The role of the glia limitans in ADP-induced pial arteriolar relaxation in intact and ovariectomized female rats

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WE RECENTLY REPORTED (20, 21) that in vivo pial arteriolar dilations elicited by the purinergic P2Y1 agonist ADP are partially dependent on endothelial nitric oxide (eNOS) synthase (eNOS) in gonadally intact rats. However, in ovariectomized (Ovx) females, the NO dependency was lost, but the magnitude of the response remained unchanged. This was due to an increase in the contribution from an NO-independent (NOS inhibitor insensitive), but still endothelium-dependent, endothelium-derived hyperpolarizing factor (EDHF). Additional findings indicated that the apparent EDHF component involved gap junctions (20). Irrespective of the endothelium-related mechanism, ADP reactivity was completely lost in intact and Ovx rats (20). We hypothesized that pial arteriolar ADP reactivity in intact and Ovx females is unlikely to include the GL and gap junctions. In intact and Ovx females, when the gap junction blocker, Gap 27 (G-nitro-L-arginine (L-NNA) and the gap junctional blocker, Gap 27 were added to the suffusate, ADP reactivity was completely lost in intact and Ovx females. The results suggest that 1) ADP-induced pial arteriolar dilations involve additive contributions from P2Y1 receptors present in both vascular endothelium and the GL and 2) the influence of the GL component is not altered by ovariectomy and the GL junctional component of the ADP response in Ovx females is unlikely to include the GL and probably resides in the vessels themselves.

METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee. Two groups of female Sprague-Dawley rats (200–250 g at arrival) were used: intact and ovariectomized (Ovx).
Ovariectomies were performed by the vendor (Charles River) 1 wk before shipment. Studies were performed at ∼4 wk after the date of arrival. Pial arteriolar reactivities were evaluated through closed cranial windows placed on the day preceding study (19). For window placement, the rats were anesthetized with halothane, intubated, paralyzed with a short-acting muscle relaxant (vecuronium), and mechanically ventilated with a 0.8% halothane-70% N2O-30% O2 gas mixture. After window preparation, 300 μl of an artificial cerebrospinal fluid (aCSF) solution with or without l-α-AAA (2 mM) was injected into the space under the cranial window (19). The cranial window access ports were then plugged, and the skin overlying the skull was sutured together. The animal was permitted to recover from anesthesia induction with halothane and paralysis (curare), the rats were tracheotomized and mechanically ventilated. Bilateral femoral arterial and venous catheters were inserted under 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 cannulae were connected. Halothane was discontinued, and a 10 μg/kg fentanyl bolus was given intravenously. During the study, the rats were maintained with fentanyl (25 μg·kg⁻¹·h⁻¹ iv) and 70% N2O-30% O2. The space under the window was filled with aCSF (pH 7.35–7.40) that was equilibrated with a gas mixture consisting of 20% O2-5% CO2-balance of N2. The 37°C aCSF solution was suffused at 0.5 ml/min. Body temperature was controlled at 37°C, and mean arterial pressure and intracranial pressure were monitored continuously during the experiment. Measurements of the diameters of pial arterioles (25–50 μm) were made using video microscopy (see Ref. 18). In all experiments, the initial diameter measurements were made 1 h posthalothane and after 40-min drug-free aCSF suffusion.

The rats were divided into four principal experimental subgroups: intact vehicle; intact l-αAAA; Ovx vehicle; and Ovx l-α-AAA. For the intact females, after measurement of baseline diameter values, ADP, at concentrations of 10 and 100 μM, was suffused into the space under the cranial window (5 min each concentration). After 10 min of drug-free aCSF suffusion, a baseline measurement was made, and the NO donor S-nitroso-N-acetyl penicillamine (SNAP) was sequentially suffused at 0.1 and 1.0 μM (5 min at each dose). After a return to baseline, l-NNa (1 mM) was added to the aCSF and suffused for 60 min, and the responses to ADP and SNAP were again evaluated. Time control rats, from vehicle and l-α-AAA-treated groups, where l-NNa was omitted from the aCSF, were also studied. No changes in ADP or SNAP reactivity were noted in these animals (data not shown). In the Ovx rats, a similar protocol was used, but with one additional step. Thus the responses to ADP (10 and 100 μM) and SNAP (0.1 and 1.0 μM) were evaluated as in the intact females; first in the absence and then in the presence of l-NNa. Subsequently, a suffusion of the Cx43 and Cx37 gap junction inhibitory peptide, Gap 27 (300 μM), was initiated and maintained for 1 h before reevaluation of ADP responses. No changes in ADP or SNAP reactivities were seen in time controls, where l-NNa and l-NNa + Gap 27 were omitted from the aCSF solution (not shown).

Two additional experimental protocols were performed. In the first, we sought to obtain further evidence in support of the hypothesis that the pial arteriolar vasodilating response to ADP, in intact and Ovx females, is a reflection of the additive contributions from vascular endothelium and the GL. To that end, l-α-AAA-treated rats were subjected to a light plus dye (L/D) endothelial injury strategy, utilized in several previous studies in our laboratory (e.g., Ref. 20). For these experiments, at 24 h after l-α-AAA treatment, ADP (10 and 100 μM) and SNAP (0.1 and 1.0 μM) reactivities were measured. Subsequently, the animals were subjected to L/D injury, where mercury light was passed through a filter that allowed transmission only at

### Table 1. Initial pial arteriolar diameters and arterial blood variables at start and end of experiments

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Pial arteriolar diameter, μm</th>
<th>PacO2, mmHg</th>
<th>pHa</th>
<th>MABP, mmHg</th>
<th>PacO2, mmHg</th>
<th>pHa</th>
<th>MABP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Vehicle</td>
<td>37.0±2.5</td>
<td>39.2±2.4</td>
<td>7.39±0.02</td>
<td>132±3</td>
<td>34.8±1.3</td>
<td>7.37±0.02</td>
<td>135±6</td>
</tr>
<tr>
<td>Intact l-α-AAA</td>
<td>37.9±1.1</td>
<td>37.2±4.6</td>
<td>7.41±0.03</td>
<td>120±4</td>
<td>34.0±1.6</td>
<td>7.41±0.02</td>
<td>128±11</td>
</tr>
<tr>
<td>Ovx Vehicle</td>
<td>37.1±1.2</td>
<td>36.8±3.4</td>
<td>7.41±0.02</td>
<td>127±5</td>
<td>31.8±1.7</td>
<td>7.39±0.02</td>
<td>138±4</td>
</tr>
<tr>
<td>Ovx l-α-AAA</td>
<td>38.9±2.1</td>
<td>35.3±1.0</td>
<td>7.38±0.01</td>
<td>125±4</td>
<td>32.5±0.7</td>
<td>7.39±0.01</td>
<td>138±2</td>
</tr>
<tr>
<td>Intact MRS-2179</td>
<td>41.7±1.6</td>
<td>37.8±3.0</td>
<td>7.41±0.01</td>
<td>127±5</td>
<td>36.0±2.0</td>
<td>7.41±0.01</td>
<td>125±5</td>
</tr>
<tr>
<td>Ovx MRS-2179</td>
<td>35.5±1.4</td>
<td>37.2±1.2</td>
<td>7.41±0.01</td>
<td>127±2</td>
<td>35.6±2.0</td>
<td>7.41±0.01</td>
<td>131±6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–5 animals in each group. PacO2, arterial PacO2; pHa, arterial pH; MABP, mean arterial blood pressure; Ovx, ovariectomized; l-α-AAA, l-α-aminoacidic acid.
RESULTS

In all experiments, the physiological variables were within normal limits. Thus $P_{\text{aCO}_2}$, pH, and mean arterial blood pressure in these groups did not show any significant differences when comparing initial and final values over the course of the experiments (Table 1). Not included in Table 1 are the arterial $P_{\text{aO}_2}$ values, which were maintained at $>100$ mmHg in all rats studied. Also provided in Table 1 are the initial pial arteriolar diameters.

No changes were seen in the pial arteriolar responses to the NO donor, SNAP, in vehicle- or l-$\alpha$-AAA-treated intact and Ovx female rats (Fig. 1). The absence of any change in vascular reactivity to SNAP suggests that neither ovariectomy nor l-$\alpha$-AAA alters vascular smooth muscle function. The absence of any effect of l-$\alpha$-AAA (1 mM, topical application) confirms that SNAP acts independently of endogenous generation of NO.

Pial arteriolar responses to suffusions of ADP (10 and 100 $\mu$M) in vehicle-treated and l-$\alpha$-AAA-treated intact females, in the absence and presence of l-$\alpha$-NNa, are shown in Fig. 2. As previously shown by us, in the intact females, NOS inhibition reduced the ADP response by $\sim 50\%$. In rats exposed to topicaly applied l-$\alpha$-AAA for 24 h, ADP reactivity was reduced, relative to vehicle controls, by 33% (100 $\mu$M ADP) to 70% (10 $\mu$M ADP) in the absence of NOS inhibition. Moreover, on addition of l-$\alpha$-NNa, ADP reactivity was reduced substantially further, by 82–100% from the initial response observed in the vehicle group. These seemingly additive effects of l-$\alpha$-NNa and l-$\alpha$-AAA suggest that NO and the GL support separate components of the ADP response in intact females.

Pial arteriolar responses to ADP in vehicle-treated and l-$\alpha$-AAA-treated Ovx females, in the absence and presence of l-$\alpha$-NNa and l-$\alpha$-NNa + Gap 27, are summarized in Fig. 3. As previously shown by us (20, 21), although pial arteriolar reactivity to ADP is not significantly different in Ovx vs. intact females (see Fig. 2), NOS inhibition has no effect on the ADP response in Ovx rats (consistent with the loss of eNOS dependency). In Ovx females treated with l-$\alpha$-AAA, the pial arteriolar response to ADP was reduced by 35–90% from the response seen in vehicle-treated Ovx females, with no further changes on addition of l-$\alpha$-NNa. However, when the gap junctional inhibitor Gap27 (which preferentially affects Cx43- and Cx37-containing gap junctions) was applied, ADP reactivity at the lower concentration completely disappeared and was mark-

Fig. 3. Pial arteriolar responses to suffusions of ADP (10 and 100 $\mu$M) in vehicle-treated ($n=5$) and l-$\alpha$AAA-treated Ovx ($n=5$) females. Also presented are the responses to ADP after NOS inhibition, via L-NNA, in the absence and presence of the gap junctional blocker Gap 27 (300 $\mu$M, given topically). Values are means ± SE. *$P<0.05$ vs. initial. †$P<0.05$ vs. vehicle.

Fig. 4. Pial arteriolar responses to suffusions of ADP (10 and 100 $\mu$M), in l-$\alpha$AAA-treated intact (left, $n=4$) and Ovx ($n=4$) females (right), before and after light plus dye (L/D) endothelial injury. Values are means ± SE. *$P<0.05$ vs. l-$\alpha$-AAA alone.
edly repressed (by >80% from the initial response in the vehicle group) at the higher ADP concentration. It should be noted that Gap 27 has no effect on pial arteriolar reactivity to ADP in intact females (20). The effects of GL injury and gap junctional blockade seem to be generally additive, although the combination of GL injury and gap junctional blockade appears to exceed what one might expect if the effects of L-α-AAA and Gap 27 were completely independent of one another. Nevertheless, the mostly additive nature of these results suggests that the gap junctional dependency and astrocytic (GL) influence, to a large degree, involve separate components of the pial arteriolar response to ADP in the Ovx female. The fact that the gap junctional component does not seem to be substantially affected by injury to the GL suggests that the gap junctions involved in the ADP response in Ovx females are mostly nonastrocytic. A likely location for these gap junctions would be within the vessels themselves.

As a further demonstration that, irrespective of hormone status, ADP-induced pial arteriolar dilation involves the additive effects of an endothelium-dependent and an astrocyte-dependent component, additional rats were studied where the ADP response was evaluated first at 24 h after L-α-AAA-induced GL injury and, subsequently, after acute L/D-induced endothelial injury. These data are provided in Fig. 4. Similar to the results presented earlier, L-α-AAA treatment was accompanied by ADP responses that were 40–70% lower than the control responses (control values given in Figs. 2 and 3). On L/D treatment, the response at the lower ADP concentration (10 μM) completely disappeared, and the response at 100 μM ADP was reduced to ~10% of the control value. The absence of any changes in the pial arteriolar responses to SNAP (Fig. 5) confirms that the L/D procedure did not injure vascular smooth muscle.

The P2Y1 receptor dependency of ADP-induced pial arteriolar relaxation is illustrated by results summarized in Fig. 6. Thus ADP responses were almost completely eliminated in the presence of MRS-2179 in both intact and Ovx females. The selectivity of the MRS-2179 effect was supported by the absence of any significant changes in the magnitude of the pial arteriolar relaxations elicited by the P1 agonist adenosine (Fig. 7).

**DISCUSSION**

There were several key findings in these studies. First, the GL has a profound influence on ADP-induced pial arteriolar dilation in female rats. Second, that influence is not altered by changes in hormone status. Third, the GL contribution in females involves the NO-insensitive and endothelium-independent component of the ADP response. Accordingly, in the intact female, the NO-dependent and the astrocyte-derived elements, in an additive manner, seem to account for nearly all of the pial arteriolar response to ADP. Furthermore, because the endothelium-dependent and the L-NNA-inhibitable components completely overlap (20) and because evidence indicates that pial arteriolar ADP-induced dilations in rats are not influenced by neuronal NOS (nNOS) (17), the “non-GL” component, in all likelihood, is eNOS. In the Ovx female, the NO-dependent component is replaced by a mechanism related to gap junctions (20, 21). Thus, in contrast to hormonally normal females, virtually all of the ADP response in the Ovx female can be attributed to the mostly additive actions of a gap junctional element and an astrocyte-associated element.
lar to the non-GL component indicated in intact females, the gap junctional element appears to involve the endothelium. This is based upon the parallel observations in the current study that either gap junctional blockade or endothelial injury, when added on top of L-α-AAA-induced GL injury, elicits a nearly complete loss of ADP reactivity.

In a recently published report (19), we established that L-α-AAA selectively damages the GL while (functionally) sparing vascular cells and neurons. In that study, we also obtained evidence that the GL plays an important role in supporting hypercapnia-induced pial arteriolar dilation. Additional findings indicated that Cx43, and presumably gap junctions, participated in that response. However, in that instance, results suggested that gap junctions residing in astrocytes were involved.

Although both involve multicellular influences, there are major differences in stimulus/vascular response coupling when comparing in vivo pial arteriolar responses to hypercapnia vs. ADP. One obvious difference is that ADP acts via a purinergic receptor (mostly P₂Y₁; see Ref. 16), whereas hypercapnia does not. As such, one might look to the sites of P₂Y₁ expression to derive clues as to the mechanisms at play in an ADP response that includes a vascular, as well as an extravascular, component. Of particular relevance to the present investigation, P₂Y₁ receptors have been shown to be well expressed in rodent astrocytes (e.g., Ref. 22). Endothelial cells are reported to be another site of P₂Y₁ expression, which is consistent with the purported role of this receptor subtype in cerebral endothelium-derived NO generation (7). In the brain, evidence points to the endothelial presence of P₂Y₁ message in arteries (11), although immunohistochemical analysis has not confirmed endothelial expression of P₂Y₁ protein (14). Nevertheless, in the present study, P₂Y₁ blockade with the selective antagonist MRS-2179 was associated with a virtually complete loss of ADP reactivity in pial arterioles. Because, in female rats, endothelium (see Fig. 4 and Ref. 20) and astrocytes appear to contribute roughly equally (i.e., 50/50) to the pial arteriolar ADP response, the results obtained with P₂Y₁ antagonist applications would seem

![Fig. 7. Pial arteriolar responses to adenosine (ADO) suffusions (10 and 100 μM) in intact (left) and Ovx females (right) before and after topical application of the P₂Y₁ antagonist MRS-2179 (10 μM). Values are means ± SE.](http://ajpheart.physiology.org/)

![Fig. 8. Postulated mechanisms of ADP-induced dilation of pial arterioles in intact and Ovx females. In both groups, the vasodilating response is the result of the additive contributions from P₂Y₁-dependent endothelial and astrocytic (glia limitans; GL) components. The major difference between intact and Ovx females resides in the endothelial component. Thus, in the intact female, ADP engagement of the receptor stimulates production of endothelial NOS (eNOS)-derived NO. On the other hand, in the Ovx female, even though ENOS function is impaired, there is no loss in ADP reactivity, because P₂Y₁ activation in endothelium now elicits a response that is endothelium-derived hyperpolarizing factor-like (21) and gap junction dependent (20). In both groups, ADP-induced activation of P₂Y₁ receptors in the underlying astrocytes accounts for ~50% of the pial arteriolar response to ADP. We suspect that this initiates a process capable of eliciting vascular smooth muscle relaxation. The fact that the gap junctional component does not seem to be appreciably affected by injury to the GL suggests that the gap junctions involved in Ovx females are nonastrocytic. Moreover, on the basis of results from experiments employing endothelial injury (Fig. 4 and Ref. 20), the gap junctions are likely to reside in the vascular compartment and may include interendothelial and/or myoendothelial communication.)
to support the presence of endothelial P2Y1 receptors, at least in pial vessels. This apparent incongruity between results obtained using pharmacological and immunohistochemical approaches may simply be a function of an insufficient sensitivity to P2Y1 antibodies, although we cannot completely discount the possibility, however unlikely (1), that MRS-2179 may be interacting with another P2Y variant or that P2Y1 expression may be relatively greater in pial arterioles compared with the rest of the cerebral vasculature. The similar efficacies of MRS-2179 and combined endothelial and GL injury in effecting a nearly total blockade of pial arteriolar ADP reactivity also imply that the P2Y1 receptors involved reside almost exclusively in endothelium and the GL. This leaves little, if any, room for contributions from the P2Y1 receptors that appear to be present in neurons (12) and cerebral vascular smooth muscle (11). Furthermore, the finding that L-α-AAA- and L/D-induced injuries do not damage cortical neurons and pial arteriolar smooth muscle (Fig. 5 and Ref. 19), yet ADP reactivity is lost nonetheless, makes it even less likely that P2Y1 receptors in those cells contribute in any meaningful way.

The fact that application of the P2Y1 antagonist was associated with a loss of ADP reactivity in both intact and Ovx females suggests that activation of endothelial P2Y1 receptors not only can stimulate eNOS-derived NO generation but also, under the “right” circumstances, is capable of promoting a gap junction-related vasodilation that has characteristics of an EDHF (20) (see also Ref. 6). We can only speculate as to what common factor links the endothelium-dependent portion of the P2Y1-mediated ADP response in intact vs. Ovx females. The metabolotropic, heterotrimeric G protein-coupled P2Y1 receptor is known to promote intracellular Ca2+ mobilization. This appears to occur via activation of phospholipase C and inositol 1,4,5-trisphosphate-mediated release of Ca2+ from intracellular stores, with the increase in intracellular Ca2+ ([Ca2+]i) being sustained by capacitative Ca2+ entry (9). Thus it is likely that ADP engagement of the endothelial P2Y1 receptor promotes eNOS activation, or, in the presence of repressed eNOS function [e.g., estrogen-depleted states (13)], increased activity of a gap junction-dependent, EDHF-like mechanism (20). Both “targets” would require acute upstream elevations in endothelial [Ca2+]i (see Refs. 10 and 17).

The mechanistic features of astrocytic P2Y1 receptor contributions to ADP-induced pial arteriolar dilation are even less clear. However, it is probably safe to assume that this also involves mobilization of [Ca2+]i, because P2Y1 receptor activation has been shown to elicit Ca2+ wave propagation between adjacent astrocytes (4). While this implies a Ca2+-dependent astrocyte-derived factor, the nature of this apparent factor is unknown. Certainly, paracrine factors generated by Ca2+-activated enzymes must be given serious consideration. Candidate enzymes include nNOS and the Ca2+-dependent PLA2 isofoms. NO generated via nNOS action seems unlikely, in light of recent findings from our laboratory showing that topical application of the neuronal NOS-selective inhibitor AR-R17477 (at least in male rats) had no effect on pial arteriolar responses to ADP suffusions (17). With regard to PLA2, one might consider the downstream metabolism of a principal product of PLA2 action, i.e., arachidonic acid (AA). Indeed, evidence exists linking P2Y1 receptor activation to AA release in astrocytes (3). There are well-established vasodilating substances generated via AA metabolic pathways. These include cyclooxygenase-1 (COX-1) products, like prostacyclin, or products of the cytochrome P-450/2C11 epoxygenase. However, in earlier studies, we failed to observe any effects of COX-1 or epoxygenase inhibitors on the ADP response of pial arterioles (21). Nevertheless, whereas the absence of any effect of epoxygenase inhibition would be consistent with a lack of any influence from de novo epoxide generation, it does not negate the participation of epoxides released from preformed pools. There are additional PLA2-related candidates one might consider. The possibility exists that AA itself, in the absence of further metabolism, may promote vasodilation, for example, via interaction with AA-sensitive membrane ion channels (2). AA has also been shown to be a potent vasodilator when applied directly to cerebral arteries and arterioles in vivo (e.g., Refs. 5 and 15). However, that AA action appears to be attributable to its metabolism via the cyclooxygenase or lipoxygenase pathway. Although a COX-1-generated AA metabolite has been essentially eliminated as a potential mediator of ADP-induced pial arteriolar relaxation in our animals, it may be of some value, in future studies, to examine whether a lipoxygenase pathway product or AA itself is involved.

In conclusion, the GL appears to have a significant influence on ADP-induced pial arteriolar dilation in female rats, irrespective of hormone status. That contribution involves the NO-insensitive component of the ADP response. In fact, in the intact female, the eNOS-dependent and the astrocyte-derived elements, in an additive manner, seem to account for nearly all of the pial arteriolar response to ADP (Fig. 8A). In the Ovx female, the NO-dependent component is replaced by a mechanism related to gap junctions and EDHF. Thus a substantial portion of the ADP response in the Ovx female can be accounted for (additively) by a gap-junctional (EDHF) element and an astrocytic element (Fig. 8B). In contrast to the pial arteriolar response to hypercapnia (19), the gap junctions involved would appear to be nonastrocytic (probably vascular). In either case, it appears that astrocytes comprising the GL contribute to ADP-induced pial arteriolar relaxation through a P2Y1 receptor-initiated mechanism. Whether that mechanism involves a diffusible or a mechanical factor (or something else) remains to be established.

GRANTS
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REFERENCES


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ADP-INDUCED CEREBRAL VASODILATION

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