = 7–8 in each group) were assessed in three trials divided by 5–10-min intervals. CBF responses to whisker stimulation were repeated in the presence of the following inhibitors administered topically onto the brain surface of separate groups of animals: N\textsuperscript{\textendash}nitro-l-arginine methyl ester (a pharmacological inhibitor of NO synthase, L-NAME; 10\textsuperscript{\textendash}4 mol/l for 20 min; Sigma Aldrich) (79); the potent, selective, competitive P2Y\textsubscript{1} purinergic receptor antagonist MRS2179 (2'\textendash-deoxy-N6-methyladenosine 3',5'\textendash-bisphosphate ammonium salt; 6 × 10\textsuperscript{\textendash}5 mol/l for 20 min; K\textsubscript{i} = 100 nmol/l; Cayman Chemicals, Ann Arbor, MI) (45, 74); and fluoroacetate (the precursor of fluorocitrate, a metabolic toxin used to inhibit astrocyte function, 3 × 10\textsuperscript{\textendash}4 mol/l for 20 min; Sigma Aldrich) (42). In a separate series of experiments (n = 8 in each group), CBF responses to topical administration of ATP (10\textsuperscript{\textendash}5 mol/l) and ACh (10\textsuperscript{\textendash}5 mol/l) were assessed in the presence and absence of L-NAME (79). Changes in CBF are expressed as percentage changes from baseline (40, 79).

Statistical analysis. Statistical analysis was carried out by unpaired t-test, one-way ANOVA, or two-way ANOVA for repeated measures followed by Bonferroni multiple-comparison test, as appropriate, using Prism 5.0 for Windows (GraphPad Software, La Jolla, CA). A P value <0.05 was considered statistically significant. Data are expressed as means ± SE.

RESULTS

Endothelial NO contributes to neurovascular coupling. Changes in CBF in the whisker barrel cortex in response to contralateral whisker stimulation were significantly attenuated in eNOS\textsuperscript{−/−} mice (Fig. 1A), indicating that deficiency in endothelial NO synthesis leads to neurovascular uncoupling, mimicking the aging phenotype (79). We found that in control animals administration of the NO synthase inhibitor L-NAME also significantly decreased CBF responses in the barrel cortex elicited by contralateral whisker stimulation (Fig. 1A). Topical application of the endothelium-dependent vasodilator agent ACh (10\textsuperscript{\textendash}5 mol/l) (63) resulted in a significant increase in CBF in the barrel cortex of control mice (Fig. 1B). ACh-induced CBF responses were significantly attenuated both by inhibition of NO synthesis by treatment with L-NAME and by genetic depletion of eNOS (Fig. 1B).

To provide additional evidence for the contribution of eNOS to neurovascular coupling responses, we utilized a novel mouse model of eNOS overexpression, developed by the Baur laboratory (Fig. 2A). In the brains of eNOS TG animals, transgene expression was confirmed by Western blot analysis of eNOS (Fig. 2B). Cerebral vessels isolated from eNOS TG mice exhibited increased dilation in response to the NO-dependent vasodilators ACh and ATP (Fig. 2, C and D, respectively), providing functional validation for the model. We found that overexpression of eNOS significantly enhanced the L-NAME-sensitive, NO-mediated portion of the CBF response (calculated based on the percentage decline in CBF in the presence of L-NAME) measured above the barrel field of the primary somatosensory cortex in response to whisker stimulation (Fig. 2E), providing additional support for the contribution of endothelium-derived NO to neurovascular coupling responses.

Role of P2Y\textsubscript{1} receptors in ATP-mediated and endothelial NO-dependent cerebromicrovascular responses. Topical administration of ATP elicited significant increases in CBF in the barrel field of the primary somatosensory cortex, which were inhibited by L-NAME and the potent, selective P2Y\textsubscript{1} receptor antagonist MRS2179 (Fig. 3A). In eNOS\textsuperscript{−/−} mice, ATP-induced CBF responses were significantly impaired and were unaffected by MRS2179 (Fig. 3A). These findings indicate that, in the mouse somatosensory cortex, P2Y\textsubscript{1} receptor-dependent activation of eNOS mediates ATP-induced cerebromicrovascular responses.

We found that in control mice topical administration of MRS2179 also significantly attenuated CBF responses induced by contralateral whisker stimulation (Fig. 2B). In the presence of MRS2179, there was no difference between whisker stimulation-induced CBF responses in control and eNOS\textsuperscript{−/−} mice (Fig. 3B), suggesting that P2Y\textsubscript{1} receptor-dependent activation of eNOS plays an important role in neurovascular coupling responses in the mouse cortex. We also found that administration of the glial-specific metabolic toxin fluoroacetate eliminated the difference between whisker stimulation-induced CBF responses in control and eNOS\textsuperscript{−/−} mice (Fig. 3C), consistent with the proposed role of eNOS in glio-endothelial coupling.

DISCUSSION

Here we demonstrate that both genetic depletion of eNOS and pharmacological inhibition of NO synthesis lead to profound neurovascular dysregulation, characterized by impaired CBF responses induced by synaptic activity (Fig. 1).
Our results extend findings by previous investigations using different pharmacological inhibitors of NO synthesis (29, 70, 79) and disruption of the cerebrovascular endothelium using the light-dye technique (9). Neurovascular uncoupling associated with experimentally induced impairment of endothelial NO mediation mimics impairment of functional hyperemia observed in aging (79) and pathophysiological conditions associated with accelerated cerebrovascular aging, including hypertension (40), obesity (82, 83), and Alzheimer’s disease (28). The aforementioned cardiovascular risk factors were shown to decrease the bioavailability of NO in the microcirculation by promoting oxidative stress, uncoupling eNOS, and/or by upregulating asymmetric dimethylarginine, an endogenous inhibitor of NO synthase (55, 79, 88).

Impairment of a key homeostatic mechanism matching energy supply with the needs of active neuronal tissue is predicted to have deleterious effects on brain function. Indeed, there is strong evidence that, in elderly patients, cerebrovascular dysfunction and impaired neurovascular coupling (69, 76, 96) are associated with decline in higher cortical functions including cognition. Previous studies demonstrate that, compared with wild-type C57BL/6 mice, eNOS−/− mice also exhibit impaired working memory performance (3, 20). Pharmacological inhibition of NO synthesis in experimental animals was also reported to promote cognitive dysfunction (5, 11, 18, 62), mimicking aspects of the aging phenotype (73).

Interestingly, previous studies have demonstrated that there are significant strain-dependent differences in eNOS expression and NO-mediated microvascular responses in mice (6). Thus, it is predicted that the relative importance of endothelium-derived NO in neurovascular coupling responses in mice may also be strain specific. This concept is supported by the previous observations (4) that, in the SV-129 mouse model, in which NO-dependent vasodilation (33, 64) and short-term memory (92) are compromised compared with those in C57BL/6 mice, genetic depletion of eNOS has only a marginal effect on functional hyperemia. Furthermore, SJL mice, which exhibit impaired NO-mediated vasodilation due to oxidative stress associated with a spontaneous mutation in superoxide dismutase 2 (10), also show impaired hippocampal learning and memory (8).

Because there is growing evidence that impaired endothelial function associated with aging and various pathophysiological conditions can be rescued, the cerebral microvasculature emerges as a potential therapeutic target for treating elderly patients with different forms of cognitive impairment. This concept is supported by previous findings that treatment of aged mice with resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is known to exert multifaceted endothelial protective effects (12–16, 46, 58, 78, 84 – 86), rescues neurovascular coupling responses (79) and improves cognitive function (51). Because the microvascular endothelium is directly exposed to the bloodstream, several drugs that do not readily cross the...
blood-brain barrier yet exert protective effects on the microvascular endothelium could be potentially exploited to improve neurovascular coupling and thereby cognitive function in the elderly.

This is the first study to suggest that upregulation of eNOS increases NO mediation of functional hyperemia in response to neuronal activation (Fig. 2). This observation provides additional support for the concept that endothelium-derived NO plays an important role in neurovascular coupling. The functional consequences of increased NO bioavailability in this model remain unclear. Further studies are needed to determine whether overexpression of eNOS is associated with improved cognitive function and/or whether these mice exhibit resistance to cognitive decline induced by cardiovascular risk factors.

Over the past decade, a number of important studies have identified astrocytes as key intermediaries in neurovascular coupling (25). Although because of methodological challenges there are still many controversies in the field (7, 60), there is substantial evidence supporting the view that neuronal activity-dependent Ca\(^{2+}\) signals in astrocytic processes, which precede the onset of functional hyperemia, trigger the release of a number of astrocyte-derived vasoactive mediators (26, 36, 52).

Previous findings that eNOS-deficient mice exhibit impaired cortical vasodilation in response to astrocyte Ca\(^{2+}\) uncaging compared with wild-type mice (70) suggest that mediators released from the astrocytes play a critical role in activation of eNOS in cerebromicrovascular endothelial cells. This finding is also substantiated by the observation that the astrocyte-specific metabolic inhibitor fluoroacetate eliminates the difference between CBF responses in control and eNOS\(^{-/-}\) mice (Fig. 3). Interestingly, there are reports that NO may also regulate astrocytic Ca\(^{2+}\) signaling (49), raising the possibility that a bidirectional communication may exist between the vascular endothelium and the astrocytic network during functional hyperemia.

Purinergic signaling represents one of the most important pathways by which astrocytes communicate with other cells, including neurons and neighboring astrocytes (71). There is strong morphological (electron microscopy) and biochemical evidence suggesting that ATP, as an astrocyte-specific metabolic inhibitor fluoroacetate eliminates the difference between CBF responses in control and eNOS\(^{-/-}\) mice (Fig. 3). Interestingly, there are reports that NO may also regulate astrocytic Ca\(^{2+}\) signaling (49), raising the possibility that a bidirectional communication may exist between the vascular endothelium and the astrocytic network during functional hyperemia.

Purinergic signaling represents one of the most important pathways by which astrocytes communicate with other cells, including neurons and neighboring astrocytes (71). There is strong morphological (electron microscopy) and biochemical evidence suggesting that ATP, as an astrocyte-specific metabolic inhibitor fluoroacetate eliminates the difference between CBF responses in control and eNOS\(^{-/-}\) mice (Fig. 3). Interestingly, there are reports that NO may also regulate astrocytic Ca\(^{2+}\) signaling (49), raising the possibility that a bidirectional communication may exist between the vascular endothelium and the astrocytic network during functional hyperemia.
ing synaptic activation, astrocyte-derived ATP may induce vasodilation by activating multiple cellular pathways. The finding that administration of a potent P2Y1 receptor antagonist significantly attenuates whisker simulation-induced CBF increases in the mouse somatosensory cortex (Fig. 3B) supports the concept that astrocyte-derived ATP contributes to the neurovascular coupling responses (2, 54, 66, 89, 91, 93) and that P2Y1 receptors play an important role in gliovascular coupling. Importantly, P2Y1 receptors are abundantly expressed on cerebromicrovascular endothelial cells (90), and previous studies in cultured endothelial cells (17) and in isolated cerebral arterioles (95) demonstrate that ATP activates eNOS via P2Y1 receptors, triggering endothelial production of NO. The observations that in eNOS−/− mice both ATP-induced and whisker stimulation-induced CBF responses are blunted and both responses are unaffected by MRS2179 (Fig. 3, A and B) extend the aforementioned findings, suggesting that predominantly endothelial P2Y1 receptors mediate ATP-induced eNOS activation in the somatosensory cortex. As predicted by this hypothesis, ATP-induced vasodilation is significantly increased in eNOS-overexpressing mice (Fig. 2). Previous studies showed that ATP can be hydrolyzed in the extracellular space into adenosine (23), which can directly dilate cerebral arterioles via adenosine A2A receptors located on smooth muscle cells (2, 89). Although this mechanism could contribute to the residual, P2Y1 receptor-independent portion of ATP-induced CBF responses, recent evidence suggests that astrocyte-derived ATP, rather than its breakdown products, is a primary mediator of neurovascular coupling (91). Additional cellular and molecular mechanisms linking neuronal activation to endothelial synthesis of NO may involve activation of eNOS via adrenergic receptors (1), glutamate receptors (43, 70), and/or eicosanoid receptors. Previous studies showed that arteriolar dilation induced by neuronal activation can propagate proximally in an endothelium-dependent manner (35) and that ATP is capable to initiate propagated dilation in peripheral vessels (24), suggesting that purinergic glio-endothelial coupling mechanisms may contribute to the endothelium-dependent component of conducted vasodilation in the brain.

Because with advanced age expression of P2Y1 receptors is downregulated (48) and astrocyte ATP generation is decreased (41), future studies should determine the role of purinergic mechanisms in age-related glio-endothelial uncoupling. There are also studies showing that cardiovascular risk factors, including hypertension (27) and diabetes mellitus (37), may also affect vascular P2Y1-dependent pathways. Thus further studies are warranted to elucidate the role of dysregulation of purinergic glio-endothelial coupling responses in these pathophysiological conditions as well.

Limitations of the study. In the present study, we have not investigated the putative role of purinergic nerves in neurovascular coupling. The P2Y1 receptor is also involved in astrocytic communication in both physiological (38) and pathological conditions (19). It should be noted that pharmacological blockade of P2Y1 receptors likely also disrupts these astrocytic pathways as well, which may also affect the neurovascular coupling response. Because likely different cellular mechanisms contribute to the different phases of the CBF response upon initiation of neuronal stimulation, further studies could provide additional valuable data by analyzing the effects of inhibitors to the early and later phases of the CBF response separately.

Conclusions. In conclusion, our results add to the growing evidence that purinergic glio-endothelial coupling mechanisms are activated during neuronal activity, which contribute to functional hyperemia in the mouse cortex. We propose that ATP-mediated activation of P2Y1 receptors plays an important role in eNOS-dependent increases in CBF during neuronal activation (Fig. 3D). It is an important aspect of the proposed model that purinergic glio-endothelial coupling mechanisms provide a physiological link between astrocytic metabolism and endothelium-dependent vasodilation of cerebral microvessels. Our findings, taken together with the results of earlier studies (reviewed in Refs. 28 and 61), point to potential additive benefits of interventions preventing metabolic dysfunction of astrocytes and promoting microvascular health for prevention of cognitive decline in the elderly.

GRANTS
This work was supported by grants from the American Heart Association (to P. Toth, A. Csiszar, Z. Tuczeck, S. Tarantini, and Z. Ungvari), the Arkansas Claude Pepper Older Americans Independence Center at University of Arkansas Medical Center (P30 AG028718), the Oklahoma Center for the Advancement of Science and Technology (to A. Csiszar, Z. Ungvari, and W. Sonntag), the Bolyai Research Scholarship of the Hungarian Academy of Sciences (to P. Toth), the National Center for Complementary and Alternative Medicine (RO1-AT006526 to Z. Ungvari), the National Institute on Aging (RO1-AG047879 to A. Csiszar, RO1-AG038747 to W. Sonntag, RO1-NS056218 to A. Csiszar and W. Sonntag, RO0-AG031182 and RO1-AG043483 to J. Baur), and the National Institute of Diabetes and Digestive and Kidney Diseases (RO1-DK098656 to J. Baur). We thank the University of Pennsylvania Diabetes Research Center (DRC) for the use of the Transgenic and Chimeric Mouse Core (P30-DK19525).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES
Purinergic Glia-Endothelial Coupling


