Increased Angiotensin II Contraction of the Uterine Artery at Early Gestation in a Transgenic Model of Hypertensive Pregnancy Is Reduced by Inhibition of Endocannabinoid Hydrolysis

Victor M. Pulgar, Liliya M. Yamaleyeva, Jasmina Varagic, Carolynne M. McGee, Michael Bader, Ralf Dechend, Allyn C. Howlett, K. Bridget Brosnihan

Abstract—Increased vascular sensitivity to angiotensin II (Ang II) is a marker of a hypertensive human pregnancy. Recent evidence of interactions between the renin–angiotensin system and the endocannabinoid system suggests that anandamide and 2-arachidonoylglycerol may modulate Ang II contraction. We hypothesized that these interactions may contribute to the enhanced vascular responses in hypertensive pregnancy. We studied Ang II contraction in isolated uterine artery (UA) at early gestation in a rat model that mimics many features of preeclampsia, the transgenic human angiotensinogen×human renin (TgA), and control Sprague–Dawley rats. We determined the role of the cannabinoid receptor 1 by blockade with SR171746A, and the contribution of anandamide and 2-arachidonoylglycerol degradation to Ang II contraction by inhibiting their hydrolyzing enzyme fatty acid amide hydrolase (with URB597) or monoacylglycerol lipase (with JZL184), respectively. TgA UA showed increased maximal contraction and sensitivity to Ang II that was inhibited by indomethacin. Fatty acid amide hydrolase blockade decreased Ang II MAX in Sprague–Dawley UA, and decreased both Ang II MAX and sensitivity in TgA UA. Monoacylglycerol lipase blockade had no effect on Sprague–Dawley UA and decreased Ang II MAX and sensitivity in TgA UA. Blockade of the cannabinoid receptor 1 in TgA UA had no effect. Immunolocalization of fatty acid amide hydrolase and monoacylglycerol lipase lipase showed a similar pattern between groups; fatty acid amide hydrolase predominantly localized in endothelium and monoacylglycerol lipase in smooth muscle cells. We demonstrated an increased Ang II contraction in TgA UA before initiation of the hypertensive phenotype. Anandamide and 2-arachidonoylglycerol reduced Ang II contraction in a cannabinoid receptor 1–independent manner. These renin–angiotensin system–endocannabinoid system interactions may contribute to the enhanced vascular reactivity in early stages of hypertensive pregnancy. (Hypertension. 2014;64:00-00.) ● Online Data Supplement

Key Words: endocannabinoids • fatty-acid amide hydrolase • high-risk pregnancy • monoacylglycerol lipase

Preeclampsia is a common disorder of pregnancy that manifests with hypertension and proteinuria. The renin–angiotensin system (RAS) plays an important role in the normal and pathological regulation of the female reproductive system1 and an increased response to activation of the RAS is characteristic of pregnancies at risk of developing preeclampsia. Thus, since the pioneering work of Gant et al.,2,3 an increased sensitivity to angiotensin II (Ang II), one of the main agonists of the RAS, was early recognized as a marker for the development of a hypertensive pregnancy.4

The transgenic female rat containing the human angiotensinogen gene mated with the male transgenic containing human renin (hREN), the hAGT×hREN rat (TgA) mimics many features of human preeclampsia. This model shows increased blood pressure, proteinuria, and placenta alterations of edema and necrosis in the last half of gestation.5 Mean blood pressure increases abruptly ≥10 days before delivery reaching values of 160±10 mm Hg.6 Among the vascular effects observed in this model, a prostanoid-mediated endothelial dysfunction of the uterine artery (UA) has been described at late gestation.5,7

The endocannabinoid system is composed of mediators such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), cannabinoid receptors (CB1, CB2, and non-CB/CB2), and enzymes in charge of synthesis or hydrolysis of these mediators, that is, fatty acid amide hydrolase (FAAH) for hydrolysis of AEA and monoacylglycerol lipase (MAGL) for...
2-AG. Thus, AEA and 2-AG act as endogenous ligands for cannabinoid receptors. Endocannabinoids possess vasoactive, mitogenic, and differentiating properties and are implicated in placentation and in several pregnancy disorders including preeclampsia. Endocannabinoids participate in the regulation of angiogenesis during implantation and decidualization, control myometrial contractility, and regulate uterine and umbilical blood flow. All these events are determinants of a successful pregnancy and may have devastating consequences when altered.

Once generated, the actions of AEA and 2-AG are terminated by specific reuptake and subsequent degradation by either FAAH or MAGL, respectively. The ability to manipulate in vivo endocannabinoid levels by blocking their degradation makes these enzymes attractive therapeutic targets for several pathologies where AEA and 2-AG are involved, thus emphasizing the relevance of studying FAAH and MAGL blockers. Notably, the localization and expression pattern of FAAH in the blastocyst suggests a role for this enzyme in limiting AEA levels as a protection for a successful implantation. Data also suggest that these metabolizing enzymes regulate endocannabinoid levels in maternal tissues during pregnancy.

Recent evidence of interactions between the RAS and endocannabinoid system suggests a regulatory role for endocannabinoids in modulating Ang II vascular responses. Thus, the contraction to Ang II in mice gracilis arteries is increased by blockade of the CB1 receptor suggesting a vasodilatory role of endocannabinoids modulating contraction to Ang II in arteries from the systemic circulation.

In this study, we compared the Ang II–mediated contraction of isolated UA from TgA and Sprague–Dawley (SD) rats at early gestation and the effects of blocking AEA and 2-AG hydrolysis, by inhibiting FAAH or MAGL. We hypothesize that prostanoids have a greater impact in modulating Ang II contractions in preeclamptic rats and that blocking endocannabinoid degradation antagonizes Ang II contraction.

### Material and Methods

#### Animals
Female human angiotensinogen transgenic rats, when mated with a hren transgenic male (human angiotensinogen×hREN), develop hypertension and proteinuria in the second half of pregnancy. Seven-day pregnant preeclamptic human angiotensinogen×hREN (TgA, n=9) and 7-day pregnant SD (n=6) rats were used. All experiments were performed in accordance with the guidelines of the Wake Forest School of Medicine Institutional Animal Care and Use Committee (see online-only Data Supplement for details).

#### Vascular Reactivity Experiments
Segments of the main UA, a maximum of 2 mm in length, were mounted between an isometric force transducer (Kistler Morec DSC 6, Seattle, WA) and a displacement device on a myograph (Multi Myograph, Model 620M Danish Myo Technologies, Aarhus, Denmark), using 2 stainless steel wires (diameter 40 μm), as described previously. In a subgroup of arteries from the control and TgA rats, the endothelium was destroyed by passing a human hair through the lumen (see online-only Data Supplement for details).

#### Response to Potassium Chloride
After equilibration, to test the viability of the arterial preparations and determine the response to nonreceptor-mediated contraction, UA segments were exposed to 75 mmol/L potassium chloride in Krebs-Henseleit Buffer for 5 minutes, and after washing the incubation was repeated twice. Contraction measured at the third incubation was recorded as maximal contraction to potassium chloride ($R_{\text{max}}$).

#### Response to Ang II
After washing and resting for 30 minutes, UA segments were exposed to a cumulative concentration–response curve of Ang II by exposing arteries to eleven (10$^{-5}$−10$^{-9}$ mol/L) increasing concentrations in fourth-log steps, with each subsequent dose being introduced only after a steady response had been reached (2 minutes). Because at higher concentrations of ligand the Ang II contraction decreased as a result of desensitization, only doses as high as 10 nmol/L were used. In parallel experiments, different arterial segments were demed or preincubated for 15 minutes with the cyclooxygenase inhibitor indo- methacin (10$^{-5}$ mol/L) or the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (10$^{-4}$ mol/L). Additional arterial segments were preincubated for 15 minutes with the FAAH blocker UR85017 (13′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)−cyclohexylcarbamate) at 1×10$^{-6}$ mol/L or the MAGL blocker JZL184 (4-nitrophenyl−4-[(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine−1-carboxylate) at 1×10$^{-6}$ mol/L. Some TgA UAs were preincubated with the CB1 receptor blocker SR141716A at 1×10$^{-6}$ mol/L or SR141716A at 1×10$^{-5}$ mol/L plus UR85017 at 1×10$^{-6}$ mol/L.

#### Immunohistochemistry
Expression of FAAH and MAGL in UA was detected by immunostaining using commercial antibodies. For details see the online-only Data Supplement. Images were acquired at ×400 magnifications using a Leica DM 4000B upright microscope (Leica Microsystems, Bannockburn, IL). Illumination settings were held constant for image capture sessions (Retiga 1300R CCD Digital camera, QImaging, Surrey, BC, Canada; SimplePCI v6 software Cranberry Twp, PA). Regions of interest were defined using the open source Fiji software (ImageJ, National Institutes of Health; http://fiji.sc/Fiji) covering smooth muscle and endothelial layers in each arterial segment. Density of the staining in 5 regions of interest per segment was quantified following the reciprocal intensity method.

#### Data Analysis
All data analysis was performed using the GraphPad Prism v5 statistical analysis package (GraphPad Software Inc, La Jolla, CA). See the online-only Data Supplement for details. Data are expressed as means±SEM. One-way ANOVA with Bonferroni multiple comparisons was used to determine significant differences. A P value of <0.05 was accepted as an indication of statistical significance.

#### Results

#### Blood Pressure in TgA Transgenic Rats at Early Gestation
Mean blood pressure values were not different between SD (n=6) and TgA (n=7) animals at 7 days of gestational age (98.8±2 versus 97±3 mm Hg; P>0.05).

#### Contractile Response to Ang II in UA at Early Gestation in Control Rats
Optimal diameters of the isolated UA segments used in these studies were not different between control and TgA (339±13 versus 317±4 μm; P>0.05). Maximal response to potassium chloride was increased in TgA compared with control UA (4.6±0.5 versus 3.3±0.3 mN/mm; P<0.05). In control arteries from SD rats, Ang II (10$^{-3}$−10$^{-7}$ mol/L) elicited a dose-dependent contraction that reached a plateau around 90% of
Arterial denudation increased maximal Ang II contraction and sensitivity (Figure 1A; Table 1; P<0.05), whereas preincubation of intact arteries with indomethacin increased Ang II sensitivity (Figure 1A; Table 1; P<0.05). Blockade of nitric oxide production with N\(^\omega\)-nitro-L-arginine methyl ester in intact arteries increased maximal contraction and sensitivity to Ang II (Figure 1A; Table 1; P<0.05).

### Contractile Response to Ang II in TgA UA at Early Gestation

In UAs from TgA rats, the contraction to Ang II was increased compared with SD controls. Maximal response and sensitivity values were higher in TgA UA (Figure 1B; Table 1; P<0.05). In denuded arteries, contraction was similar to intact arteries, whereas preincubation of intact arteries with indomethacin diminished maximal response and sensitivity compared with TgA intact. Compared with intact arteries, sensitivity to Ang II in indomethacin-treated arteries was lower in TgA (Figure 1E; Table 1; P<0.05). Blockade of nitric oxide production with N\(^\omega\)-nitro-L-arginine methyl ester in intact arteries did not alter Ang II contraction in TgA (Figure 1F; Table 1; P<0.05). Ang II contraction in SD and TgA UA was completely abolished by preincubation with losartan 10\(^{-6}\) mol/L (data not shown).

### Effects of Blocking Endogenous Production of AEA and 2-AG on Ang II Contraction

Preincubation with the FAAH blocker URB597 10\(^{-6}\) mol/L reduced maximal response to Ang II in SD UA, whereas preincubation with the MAGL blocker JZL184 10\(^{-6}\) mol/L had no effect (Figure 2A and 2B; Table 2). In contrast, both FAAH

### Table 1. Contractile Responses to Ang II in Uterine Arteries From SD and TgA Rats at Early Gestation

<table>
<thead>
<tr>
<th>Variables Measured</th>
<th>(\text{Ang II}<em>{\text{MAX}}, % K</em>{\text{MAX}})</th>
<th>(\text{pD}_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>91±5</td>
<td>8.60±0.08</td>
</tr>
<tr>
<td>TgA</td>
<td>109±4*</td>
<td>8.86±0.08*</td>
</tr>
<tr>
<td>Denuded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>150±17\†</td>
<td>9.14±0.2\†</td>
</tr>
<tr>
<td>TgA</td>
<td>108±4*</td>
<td>8.87±0.13</td>
</tr>
<tr>
<td>+ Indomethacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>111±15</td>
<td>8.79±0.08\†</td>
</tr>
<tr>
<td>TgA</td>
<td>92±7\†</td>
<td>8.54±0.03\†</td>
</tr>
<tr>
<td>+ L-NAME</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>128±8\†</td>
<td>9.28±0.17\†</td>
</tr>
<tr>
<td>TgA</td>
<td>110±13</td>
<td>8.82±0.01</td>
</tr>
</tbody>
</table>

Maximal response to Ang II (\(\text{Ang II}_{\text{MAX}}\)) is expressed as \(\% K_{\text{MAX}}\) and sensitivity as \(\text{pD}_2\). Results are shown for intact, denuded arteries and arteries preincubated with indomethacin (10\(^{-5}\) mol/L) or N\(^\omega\)-nitro-L-arginine methyl ester (L-NAME) 10\(^{-4}\) mol/L (+L-NAME) as indicated. Ang II indicates angiotensin II; SD, Sprague–Dawley; and TgA, hAGT×hREN.

*P<0.05 vs SD;
†P<0.05 vs intact arteries.
and MAGL blockade inhibited maximal response and sensitivity to Ang II (Figure 3A and 3B; Table 3) in TgA UA. The inhibitory effect of blocking FAAH on Ang II in TgA UA was not modified by concomitant blockade of the CB1 receptor with SR141716A 10−6 mol/L. We observed no effect of blocking CB1 receptor alone on Ang II contraction (Figure 4; Table 3).

Expression of MAGL and FAAH in Control and TgA UA at Early Gestation

Immunolocalization of FAAH and MAGL revealed the presence of both enzymes in UA. FAAH was localized predominantly in the endothelium (Figure 5B and 5E), whereas MAGL was detected predominantly in smooth muscle cells (Figure 5C and 5F). A similar intensity of FAAH and MAGL signals was observed in both control and TgA arteries with stronger staining for MAGL than FAAH in both groups. (Figure S1 in the online-only Data Supplement).

Discussion

For the first time we report that the transgenic hAGT×hREN rat, a rodent model that mimics many features of preeclampsia, displayed increased contraction to Ang II in the UA at early gestation, before the onset of blood pressure rise. Herein, we also present additional novel findings of functional interactions between RAS and endocannabinoid system in the uterine vasculature, with an increased role for the endocannabinoid system in early gestation, before the pathogenic syndrome is established.

Increased vascular sensitivity to Ang II was reported in human pregnancies with an increased risk of developing hypertension.2−4 We observed this effect at early gestation in rat, a rodent model that mimics many features of preeclampsia. The presence of agonistic autoantibodies to the angiotensin 1 (AT1) receptor (AT1-AA) is another characteristic of human preeclampsia that this model replicates.29 AT1-AA have been shown to modulate vascular responses in systemic and placental vessels29,30 and increase Ang II sensitivity in pregnant rats.31 The effects of AT1-AA on blood pressure and Ang II sensitivity in isolated vessels of late pregnant rats required the presence of both AT1-AA and Ang II,32 and these vascular responses are blocked by the epitope peptide AFHYEQ.32 Because AT1-AA seem to be secondary to a hypoxic/ischemic vascular disorder,33 it is less probable that AT1-AA are playing a role in the increased Ang II sensitivity in early gestation TgA UA before the pathogenic syndrome is established.

The effects of endothelium denudation or inhibition of nitric oxide production on Ang II contraction were lower in TgA than in controls, suggesting an endothelium-derived dysfunction in TgA UA, consistent with a previous report in this model at late gestation.6 Ang II contraction was dependent on prostanoid generation. Indomethacin preincubation shifted Ang II contraction to the left in arteries from control animals and to the right in arteries from TgA animals. This suggests a change in the type of prostanoids being generated in UA: from vasodilatory prostanoids in control UA to vasoconstrictor prostanoids in TgA arteries. This observation is consistent with the reported imbalance in the production of prostanoids described in preeclampsia: endothelial production of pros-tacyclin is decreased, whereas thromboxane A2 levels are increased.

Because increased sensitivity to Ang II constitutes a hallmark for the development of a hypertensive pregnancy, our results contribute to the characterization of the hAGT×hREN rat as a suitable model of preeclampsia. The presence of agonistic autoantibodies to the angiotensin 1 (AT1) receptor (AT1-AA) is another characteristic of human preeclampsia that this model replicates.29 AT1-AA have been shown to modulate vascular responses in systemic and placental vessels29,30 and increase Ang II sensitivity in pregnant rats.31 The effects of AT1-AA on blood pressure and Ang II sensitivity in isolated vessels of late pregnant rats required the presence of both AT1-AA and Ang II,32 and these vascular responses are blocked by the epitope peptide AFHYEQ.32 Because AT1-AA seem to be secondary to a hypoxic/ischemic vascular disorder,33 it is less probable that AT1-AA are playing a role in the increased Ang II sensitivity in early gestation TgA UA before the pathogenic syndrome is established.

Table 2. Effects of FAAH and MAGL Blockade on Contractile Responses to Ang II in Uterine Arteries From SD Rats at Early Gestation

<table>
<thead>
<tr>
<th>Variables Measured</th>
<th>Control</th>
<th>+URB595</th>
<th>+JZL184</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II Max (%) K Max</td>
<td>91±5</td>
<td>61±12*</td>
<td>85±20</td>
</tr>
<tr>
<td>pD2</td>
<td>8.60±0.08</td>
<td>8.68±0.14</td>
<td>8.68±0.15</td>
</tr>
</tbody>
</table>

Maximal response to Ang II (Ang II Max) is expressed as % K Max and sensitivity as pD2. Results are shown for non-treated arteries (control), FAAH blockade (URB597, 10−6 mol/L) and MAGL blockade (JZL184, 10−6 mol/L) in SD UA. Ang II indicates angiotensin II; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; and SD, Sprague–Dawley.

*p<0.05 vs control arteries.

Table 3. Effects of FAAH and MAGL Blockade on Contractile Responses to Angiotensin II in Uterine Arteries From TgA Rats at Early Gestation

<table>
<thead>
<tr>
<th>VariablesMeasured</th>
<th>TgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Ang II Max (%) K Max</td>
<td>109±4</td>
</tr>
<tr>
<td>pD2</td>
<td>8.86±0.08</td>
</tr>
</tbody>
</table>

Maximal response to Ang II (Ang II Max) is expressed as % K Max and sensitivity as pD2. Results are shown for FAAH blockade (URB597, 10−6 mol/L), MAGL blockade (JZL184, 10−6 mol/L), CB2 blockade (+SR, SR141716A 10−6 mol/L), and concomitant FAAH and CB2 blockade (+URB595+SR) in TgA UA. Ang II indicates angiotensin II; CB2, cannabinoid receptor 2; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; TgA, hAGT×hREN; and UA, uterine artery.

*p<0.05 vs control arteries.
increased, an effect proposed to be mediated by increased reactive oxygen species generation in preeclamptic pregnancies. Interestingly, prostacyclin levels decreased months before the clinical onset of preeclampsia. TgA rats seem to reproduce this imbalance before the installation of the hypertensive phenotype. Recent reports also support the contribution of CYP subfamily 2J polypeptide 2 epoxygenase to the effects on blood pressure, albuminuria, and vascular function observed in the TgA rat at late gestation.

The actions of endocannabinoids in the vasculature are complex and not explained by a single mechanism or target tissue. An endothelium-dependent vasodilatory action involving a CB receptor-dependent pathway, as well as nitric oxide generation and K⁺ channels activation has been demonstrated for endocannabinoids. Although the development of highly specific blockers of FAAH and MAGL has allowed the study of the mechanisms mediated by AEA and 2-AG, one limitation of our study is that our approach to examine endocannabinoid-mediated responses relies exclusively on pharmacological blockade. Using this approach, however, an efficient catabolism of AEA and 2-AG by their corresponding hydrolyzing enzymes has been demonstrated in vascular tissues. Although the development of highly specific blockers of FAAH and MAGL has allowed the study of the mechanisms mediated by AEA and 2-AG, one limitation of our study is that our approach to examine endocannabinoid-mediated responses relies exclusively on pharmacological blockade. Using this approach, however, an efficient catabolism of AEA and 2-AG by their corresponding hydrolyzing enzymes has been demonstrated in vascular tissues. Therefore, in the mesenteric artery AEA and 2-AG are able to relax preconstricted arteries, an effect that is potentiated by blockade of the hydrolases FAAH and MAGL. We used the enzyme blockers, URB597 and JZL184, at concentrations previously shown effective in vascular studies; that is, URB597 10⁻⁶ mol/L abolished AEA vascular actions in rat gracilis arteries. Our observations of a reduced Ang II contraction in TgA UA on blockade of FAAH or MAGL suggest the induction of a vasodilatory mechanism or the attenuation of vasoconstrictor mediators. Studies of coronary arteries showed that the vasodilatory effects of endocannabinoids, particularly AEA, are mediated by their catabolism to arachidonic acid and subsequent conversion to vasodilatory eicosanoids such as prostacyclin or epoxyeicosatrienonic acids. Interestingly, these effects in coronary arteries are not mediated by the CB₁ receptor, in agreement with the absence of effects of CB₁ receptor antagonism on the reduction of Ang II contraction induced by FAAH blockade in TgA UA. Vasodilatory effects of AEA in rat aorta, as well as AEA-mediated nitric oxide production in endothelial cells, have both been described as being independent of CB₁ or CB₂ receptors, making it possible to explain the involvement of non-CB₁/CB₂-mediated vasodilatation in the reduction of Ang II contraction that we observed after blockade of endocannabinoids degradation.

It has also been described that vasodilatory prostanooids released by endocannabinoid hydrolysis would cause vasorelaxation in part via opening K⁺ channels. Given the important effect of blockade of prostaglandin generation we observed in TgA, it is conceivable that similar mechanisms may be operating in UA. Thus, increased endocannabinoid levels, via inhibition of their degradation, would increase vasodilatory prostanooids in UA that in turn would limit Ang II contraction. Our immunohistochemistry data indicate differences in tissue localization for FAAH and MAGL in UA. FAAH was mainly localized to the cells lining the arterial lumen consistent with endothelial location and sparsely located in smooth muscle cells in SD and TgA arteries. MAGL was observed along the whole arterial wall spanning both endothelial and smooth muscle cells. Previous evidence indicated the presence of FAAH in

**Figure 3.** Effects of blocking anandamide and 2-arachidonoylglycerol hydrolysis on uterine artery (UA) angiotensin II (Ang II) contraction in transgenic hAGT-xhREN (TgA) arteries. Contraction to Ang II in isolated UA from TgA rats (○, n=9) in the presence of fatty acid amide hydrolase (URB597 10⁻⁶ mol/L, ●, n=9), CB of blockade (SR141716A 10⁻⁶ mol/L, △, n=5) or FAAH plus CB blockade (▲, n=3). See Table 3 for analysis.

**Figure 4.** Effects of blocking the CB₁ receptor and anandamide hydrolysis on uterine artery (UA) angiotensin II (Ang II) contraction in transgenic hAGT-xhREN (TgA) arteries. Contraction to Ang II in isolated UA from TgA rats (○, n=9) in the presence of fatty acid amide hydrolase (URB597 10⁻⁶ mol/L, ●, n=9), CB, blockade (SR141716A 10⁻⁶ mol/L, △, n=5) or FAAH plus CB, blockade (▲, n=3). See Table 3 for analysis.

**Figure 5.** Immunolocalization of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) in uterine artery (UA) from control and hAGT-xhREN (TgA) rats at early gestation. UA segments from control (Sprague-Dawley [SD], A–C, n=5) or TgA rats (D–F, n=5) were incubated with anti-FAAH (B and E) or anti-MAGL (C and F) antibodies. Representative pictures from each group are shown. Signals were developed by incubation with 3, 3'-diaminobenzidine (brown) and counterstained with hematoxylin (nuclei, blue). Control incubations for SD (A) and TgA UA (D) did not contain primary antibodies (scale bar, 200 μm).
bovine coronary arteries, kidney endothelial cells, and human umbilical vein endothelial cells. Recently, FAAH expression has also been reported in arterial smooth muscle cells. The localization we observed for FAAH agrees with the endothelium-dependent effects described for the FAAH blocker URB597. As a key mediator of 2-AG degradation, MAGL is ubiquitously expressed. In the vasculature, 2-AG relaxation of mesenteric artery has been shown to be endothelium-independent, in agreement with the localization in smooth muscle cells we observed in UA. In both SD and TgA UA it seems that the staining for MAGL was higher than what we observed for FAAH, and this may be related to the reported greater levels of 2-AG compared with AEA observed in the rodent uterus. Thus, a higher expression of MAGL would contribute to modulate 2-AG levels. Similar intensity of immunohistochemistry signals between arterial segments from control and TgA animals suggests that the observed differences in vascular effects are not related to different levels of expression of FAAH or MAGL in arteries from preeclamptic animals.

In terms of the possible prostanoid compounds involved in the vascular responses to endocannabinoid hydrolysis, AEA and 2-AG are also substrates of the enzyme cyclo-oxygenase-2. Thus, if endocannabinoids are not able to be metabolized by FAAH or MAGL because of their blockade, more substrate would be available for cyclo-oxygenase-2. AEA and 2-AG are oxidized by cyclo-oxygenase-2 to prostaglandins-ethanolamides (prostamides) and prostaglandins-glyceryl esters, respectively. These compounds do not interact with cannabinoid or prostaglandin receptors, suggesting additional pathways involved in their cellular function. Interestingly, the effects of URB597 on myogenic tone of mouse gracilis arteries are inhibited by indomethacin, suggesting the involvement of prostaglandins-ethanolamides and prostaglandins-glyceryl esters on endocannabinoid-derived vascular responses. In the mesenteric artery the vasodilatory responses to endocannabinoids are endothelium-dependent; however, we did not test the influence of endothelium on the responses to FAAH and MAGL blockers. Thus, the role of the prostanoids families of compounds in the regulation of the vascular actions of Ang II by endocannabinoids warrants further investigation.

Perspectives

We observed a functional interaction between RAS and endocannabinoid system in the uterine circulation. As in the clinical manifestation of preeclampsia, an increased role of prostanoids in the vasculature may help explain the enhanced effects of endocannabinoids we observed on Ang II contraction. The vascular response to Ang II is a clinical target for antihypertensive therapies, and because FAAH inhibitors have no hemodynamic effects under normotensive conditions, our results also point to the use of endocannabinoid degradation blockers as effective pharmacotherapies for hypertension.

Acknowledgments

We gratefully acknowledge grant support in part provided by Unifi, Inc Greensboro, NC and Farley-Hudson Foundation, Jacksonville, NC.

Sources of Funding

This work was funded by the National Heart, Lung, and Blood Institute P01-HL51952.

Disclosures

None.

References

19. Feledziak M, Lambert DM, Marchand-Brynaert J, Muccioli GG. Inhibitors of the endocannabinoid-degrading enzymes, or how to increase
endocannabinoid’s activity by preventing their hydrolysis. Recent Pat CNS Drug Discov. 2012;7:49–70.
43. PrantPF;HillardCJ;EdgemonWD;CampbellWB. N-arachidonylethanolamine relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor. Am J Physiol. 1998;274(1 pt 2):H375–H381.

Novelty and Significance

What Is New?

- Increased contraction to angiotensin II in the uterine artery at early gestation in a rat model of preeclampsia.
- The endogenous cannabinoids anandamide and 2-arachidonoylglycerol reduced contraction of the uterine artery to angiotensin II.

What Is Relevant?

- By increasing levels of endogenous cannabinoids, the inhibition of the enzymes fatty acid amide hydrolase and monoacylglycerol lipase would counteract enhanced contractile responses to angiotensin II.
- These effects are enhanced in a model of preeclampsia at early gestation, before the hypertensive phenotype initiates.

Summary

A functional interaction between the renin–angiotensin system and the endocannabinoid system was observed in the uterine circulation.
Increased Angiotensin II Contraction of the Uterine Artery at Early Gestation in a Transgenic Model of Hypertensive Pregnancy Is Reduced by Inhibition of Endocannabinoid Hydrolysis

Victor M. Pulgar, Liliya M. Yamaleyeva, Jasmina Varagic, Carolynne M. McGee, Michael Bader, Ralf Dechend, Allyn C. Howlett and K. Bridget Brosnihan

Hypertension. published online June 16, 2014;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2014/06/16/HYPERTENSIONAHA.114.03633

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/06/16/HYPERTENSIONAHA.114.03633.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
INCREASED ANG II CONTRACTION OF THE UTERINE ARTERY AT EARLY GESTATION IN A TRANSGENIC MODEL OF HYPERTENSIVE PREGNANCY IS REDUCED BY INHIBITION OF ENDOCANNABINOID HYDROLYSIS

Victor M. Pulgar\textsuperscript{1,2,4}, Liliya M. Yamaleyeva\textsuperscript{1}, Jasmina Varagic\textsuperscript{1}, Carolynne M. McGee\textsuperscript{1}
Michael Bader\textsuperscript{5,6}, Ralf Dechend\textsuperscript{6}, Allyn C. Howlett\textsuperscript{3}, K. Bridget Brosnihan\textsuperscript{1}

\textsuperscript{1}Hypertension & Vascular Research Center, \textsuperscript{2}Department of Obstetrics & Gynecology, \textsuperscript{3}Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC; \textsuperscript{4}Biomedical Research Infrastructure Center, Winston Salem State University, Winston-Salem, NC; \textsuperscript{5}Max Delbrück Center for Molecular Medicine, Berlin-Buch and \textsuperscript{6}Charité University Hospital Berlin, Berlin, Germany.

Short title: AEA and 2-AG modulate Ang II contraction

**Corresponding Author**

Victor M. Pulgar, PhD
Hypertension & Vascular Research Center
Wake Forest School of Medicine
Medical Center Blvd
Winston Salem, NC 27575
Phone: (336) 716-5149
Fax: (336) 761-6937
Email: vmpulgar@wakehealth.edu
Supplemental Data

MATERIAL AND METHODS

Animals
12-week-old male and female Sprague-Dawley (SD) rats were obtained from Charles River Laboratory (Wilmington, MA). Aged-matched transgenic hAGN females and hREN males were obtained from the colony maintained by the Hypertension and Vascular Research Center at Wake Forest School of Medicine, Winston Salem, NC. Breeders for the transgenic animal colony were obtained from Dr. Micheal Bader, Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany. Female hAGN transgenic rats, when mated with a hREN transgenic male (hAGN×hREN), develop hypertension and proteinuria in the second half of pregnancy (preeclamptic TgA, n=9). The animals were housed at a constant room temperature, humidity and light cycle (12:12h light dark), with a global 18% protein extruded rodent chow (Harlan Laboratories, Indianapolis, IN) and water available ad libitum. Pregnant animals were studied on day 7 of gestation, where Day 0 is defined as presence of a vaginal plug.

Blood Pressure Measurements
Blood pressure measurements were performed using an intra-arterial catheter placed into left femoral artery under 2.5% of isoflurane anesthesia. Blood pressure signals were acquired for 10 minutes using PowerLab® 8/35 and analyzed using LabChart® v7 (ADInstruments, Inc., Colorado Springs, CO). Data were averaged for each animal and reported as mean blood pressure and as mean ± SEM.

Tissue Collections
At 7 days of gestational age, animals were euthanized by decapitation. For reactivity studies both uterine horns were carefully removed and the main uterine artery (UA) was isolated and cleaned of fat and connective tissue while kept in cold Krebs-Henseleit Buffer (KHB) containing (in mmol/l): NaCl 118, KCl 4.47, NaHCO3 25, KH2PO4 1.2, MgSO4 1.2, CaCl2 ·2H2O 2.5, glucose 5.5. For immunohistochemistry studies, some UA segments were incubated in buffered formalin for 24 h and then stored in ethanol 70%. Arteries were sectioned in rings of 5 µm (AML Laboratories, Baltimore, MD).

Vascular Reactivity
The myograph organ bath (5 ml) was filled with KHB maintained at 37°C and aerated with 95% O2/5% CO2. The vessels were washed and incubated for 30 min before the normalization procedure was performed. Each arterial segment was stretched in a stepwise manner and the internal circumference and corresponding wall tension at each stretch were calculated and plotted to produce a resting wall tension–internal circumference curve for that particular artery using the DMT Normalization Module (ADInstruments). Arterial segments were normalized to 0.9•L100, with L100 being the internal circumference the vessels would have if they were exposed to a transmural pressure of 100 mm Hg, as described 27. Optimal diameters (OD) were calculated as OD=0.9•L100/π. After obtaining the optimal diameter, a 30-min equilibration period preceded the addition of test substances.
Immunohistochemistry

Fixed arterial rings were deparaffinized, rehydrated and unspecific binding sites were blocked with 10% normal goat serum, 0.1% Triton X-100 and 0.1% BSA in phosphate buffered saline (PBS) for 30 min at room temperature. Sections were incubated with primary antibodies (mouse monoclonal anti-FAAH 1:300 dilution; rabbit polyclonal anti-MAGL 1:200 dilution, both from Abcam, Cambridge, MA) overnight. After washing 3 times in PBS incubations with the corresponding secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG, both at 1:400 dilutions) were performed in blocking solution during 1 h at room temperature. Signals were developed using the ABC Vectastain kit (Vector Labs, Burlingame, CA) and sections were counterstained with hematoxylin.

Drugs

Angiotensin II, Nω-nitro-L-arginine methyl ester (L-NAME) and indomethacin were purchased from Sigma (St Louis, MO). Stock solutions for Ang II and L-NAME were prepared in distilled water. Indomethacin was dissolved in 50 mM NaCO₃ in KHB. URB597 and JZL184 (Cayman Chemical Co, Ann Harbor, MI) were dissolved in DMSO with the final DMSO concentration in the bath being 0.03%. Preliminary experiments showed no effects of 0.03% DMSO on vascular responses. SR141716A (Cayman Chemical Co, Ann Harbor, MI) stock solutions were prepared in ethanol. All other chemical reagents were purchased from Sigma.

Data Analysis

Maximal contractile responses to KCl were expressed in absolute values, whereas maximal responses to Ang II were expressed as a percent of the maximal response induced by KCl (%KMAX). Concentration-response curves for Ang II were analyzed as previously described 25. Concentration-response curves for Ang II were analyzed as previously described by fitting individual experimental data to a logistic curve to determine the maximal response and sensitivity. The curve was of the form \( Y = \text{bottom} + \left( \frac{\text{top} - \text{bottom}}{1 + 10^{(\text{LogEC}_{50} - X) \times \text{Hill Slope}}} \right) \) where \( X \) is the logarithm of the concentration and \( Y \) is the response; the sensitivity values reported are derived from these fits. The contractile response to KCl was expressed in mN/mm as units of arterial wall tension (AWT) (AWT = force / 2 × length of vessel). For each arterial segment, the maximal response to KCl was calculated, and the response to Ang II was expressed as %KMAX. Sensitivity was expressed as pD₂ (pD₂ = -log [EC₅₀]) with EC₅₀ being the concentration of agonist producing 50% of the maximal response.
RESULTS

Supplemental Figure S1

Quantification of immunohistochemistry signals in SD and TgA UA.
Higher intensity of signal for FAAH as compared to MAGL staining in SD (n=5) and TgA (n=5) UA. There were no group differences. a.u. arbitrary units. * p<0.05 vs FAAH