RESEARCH ARTICLE | Cell-to-Cell Communication and Signaling Pathways

Myoendothelial gap junctions mediate regulation of angiopoietin-2-induced vascular hyporeactivity after hypoxia through connexin 43-gated cAMP transfer

Jing Xu, Guangming Yang, Tao Li, and Liangming Liu

State Key Laboratory of Trauma, Burns and Combined Injury, Second Department of Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing, People’s Republic of China

Submitted 29 December 2016; accepted in final form 15 June 2017

Xu J, Yang G, Li T, Liu L. Myoendothelial gap junctions mediate regulation of angiopoietin-2-induced vascular hyporeactivity after hypoxia through connexin 43-gated cAMP transfer. Am J Physiol Cell Physiol 313: C262–C273, 2017. First published June 21, 2017; doi:10.1152/ajpcell.00369.2016.—Angiopoietin-2 (Ang-2) contributes to vascular hyporeactivity after hemorrhagic shock and hypoxia through upregulation of inducible nitric oxide synthase (iNOS) in a vascular endothelial cell (VEC)-specific and Ang-2/Tie2 receptor-dependent manner. While iNOS is primarily expressed in vascular smooth muscle cells (VSMCs), the mechanisms of signal transfer from VECs to VSMCs are unknown. A double-sided coculture model with VECs and VSMCs from Sprague-Dawley rats was used to investigate the role of myoendothelial gap junctions (MEGJs), the connexin (Cx) isoforms involved, and other relevant mechanisms. After hypoxia, VSMCs treated with exogenous Ang-2 showed increased iNOS expression and hyporeactivity, as well as MEGJ formation and communication. These Ang-2 effects were suppressed by the MEGJ inhibitor 18-glycyrrhetic acid (18-GA), Tie2 siRNA, or Cx43 siRNA. Reagents antagonizing CAMP or protein kinase A (PKA) in VECs inhibited Cx43 expression in MEGJs, decreasing MEGJ formation and associated communication, after hypoxia following Ang-2 treatment. The increased cAMP levels in VSMCs and transfer of Alexa Fluor 488-labeled cAMP from VECs to VSMCs, after hypoxia following Ang-2 treatment, was antagonized by Cx43 siRNA. A CAMP antagonist added to VECs or VSMCs inhibited both increased iNOS expression and hyporeactivity in VSMCs subjected to hypoxia following Ang-2 treatment. Based on these findings, we propose that Cx43 was the Cx isoform involved in MEGJ-mediated VEC-dependent regulation of Ang-2, which induces iNOS protein expression and vascular hyporeactivity after hypoxia. Cx43 was upregulated by cAMP and PKA, permitting cAMP transfer between cells.

VASCULAR HYPOREACTIVITY, defined as decreased vascular reactivity to vasoactive reagents, has been observed in severe clinical cases such as trauma and shock and significantly influences patient progress and prognosis (23, 39). We have previously shown that angiopoietin-2 (Ang-2), an angiogenesis regulator (28), was significantly upregulated after hemorrhagic shock or hypoxia and was involved in the development of vascular hyporeactivity by promoting inducible nitric oxide synthase (iNOS) expression in a Tie2 receptor-dependent manner (38). However, iNOS is primarily expressed in vascular smooth muscle cells (VSMCs), not vascular endothelial cells (VECs). In contrast, Ang-2 is well known to be secreted from the Weibel-Palade bodies of VECs and act on the Tie2 receptor, which is specifically located on the VEC membrane. This led to the question of how Ang-2-mediated signals transfer from VECs to VSMCs.

Myoendothelial gap junctions (MEGJs), comprising connexin (Cx) proteins, are hydrophilic membrane channel structures between adjacent VECs and VSMCs that provide contact between the two cell types. MEGJs allow passage of small molecules, including second messengers, and electrical currents between VECs and VSMCs. Thus they play a critical role in transverse signaling in the vascular bed during processes such as angiogenesis, vascular cell growth and differentiation, vascular metabolism, and maintenance of vascular resistance (10, 26).

In previous studies, MEGJs participated in VEC-dependent vascular contraction and relaxation. For example, MEGJ-mediated communication contributed to smooth muscle cell hyperpolarization and dilation induced by endothelium-dependent hyperpolarizing factor (EDHF) in the middle cerebral artery of rats (30). Additionally, the gap junction inhibitory peptides 40Gap27, 37,43Gap27, and 43Gap26 decreased endothelium-dependent contractions in the aortas of spontaneously hypertensive rats (32). These findings suggest that MEGJ-mediated communication helps regulate signal transfer from VECs to VSMCs and subsequently VSMCs contractile function.

Although MEGJs may mediate via Ang-2 VEC-dependent regulation of iNOS expression and vascular hyporeactivity in VSMCs, the Cx isoforms involved in the process and the detailed mechanisms remain unknown. Only one relevant study has shown that the soluble and stable form of angiopoietin-1 (Ang-1), cartilage oligomeric matrix protein (COMP)-Ang-1, could inhibit Cx43 expression, promote angiogenesis, and suppress inflammation in the sciatic nerves of ob/ob mice (17). Both Ang-1 and Ang-2 are important members of the angiopoietin family, sharing a similar overall structure and binding to the Tie2 receptor competitively with comparable affinities. However, they show opposing effects on the regulation of blood vessel function (28). Based on these previous findings, we hypothesized that Ang-2 regulates Cx in MEGJs, to change its communication function to enable transfer of
vascular contraction regulatory information from VECs to VSMCs.

To address this hypothesis, we used cultured rat VECs and VSMCs in a double-sided coculture model. We investigated the intermediary role of MEGJs in the transfer of Ang-2 signals from VECs to VSMCs after hypoxia, which induced iNOS expression in VSMCs and vascular hyporeactivity. Furthermore, we identified the Cx isofrom involved and further elucidated the relevant mechanisms.

MATERIALS AND METHODS

Ethical Approval

This research used protocols approved by the Research Council and Animal Care and Use Committee of the Research Institute of Surgery, Daping Hospital, Third Military Medical University (Chongqing, China) and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell Culture and the Double-Sided Coculture System

Male and female Sprague-Dawley rats (200–250 g) were used to isolate VECs and VSMCs. The rats were euthanized by cervical dislocation following 1–4% isoflurane anesthesia, and the VECs and VSMCs were obtained from the superior mesenteric arteries by an explant technique as reported previously (27, 38). VECs and VSMCs between passages 2 and 4 from different cell batches were used for independent experiments.

The double-sided coculture model, with VECs and VSMCs grown on each side of the membrane, was constructed as described by Isakson and Duling (13). Briefly, a polyester Transwell insert (Corning, Corning, NY), precoated with FBS, was seeded with VSMCs on its reverse side at 1 × 10^5 cells/insert. After 1 day of culture, the insert was turned over, inserted into a six-well plate, and seeded with VECs on its upper side at 2 × 10^5 cells/insert. This coculture system was cultured for 72 h in a 5% CO_2-95% air incubator at 37°C to form monolayers of VECs and VSMCs on each side. Antibodies against vascular endothelial (VE)-cadherin and α-smooth muscle (SM) actin were used for immunocytochemistry to detect VECs and VSMCs, respectively.

For hypoxia treatment, the cells were put into a hypoxia compartment, bubbled with hypoxic gas (95% N_2-5% CO_2) at 3 l/min for 15 min, followed by a 10-min rest. This procedure was repeated five times until the O_2 concentration was below 0.2% (38, 40). The cells were then cultured in the hypoxia conditions for the indicated duration.

Measurement of VSMC Contractile Reactivity in the Coculture System

After 3 days of culture, the cells were treated with antagonists and/or hypoxia. Then, fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used to measure VSMC contractile reactivity induced by the agonist norepinephrine (NE) (38). To exclude FITC-BSA leakage, such as that caused by change in barrier function, and to better show the NE-induced VSMCs contraction, the contractile reactivity value for each sample was calculated using two inserts: a control coculture insert (without NE treatment) and an experimental coculture insert (with NE treatment). First, FITC-BSA (10 mg/ml) was added to the upper well (VEC side) of the coculture system. Then, NE (1 × 10^{-9} mol/L, in the experimental sample) or medium (the same volume as NE, in the control sample) was added to the lower well (VSMC side). Eight aliquots from the medium were collected from the lower well at 15-min intervals. Fluorescence intensity values of these aliquots were read, and the cumulative fluorescence permeation rate after NE treatment (normalized to the value of the control system without NE treatment) was calculated, reflecting the VSMC contractile reactivity.

Isolation of Protein Fractions from VECs, VSMCs, and MEGJs

After 3 days of culture, cells in the coculture system were treated with antagonists and/or hypoxia. The VECs and VSMCs fractions were obtained by scraping the cell monolayers into lysis buffer. The scraped Transwell membranes were then sheared into pieces and vortexed in lysis buffer to collect the MEGJ fraction. After 30 min of lysis, the extracted proteins were collected by centrifuging the lysates at 12,000 g for 15 min at 4°C, as described by Heberlein et al. (9).

Protein Expression Analysis

Protein expression was analyzed by standard Western blotting. The protein lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. These membranes were then incubated with primary and secondary antibodies sequentially. Protein bands were visualized by chemiluminescence, and their optical density values were determined. Signals of iNOS protein bands were normalized to those of β-actin. Signals of Cx43 and Cx40 protein bands from MEGJ samples were normalized to those of GAPDH, as described previously (9).

Immunocytochemistry

Immunocytochemistry was used to identify VECs and VSMCs and to analyze iNOS expression. After 3 days of culture and hypoxia treatment, each coculture system was washed with PBS, fixed in 4% parafomaldehyde for 30 min at 37°C, and then permeabilized and blocked by 0.25% Triton X-100 and 1% BSA for 30 min. Next, the indicated primary antibody (anti-rat VE-cadherin, anti-α-actin, or anti-rat iNOS) and its corresponding secondary antibody were sequentially added to the correct side of the coculture system. Finally, stained samples were washed and immunoflourescence was observed by fluorescence microscopy.

MEGJ Formation

MEGJ formation was examined by immunocytochemistry, as described previously (9). After treatment with antagonists and/or hypoxia, the cells in the coculture system were washed with PBS and fixed by 4% paraformaldehyde at 37°C for 30 min. The membranes were then removed from the Transwell inserts and cut into 10-µm thick frozen sections. The frozen sections were permeabilized and blocked by 0.25% Triton X-100 and 1% BSA for 30 min, followed by incubation with FITC-labeled phalloloidin at 37°C for 30 min. Images were obtained by a fluorescence microscope. MEGJs were stained with phalloloidin-labeled F-actin in the micropores across the Transwell membranes, and MEGJ formation was densitometrically quantitated by pixel intensity per square millimeter of the F-actin in the pore area across the membrane.

MEGJ Communication

MEGJ communication was monitored by dye transfer, based on methods described by Isakson and Duling (13). Briefly, VECs in the coculture system were loaded with sulforhodamine B (1 mg/ml) by a pinocytic uptake method, using Influx Pinocytic Cell-Loading Reagent. The coculture samples were allowed to stabilize for 10 min. Then, the Transwell membranes were collected, placed on a microscope slide and imaged with a Leica TCS-SP confocal microscope (Wetzlar, Germany) in the Z-stack scan mode. The Z-stack scan was from the VECs side to the VSMCs side along the micropore length axis in 0.13-µm steps. The sulforhodamine B intensity difference between the XY sections of the two sides was used to indicate dye transfer and MEGJ communication.
Fig. 1. Differences in inducible nitric oxide synthase (iNOS) protein expression in vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) after hypoxia and angiopoietin-2 (Ang-2) treatment. Immunocytochemistry staining of α-smooth muscle (α-SM) actin and vascular endothelial (VE)-cadherin was used to characterize the coculture models (A, n = 3, ×400). In the coculture system, iNOS protein expression after hypoxia showed a higher increase in VSMCs than in VECs, as demonstrated by immunofluorescent staining (B, n = 3, ×200) and Western blotting (C, n = 3). The increased iNOS expression in VSMCs after hypoxia was maintained by exogenous Ang-2 and suppressed by Ang-2 siRNA (D, n = 3). Scale bars = 50 μm (A) and 100 μm (B). *P < 0.05 vs. normal control of VECs; #P < 0.05 vs. normal control of VSMCs; @P < 0.05 vs. 4 h hypoxia group of VSMCs.
cAMP Transfer

Alexa Fluor 488 cAMP was used to show direct transfer of cAMP across the MEGJs, based on the method of Bopp et al. (2). VECs in the coculture systems were loaded with Alexa Fluor 488 cAMP (125 µmol/l) using Influx Pinocytic Cell-Loading Reagent and stabilized for 10 min. Then, the Transwell membranes were collected and imaged with a Leica TCS-SP confocal microscope in the Z-stack scan mode, similar to the detection of MEGJ communication. The Alexa Fluor 488 cAMP intensity difference between the XY sections of the VECs and VSMCs sides was used to evaluate cAMP transfer across the MEGJs.

cAMP Concentration and Protein Kinase A Activity

The cAMP concentration and protein kinase A (PKA) activity were measured with a cAMP enzyme immunoassay kit and PKA kinase activity kit, respectively, according to the manufacturers' instructions.

Antibodies and Reagents

Anti-VE-cadherin was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin and anti-GAPDH were from Boster Biological Technology (Pleasanton, CA). Anti-β-actin, anti-Cx43, anti-iNOS, the MEGJ inhibitor 18α-glycyrrhetinic acid (18α-GA), the protein kinase C (PKC) inhibitor staurosporine, the PKA inhibitor H-89, the protein kinase G (PKG) inhibitor KT-5823, the cAMP inhibitor Rp-8-Br-cAMP, cAMP enzyme immunoassay kit, exogenous Ang-2, FITC-BSA, and sulforhodamine B were from Sigma-Aldrich (St. Louis, MO). Anti-Cx40 and Src inhibitor SU6656 were from Merck (Darmstadt, Germany). Alexa Fluor 488 cAMP, Influx Pinocytic Cell-Loading Reagent, Cx43, Cx40, Tie2, and Ang-2 siRNAs, and FITC-labeled phalloidin were from Thermo Fisher (Waltham, MA). The PKA kinase activity kit was from Enzo Life Sciences (New York, NY).

Data analysis

Data are presented as means ± SD of n samples from independent experiments. The results were analyzed for statistical significance using Student’s t-test or one-way ANOVA, followed by post hoc Tukey’s test. Differences were considered significant at P < 0.05.

RESULTS

iNOS Protein Expression in VECs and VSMCs After Hypoxia and Ang-2 Treatment

Vascular cell coculture model. In the double-sided coculture model, the growth of VECs and VSMCs on each side of the

<table>
<thead>
<tr>
<th>Normal</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (42kDa)</td>
<td>0.225</td>
<td>0.45</td>
<td>0.675</td>
<td>0.9</td>
</tr>
<tr>
<td>Cx43 (43kDa)</td>
<td>0.175</td>
<td>0.35</td>
<td>0.525</td>
<td>0.7</td>
</tr>
<tr>
<td>Cx40 (40kDa)</td>
<td>0.125</td>
<td>0.25</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>GAPDH (37kDa)</td>
<td>0.125</td>
<td>0.25</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Protein expression of Cx43 (β-actin)

<table>
<thead>
<tr>
<th>Normal</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43 in VECs</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Cx43 in VSMCs</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Protein expression of iNOS (β-actin)

<table>
<thead>
<tr>
<th>Normal</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS (131kDa)</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>β-actin (42kDa)</td>
<td>0.125</td>
<td>0.25</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

120 min accumulative infiltration of FITC-BSA (%)

<table>
<thead>
<tr>
<th>Normal</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-BSA (%)</td>
<td>0.125</td>
<td>0.25</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Fig. 2. Protein expression of connexin (Cx) isoforms and iNOS expression, as well as VSMC contractile reactivity after hypoxia. The protein expression of Cx40 and Cx43 in myoendothelial gap junctions (MEGJs) (A, n = 3), and of iNOS in VSMCs (C, n = 3) gradually increased, while the VSMCs contractile reactivity (D, n = 4–5) decreased after hypoxia. The protein expression of Cx43 in VECs showed a 2-phase change after hypoxia, increased after 1 h, and then slightly decreased gradually; the expression of Cx43 in VSMCs was maintained at an increase after hypoxia (B, n = 3). *P < 0.05 vs. normal control.
membrane was confirmed by immunocytochemistry. The results showed expression of α-SM actin, but not VE-cadherin, on the reverse side of the membrane, suggesting specific growth of VSMCs. On the upper side of the membrane, VE-cadherin staining was observed, but not α-SM actin, suggesting specific growth of VECs (Fig. 1A).

* Differences in iNOS expression. In our coculture system, the immunofluorescent staining of iNOS was hardly detectable in normal VECs and VSMCs. However, after 4 h of hypoxia, it increased much more in VSMCs than in VECs (Fig. 1B). Western blotting showed similar changes in iNOS protein expression in VECs and VSMCs (P < 0.05, Fig. 1C). The increased iNOS protein expression in VSMCs after 4 h of hypoxia was maintained at a high level by exogenous Ang-2 (200 ng/ml) and was suppressed by Ang-2 siRNA (25 nmol/l) (P < 0.05, Fig. 1D; exogenous Ang-2 or Ang-2 siRNA was added to the VEC side of the coculture system before hypoxia).

Role of MEGJs in Ang-2-Regulated iNOS Protein Expression in VSMCs and Vascular Hyporeactivity After Hypoxia and Identification of the Cx Isoform Involved

Protein expression of Cx isoforms and iNOS expression, as well as VSMC contractile reactivity after hypoxia. In the double-sided coculture system, the protein expression of Cx40 and Cx43 in MEGJs (P < 0.05, Fig. 2A) and of iNOS in VSMCs (P < 0.05, Fig. 2C) gradually increased, while the VSMC contractile reactivity gradually decreased, after hypoxia (P < 0.05, Fig. 2D). The protein expression of Cx43 in VECs showed a two-phase change after hypoxia, increased after 1 h, and then slightly decreased gradually; its expression in VSMCs was maintained at an increase after hypoxia (P < 0.05, Fig. 2B), demonstrating the increased expression of Cx43 in the VECs precedes that occurring in the MEGJ and VSMC but falls to a steady elevated level as expression in the MEGJ and VSMC rise with time.

Effects of MEGJs and Cx isoform antagonists on iNOS expression in VSMCs and on VSMC contractile reactivity. The nonspecific MEGJ inhibitor 18α-glycyrrhetinic acid (18α-GA) or the specific Cx43 siRNA (50 nmol/l) or Tie2 siRNA (25 nmol/l) was added to the VEC side of the coculture system before hypoxia. These suppressed iNOS expression in VSMCs (P < 0.05, Fig. 3A) and improved VSMC contractile reactivity (P < 0.05, Fig. 3B), after 4 h of hypoxia following Ang-2 treatment. In contrast, Cx40 siRNA (25 nmol/l) had no significant effects.

Effects of Cx isoform antagonists on MEGJ formation and cell-cell communication. In previous studies, fluorescent phalloidin was used to show MEGJ formation, and sulforhodamine B transfer was used to show MEGJ-mediated communication (9, 13). Using these methods, we found that MEGJ formation increased after 4 h of hypoxia following Ang-2 treatment. This effect was suppressed by Cx43 siRNA or Tie2 siRNA, which were added to the VEC side of the coculture system before hypoxia (P < 0.05, Fig. 4A). Furthermore, MEGJ communication substantially increased after 4 h of hypoxia following Ang-2 treatment (P < 0.05). This was inhibited by Cx43 siRNA or Tie2 siRNA, which were added to the VEC side of the coculture system before hypoxia (P < 0.05, Fig. 4B). Neither phalloidin fluorescence nor dye transfer was significantly changed by Cx40 siRNA.

Mechanism Involved in the Intermediary MEGJ Effects

Regulatory molecule involved in the Ang-2-induced regulation of iNOS expression in VSMCs and VSMC contractile reactivity. H-89 (10 μmol/l), staurosporine (0.1 μmol/l), KT-5823 (1 μmol/l), and SU6656 (2 μmol/l) are antagonists of PKA, PKC, PKG, and Src, respectively. These have been previously reported to regulate Cx43 expression, phosphorylation, or communication functions (15). Each antagonist was separately added to the VEC side of the coculture system. Their effects on iNOS expression in VSMCs and on VSMC contractile reactivity after 4 h of hypoxia following Ang-2 treatment were determined to identify the relevant regulatory molecules. The results showed that only the PKA antagonist decreased iNOS protein expression in VSMCs (P < 0.05, Fig. 5A) and suppressed VSMC hyporeactivity (P < 0.05, Fig. 5B).

Changes in the cAMP-PKA pathway in VECs after hypoxia and Ang-2 treatment. In the double-sided coculture system, the cAMP concentration and PKA activity in VECs increased after 4 h of hypoxia (P < 0.05). Exogenous Ang-2 maintained the
A

Normal control          4h hypoxia

ANG2+4h hypoxia

ANG2                       Tie2 SiRNA

ANG2+4h hypoxia

Cx40 SiRNA              Cx43 SiRNA

Normal control          4h hypoxia

VECs                       VSMCs

ANG2+4h hypoxia

VECs                       VSMCs

Tie2 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs

Cx40 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs

Cx43 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs


Intensity Difference of
Sulforhodamine B
between
VECs and VSMCs

ANG2+4h hypoxia

B

Normal control

VECs                       VSMCs

4h hypoxia

VECs                       VSMCs

ANG2+4h hypoxia

VECs                       VSMCs

Tie2 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs

Cx40 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs

Cx43 SiRNA+ANG2+4h hypoxia

VECs                       VSMCs

Intensity Difference of
Sulforhodamine B
between
VECs and VSMCs

ANG2+4h hypoxia
cAMP concentration and PKA activity at a high level, but these effects were suppressed by Tie2 siRNA, which was added to the VEC side of the system before hypoxia (P < 0.05, Fig. 5C).

**Effects of cAMP-PKA pathway antagonists on Cx43 protein expression in MEGJs and on MEGJ formation and communication.** The cAMP or PKA antagonist, Rp-8-Br-cAMP (100 μmol/l), or H-89 (10 μmol/l), respectively, was added to the VEC side of the coculture system before hypoxia. These pretreatments suppressed Cx43 protein expression in MEGJs after 4 h of hypoxia following Ang-2 treatment (P < 0.05, Fig. 6A). The increased MEGJ formation (P < 0.05, Fig. 6B) and communication (P < 0.05, Fig. 6C), after 4 h of hypoxia following Ang-2 treatment, were also inhibited by these antagonists.

**Information molecule transferred across the MEGJs**

Changes in cAMP concentration in VECs and VSMCs after hypoxia and Ang-2 treatment. In separately cultured VECs and VSMCs, the cAMP levels significantly increased after 4 h of hypoxia following Ang-2 treatment. These cAMP levels were higher in VECs than in VSMCs (P < 0.05, Fig. 7A). In the double-sided coculture model with VECs and VSMCs, there was a smaller difference between the cAMP levels in VECs and VSMCs. Moreover, the increased cAMP concentration in VSMCs was inhibited by Cx43 siRNA, which was added to the VEC side of the coculture system before hypoxia (P < 0.05, Fig. 7B).

**Effects of Cx isoform antagonists on transfer of Alexa Fluor 488 cAMP across the MEGJs.** Loading Alexa Fluor 488 cAMP to show cAMP transfer, we found that labeled cAMP was significantly transferred from VECs to VSMCs after 4 h of hypoxia following Ang-2 treatment (P < 0.05). This transfer was inhibited by Cx43 siRNA, which was added to the VEC side of the coculture system before hypoxia (P < 0.05, Fig. 7C).

**Effects of cAMP antagonist added to VECs and VSMCs on iNOS protein expression in VSMCs and on vascular hyporeactivity.** The cAMP antagonist Rp-8-Br-cAMP (100 μmol/l) was added to the VECs or VSMCs in the coculture system, and the effects on iNOS protein expression in VSMCs and on VSMCs contractile reactivity were examined. The cAMP antagonist added to either side inhibited the increased iNOS protein expression in VSMCs (P < 0.05, Fig. 8A) and improved the VSMC hyporeactivity (P < 0.05, Fig. 8B) after 4 h of hypoxia following Ang-2 treatment.

**DISCUSSION**

We have previously reported that vascular contractile reactivity was substantially decreased after hemorrhagic shock or hypoxia, with increased Ang-2 levels promoting high iNOS expression and contributing to vascular hyporeactivity. The effects of Ang-2 were specific to VECs and were Tie2 receptor dependent (38). In the double-sided coculture model, iNOS expression increased more in VSMCs than in VECs, and the
increase in VSMCs was maintained by exogenous Ang-2 and was inhibited by Ang-2 siRNA, suggesting that the Ang-2 signal could be transferred from VECs to VSMCs through an unknown mechanism.

Our current study revealed that under hypoxia following Ang-2 treatment, the nonspecific MEGJ inhibitor 18α-GA or the specific Cx43 siRNA or Tie2 siRNA, suppressed the increased iNOS expression in VSMCs and the VSMCs hyporeactivity. Increased MEGJ formation and communication were also inhibited by Cx43 siRNA or Tie2 siRNA, suggesting that MEGJs mediated the VEC-dependent effect of Ang-2 on iNOS expression in VSMCs and on VSMC hyporeactivity after hypoxia. Cx43 was the responsible Cx isoform. We also found with antagonist experiments that PKA was implicated in the increased iNOS protein expression in VSMCs and the VSMC hyporeactivity. Consistently, the cAMP levels and PKA activ-
### Table

**Intensity Difference of Alexa Fluor 488 cAMP between VECs and VSMCs**

<table>
<thead>
<tr>
<th>Condition</th>
<th>VECs</th>
<th>VSMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>4h hypoxia</td>
<td>4h hypoxia</td>
<td>4h hypoxia</td>
</tr>
<tr>
<td>ANG2 +4h hypoxia</td>
<td>ANG2 +4h hypoxia</td>
<td>ANG2 +4h hypoxia</td>
</tr>
<tr>
<td>Cx40 SiRNA +ANG2 +4h hypoxia</td>
<td>Cx40 SiRNA +ANG2 +4h hypoxia</td>
<td>Cx40 SiRNA +ANG2 +4h hypoxia</td>
</tr>
<tr>
<td>Cx43 SiRNA +ANG2 +4h hypoxia</td>
<td>Cx43 SiRNA +ANG2 +4h hypoxia</td>
<td>Cx43 SiRNA +ANG2 +4h hypoxia</td>
</tr>
</tbody>
</table>

### Figure A

**cAMP Concentration (pmol/mg Protein)**

- Normal control
- 4h hypoxia
- ANG2 +4h hypoxia

### Figure B

**cAMP Concentration (pmol/mg Protein)**

- Normal control
- 4h hypoxia
- ANG2 +4h hypoxia
- Cx40 SiRNA
- Cx43 SiRNA

### Figure C

**Intensities of Alexa Fluor 488 cAMP**

- Normal control
- 4h hypoxia
- ANG2 +4h hypoxia
- Cx40 SiRNA +ANG2 +4h hypoxia
- Cx43 SiRNA +ANG2 +4h hypoxia
ity in VECs increased after hypoxia following Ang-2 treatment and their inhibitors suppressed the associated increases in Cx43 protein expression in MEGJs, and in MEGJ formation and communication. These findings suggest that Ang-2 regulated iNOS expression in VSMCs and VSMC hyporeactivity after hypoxia via a cAMP-PKA-Cx43-MEGJ communication pathway.

Next, we focused on identifying the signaling molecule passing through the MEGJs. We found that during hypoxia cAMP increased more in VECs than in VSMCs when cultured separately, while the difference was smaller in the coculture model. Moreover, the hypoxia-dependent cAMP increase in VSMCs in the coculture model was suppressed by Cx43 siRNA. Loaded Alexa Fluor 488 cAMP was transferred from VECs to VSMCs after hypoxia following Ang-2 treatment, which was inhibited by Cx43 siRNA. Furthermore, a cAMP antagonist inhibited the increase in iNOS protein expression and the VSMC hyporeactivity. These results suggest that cAMP was the signaling molecule crossing the MEGJs and mediating the VEC-dependent regulation of Ang-2 after hypoxia.

An interesting observation in our study was that iNOS production was greater in VSMCs than in VECs after hypoxia. We further showed that the signal inducing increased iNOS expression in VSMCs was transferred from VECs. In fact, the phenomenon of cell type-associated differences in iNOS expression was also reported in other studies. For example, MacNaul et al. (25) and Linscheid et al. (22) reported that under specific inflammatory conditions, such as after cytokine or lipopolysaccharide treatment, NO production switched from endothelial NOS in the endothelium to iNOS in the smooth muscle. Wen et al. (35) have reported that interleukin-1β stimulation induced iNOS gene expression in rat VECs and VSMCs, and the response in VSMCs was greater than that in VECs. After carotid angioplasty in pig, balloon injury to the blood vessel luminal surface was associated with a marked increase in iNOS, detected both by gene expression and activity, throughout the arterial media within VSMCs (1). A possible explanation of such a phenomenon is that because VECs line the intima and form a barrier between the blood and the vascular wall, they are first to receive the signals of ischemia, hypoxia, inflammation, or mechanical injury and then transfer these exogenous stimuli through the blood vessel wall and into the surrounding tissue. Ultimately, this communication process would drive the adaptive response or lead to injury.

It is well known that gap junctions (GJ) are constructed of Cx molecules. There are four Cx isoforms in the vasculature, Cx37, Cx40, Cx43 and Cx45. In this study, we only examined Cx40 and Cx43. One reason was that previous studies have indicated that although there are four Cx isoforms in the vasculature, they regulate different vascular functions. Cx40 and Cx43 have been reported to regulate vascular contraction or relaxation (6, 8, 24, 34, 37), Cx45 is involved in vasculogenesis and blood vessel maturation (18), and Cx37 contributes to vessel occlusion and narrowing (atherosclerosis) (4, 19). Another reason is that only Cx40 and Cx43 have been found to exist at the VEC and VSMC coupling sites in vitro (13) and in vivo (8, 12, 29). Therefore, in our study we examined Cx40 and Cx43 and determined that Cx43 was the isoform involved in regulating iNOS protein expression in VSMCs and vascular hyporeactivity after hypoxia.

Our results also showed that the cAMP-PKA pathway was responsible for the regulation of Cx43 expression and MEGJ formation and, therefore, for iNOS production and vascular hyporeactivity in VSMCs after hypoxia. This finding is consistent with a previous report that PKA activation promoted Cx43 movement to the plasma membrane, and in the process GJ assembly was enhanced (31). Elevation in intracellular cAMP also enhanced dye coupling via channels constructed from Cx43 (36). It was previously shown that PKA and cAMP

![Image](http://ajpcell.physiology.org/DownloadedFrom/http://ajpcell.physiology.org)
enhanced GJ communication by not only increasing Cx43 expression but also phosphorylating Cx43 (41). The role of Cx43 phosphorylation in regulation of vascular reactivity following hypoxia, and the identity of the phosphorylation site, Ser364, Ser365, Ser368, Ser369, or Ser373, will require further study.

Regarding the signaling molecule passing across the MEGJs, previous studies have shown that several second messengers, including ATP, IP3/Ca2+, cAMP, and cGMP, were permeable to some degree through each type of Cx channel (15, 16). Among these, cAMP activates the cAMP-response element binding site and induces iNOS expression at the transcriptional level (11). In addition, acetylcholine-induced EDHF-type relaxation was associated with cAMP elevation in vascular smooth muscle in isolated rabbit arteries. This effect required an intact endothelium and was attenuated by blockade of GJ with Cx43-mimetic peptides or 18α-GA, suggesting that endothelium-derived cAMP could diffuse into the arterial media via MEGJs containing Cx43 (3, 33). This background data led us to focus on cAMP in our study, determining that this messenger molecule could pass through the MEGJs and induce iNOS expression in VSMCs. Taken together, cAMP not only increased Cx43 expression in MEGJs and promoted MEGJ formation and communication, but it also permeated through the MEGJs and induced the subsequent effects on VSMCs after hypoxia.

Like the cell-specific heterogeneity (VECs vs. VSMCs) of induction of iNOS expression, we observed similar heterogeneity in cAMP production. Without MEGJs there was more cAMP production in VECs than in VSMCs. This is consistent with previous reports of differential changes in cAMP levels in VECs and VSMCs under EDHF stimulation. In that case, cAMP elevation in VSMCs required diffusion of the stimulus from the endothelium (3, 21, 33). Furthermore, our results explain well the cell-type heterogeneity of iNOS and cAMP using MEGJs and show that MEGJs are a key structure enabling intercellular transfer of cAMP from VECs to VSMCs, inducing iNOS production in VSMCs after hypoxia. However, many questions about the cell-specific mechanisms remain, such as why does cAMP in VSMCs need to be transferred from VECs, and why did it not induce much iNOS production in VECs; hence, further research is required.

Finally, the VEC and VSMC double-sided coculture model is a generally accepted model to study MEGJ in vitro. In this model, VECs and VSMCs sent projections through the pores of a Transwell membrane, made contact with each other, and successfully formed functional MEGJs (13). This model mimicked well the MEGJs in the intact vasculature, which is located in the internal elastic lamina, and is constructed by the projections of VECs and VSMCs. Although this model has been widely used to study the function and mechanism of MEGJs in different disease models (5, 7, 9, 42–44), as a simplified coculture model it has limitations compared with the real in vivo physiological system. As we know, the regulation of Cx relies on the combined effects of the complicated hormone and mechanical environments. Vascular endothelial growth factor and basic fibroblast growth factor stimulated angiogenesis and wound healing by affecting GJ and GJ-related VEC behaviors (15, 20). Similarly, Cx43 expression in VECs increased by high laminar shear stress (14, 43). As the complicated hormone and mechanical environments are hard to mimic by the present coculture model, development of more advanced techniques and models is needed to fully elucidate the role, regulation, and mechanism of MEGJ signaling.

In conclusion, our study further elucidates the detailed mechanism by which Ang-2 regulates iNOS expression in VSMCs and vascular hyporeactivity after hypoxia. Following hypoxia, upregulated Ang-2 activated cAMP and PKA via Tie2 receptors. This led to Cx43 accumulation in the MEGJs, facilitating MEGJ formation and communication, which enabled cAMP itself to pass through the MEGJ channels to induce iNOS expression and vascular hyporeactivity in VSMCs.

GRANTS

This work was supported by the National Natural Science Foundation of China (Grant nos. 81370426 and 81670445; to J. Xu) and the Natural Science Foundation Project of CQ CSTC (Grant no. cstc2013jcyjA10128; to J. Xu).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

L.L., conceived and designed research; J.X., G.Y., and T.L. performed experiments; J.X. analyzed data; J.X. interpreted results of experiments; J.X. prepared figures; J.X. drafted manuscript; L.L. edited and revised manuscript; J.X., G.Y., T.L., and L.L. approved final version of manuscript.

REFERENCES


