5-2018

Role of Local Renin-Angiotensin System in Altering Valve Interstitial Cell Phenotype

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Role of Local Renin-Angiotensin System in Altering Valve Interstitial Cell Phenotype

An undergraduate Honors College Thesis in the Department of Biomedical Engineering

University of Arkansas

Fayetteville, AR

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May 2018
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Abstract

Hypertension is a disorder prevalent in adults around the world and is a common attributable cause of heart disease and mortality [1]. Calcification is much more common in the aortic valve than the other three heart valves [2]. Aortic valve interstitial cells (VICs), the principal cell type found in the human aortic valve, have been linked to disease development when they change in phenotype to become osteoblast like cells [3]. The phenotypes of these cells play a role in the development of calcification in the aortic valves [4].

Angiotensin II is a hormone in the body that has been implicated to stimulate inflammation in heart valves and is linked to the development of heart valve disease [5][6]. Losartan (AT1R antagonist) and PD123319 (AT2R antagonist) are commercially available drugs to prevent ang-II from binding to ang-II receptors on the cells [7][8]. There are certain protein signatures (RUNX2, αSMA, Ki-67, TGFβ1, Vimentin, and Calponin) that can be observed to determine if VICs are being activated in the presence of ang-I or ang-II with and without the presence of receptor antagonists.

Cells were cultured in 10% media with six treatment groups and one control: angiotensin I (ang-I) without an inhibitor, angiotensin II (ang-II) without an inhibitor, angiotensin I + Losartan (ang-I + Los), angiotensin II + Losartan (ang-II + Los), angiotensin I + PD123319 (ang-I + PD), and angiotensin II + PD123319 (ang-II + PD). Expression of protein signatures was assessed using immunocytochemistry. Results displayed phenotypic change in the form of increased expression of protein signatures in VICs with treatment groups. Most VICs were inhibited in the presence of losartan, demonstrating that the binding of ang II to AT1R is causing increased activation of the VICs.
**Introduction:**

The heart is a vital organ that is needed for survival [9]. It consists of four chambers: the right and left atria and the right and left ventricle, and four valves: the tricuspid, pulmonary, mitral, and aortic. The aortic valve is what enables freshly oxygenated blood to flow from the left ventricle to the aorta and on to the body’s systemic circulation [9].

![Heart Diagram](image)

**Figure 1:** Image illustrating flood flow through heart [10]

Aortic valve calcification occurs when calcium deposits form on the aortic valve, causing a narrowing in the opening of the valve that reduces blood flow. When flow is restricted, it results in aortic valve stenosis. This is a common early sign of heart disease [11]. It causes the heart to work harder to pump blood to the body [10].

![Healthy and Aortic Valve Diagram](image)

**Figure 2:** Healthy and aortic and aortic valve stenosis visualization [12]
Calcification of the aortic valve is much more common than calcification of the other three heart valves. Calcific aortic stenosis is the 3rd most prevalent cardiovascular disease in the United States [2]. Valvular aortic stenosis caused by calcification of cells is commonly associated with hypertension, a disease that affects 2% of the population above the age of 65 [13].

Aortic valve interstitial cells (VICs), the principal cell type found in the human aortic valve, have been linked to disease development when they change in phenotype to become osteoblast-like cells [3]. The phenotypes of these cells play a role in the development of calcification in the aortic valves [4]. The calcification of aortic valves is not due to random degeneration, but rather an active process of bone formation in the aortic valve associated with certain cell phenotypes [14]. VIC phenotypes include quiescent (qVICs), activated (aVICs), and osteoblastic (obVICs). Quiescent VICs are responsible for maintaining normal valve functions, and are normally inactive. If the cells are injured or stressed, they have a tendency to become aVICs, which can easily differentiate into diseased obVICs [15]. Active VICs are a very important part of the wound repair process, and are usually eliminated by apoptosis once they have served their purpose. If this apoptosis fails it may result in pathological fibrosis, chronic inflammation, and calcification, which leads to clinical valve diseases [5].

**Figure 3:** Illustration of how Valve Interstitial Cells can go from quiescent, to activated, and then osteoblastic/diseased

Angiotensin II (ang-II) is a hormone in the body partially responsible for regulating blood pressure, cardiac function, and plasma volume. It’s of incredible importance in hypertension and is a fundamental effector of the renin-angiotensin system [6][16]. Studies have shown that ang-II has been implicated to stimulate inflammation in heart valves and is linked to the development of heart valve disease [5][6]. Angiotensin receptor blockers are among some of the only standard evidence-based heart failure therapies [13]. All national guidelines recommend an angiotensin receptor blocker as a part of anti-hypertension therapy [1]. Recently, there has been clinical evidence that long-term blocking of ang-II conversion could have a protective effect against carcinogenesis [6].

Renin is an enzyme produced by the kidneys and acts on the protein angiotensinogen that’s formed by the liver to produce the hormone Angiotensin I (ang-I). Ang-I can be transformed into ang-II in the blood via the angiotensin-converting enzyme (ACE). This is commonly referred to as the renin-angiotensin System, or RAS [17]. Ang-II (ang-I) can then bind to two different ang-II receptors on cells that mediate the majority of its effects, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) [6].
There are currently four classes of medications that focus on various aspects of the renin-angiotensin system pathway in an effort to combat hypertension. These include angiotensin converting enzyme (ACE) inhibitors, aldosterone antagonists, direct renin inhibitors, and ang-II receptor blockers (ARBs) [1]. The two ARBs that will be utilized in this experiment are Losartan and PD123319. In general, ARBs are better tolerated and produce fewer side effects than ACE inhibitors [18]. Unfortunately, ACE inhibitors are only partially successful in inhibiting the formation of ang-II, and are related to several adverse effects such as chronic cough and angioedema [1][18].

**Losartan** (AT1R antagonist) and **PD123319** (AT2R antagonist) are commercially available drugs to prevent ang-II from binding to ang-II receptors on the cells [7][8]. AT2R expression is increased in tissues experiencing hypertension, cardiac hypertrophy, and ischemic heart disease. AT2R negatively regulates AT1R signaling, but the precise mechanism that makes this happen is poorly understood [19]. AT1R activates growth-promoting pathways and mediates ang-II effects such as vasoconstriction and increased blood pressure as well as detrimental effects such as inflammation. AT2R has been linked to producing opposite effects, such as vasodilation, hypotension, and possible inhibition of AT1R [16].

![Angiotensin Pathway with AT1R and AT2R Inhibiting Medications](image)

**Figure 4:** Illustration of ang-I to ang-II pathway with AT1R and AT2R inhibiting medications

There are certain protein signatures that can be observed to determine if VICs are being activated in the presence of ang-I or ang-II with and without the presence of receptor antagonists. If the marker Vimentin were up regulated, it would indicate more quiescent VICs [24]. If the markers αSMA, Ki-67, TGFβ1, and Calponin are up regulated, it's an indicator of increased aVICs. [5][21][20][15][22][23]. These aVICs can easily differentiate into obVICs, which regulate osteogenesis and the calcification process [15]. ObVICs secrete RUNX2, so if this marker is present in the sample cells are likely to be calcified [14][15]. It is important to look for these markers in to test whether the VICs are changing in phenotype when exposed to ang-I and ang-II.
Table 1: Table simplifying protein signature markers, their roles in the body, and what an up-regulation in results would indicate. Sources: [5][21][20][15][22][23][24][14]

<table>
<thead>
<tr>
<th>Marker</th>
<th>Role in the Body</th>
<th>Up-regulation would mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX2</td>
<td>Transcription factor necessary for bone formation</td>
<td>More osteogenic VICs</td>
</tr>
<tr>
<td>αSMA</td>
<td>Smooth muscle actin, Marker for aVIC activation</td>
<td>More activated VICs</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Protein with important function in cell division</td>
<td>More activated VICs</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Growth factor secreted by aVICs, also increases overexpression of αSMA</td>
<td>More activated VICs</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Proteins expressed in inactive VICs</td>
<td>More quiescent VICs</td>
</tr>
<tr>
<td>Calponin</td>
<td>Protein shown to regulate smooth muscle cell differentiation</td>
<td>More activated VICs</td>
</tr>
</tbody>
</table>

Objectives and Hypothesis

The objective of this research was to better understand how the renin-angiotensin system affected the phenotype of valve interstitial cells both with and without the presence of inhibitors.

There are two main questions we were hoping to answer:
1. Are VICs changing in phenotype in the presence of ang-I and ang-II?
2. Is RAS affecting the phenotype of VICs due to the binding of ang-II to AT1R or AT2R?

We hypothesized that the renin-angiotensin system will alter the phenotype of VICs. Additionally, we hypothesized that VICs will become more osteogenic in the presence of ang-II and will experience up-regulation in markers for aVICs and obVICs in its presence.

Methods:

Preparing Media

To prepare 2% media for coverslips
In order to make 500mL of media total, 2% needs to be FBS, 1% needs to be Penicillin Streptomycin, 1% needs to be HEPES, and the rest should consist of DMEM media. In order to accomplish this, 10mL of 2% FBS, 5mL of Penicillin Streptomycin, and 5mL of HEPES was added to 480mL of DMEM media.

To prepare 10% media for culture in the flask
In order to make 500mL of media total, 10% needs to be 2% FBS, 1% needs to be Penicillin Streptomycin, 1% needs to be HEPES, and the rest should consist
of DMEM media. In order to accomplish this, 50mL of 2% FBS, 5mL of Penicillin Streptomycin, and 5mL of HEPES was added to 440mL of DMEM media.

The entire 500mL media mixture was run through a filter, using an aspirator as a vacuum.

**Sterilizing cover slips**
18mm diameter cover slips were autoclaved for one hour.

**Cell Culture and Seeding**

A T175 flask confluent with porcine valvular interstitial cells from an aortic valve growing in 10% media was prepared in advance. All cell culture was performed in a biosafety cabinet, and all outside materials were sterilized with 70% ethanol before entering the sterile environment. 100µL of gelatin was placed onto each autoclaved coverslip and incubated at 37 degrees Celsius and 5% CO2 for one hour. Seven 12 well plates were set out, and 1mL of PBS was pipetted into each well. A coverslip was placed in each well, with the gelatin side face up. The PBS was aspirated from the wells are replaced three times to wash the coverslips. 2mL of .25% trypsin was pipetted into the confluent T175 flask and left to incubate for 2 minutes. The T175 flask was removed from the incubator and tapped firmly against the inside of a wrist to loosen the cells from the bottom of the flask. Cells were observed under a microscope while still in the T175 flask to ensure that they were visibly unstuck/ appeared more round than their adhered fibroblast-like state. 15mL of 10% red media was pipetted into the T175 flask and swirled around to cover the surface area of the flask several times to deactivate the trypsin. All media was then pipetted out of the T175 flask and into a 50mL centrifuge tube. The 50mL tube was centrifuged at 4 degrees Celsius for 6 minutes. The supernatant was removed and discarded by bleaching and placing it in biohazard waste. 3mL of 2% media was pipetted into the 50mL tube containing the pellet and mixed thoroughly until the pellet was dissolved.

Cell counting took place with a hemocytometer. 20µL of well-mixed sample was transferred into a small eppendorf tube to take to the microscope. 10µL of sample was pipetted into each side of the hemocytometer, and cells were counted in the center and averaged to estimate the density of the sample. 161 cells were counted on one side and 80 cells were counted on the second side. This averaged out to be about $119.5 \times 10^4$ cells per mL of solution. Accounting for the 3mL of media that the pellet was suspended in, centrifuge tube was estimated to have about $3.59 \times 10^6$ cells in the suspension. Each of the 84 coverslips needed to be seeded with 2,000 cells, so it was calculated that 168,000 cells were needed from the mixture.
84 cover slips * 2,000 cells per slip = 168,000 cells needed

This number was rounded to 200,000. The equation \( \frac{\text{cell}_{1}}{\text{volume}_{1}} = \frac{\text{cell}_{2}}{\text{volume}_{2}} \) was used to calculate the second volume number to extract the amount of cells desired. It was calculated that 167µL of the mixture was needed from the 50mL centrifuge tube to obtain the approximate amount of cells for the current procedure. It was then calculated that 8.233mL of media from the 167µL would be needed for seeding cells onto coverslips.

\[
volume_{2} = \frac{0.2 \times 10^{6} \times 3 \mu L}{3.59 \times 10^{6}} = 167 \mu L
\]

84 cover slips * 100µL of cell solution = 8.4mL

8.4mL − 0.167mL = 8.233mL of media from 167µL cell solution

100µL of cell solution was pipetted onto the prepared gelatin coverslips, being careful not to break the surface tension of the media so that all cells would remain in the center of the coverslips were placed into an incubator for 2 days.

Treatment Groups

Each of the seven 12 well plates were labeled with the date (4/3/18), the name of the cell line (VIC5), the passage number (P5), who was the primary handler for each plate, and the treatment group. There was one treatment group for each 12 well plate: control, angiotensin I (ang-I) without an inhibitor, angiotensin II (ang-II) without an inhibitor, angiotensin I + Losartan (ang-I + Los), angiotensin II + Losartan (ang-II + Los), angiotensin I + PD123319 (ang-I + PD), and angiotensin II + PD123319 (ang-II + PD).

Coverslips were treated with respective inhibitors (Losartan or PD123319) for 24 hours while the control and other non-inhibitor groups incubated in 2% media. Afterwards, coverslips were treated with ang-I \((10^{-5})\) or ang-II \((10^{-5})\) for 2 days. The cells had a total of 5 days on the coverslips before fixing.

12.375mL of 2% media and 125µL of Losartan \((10^{-4})\) was added into the 15mL “Los” tube. 12.375mL of 2% media and 125µL of PD123319 \((10^{-4})\) was added into the 15mL “PD” tube. Each 15mL tube was vortexed and placed in a water bath. 500µL of fluid from the “Los” tube was applied to each treatment group well that was labeled with Losartan, 24 wells in total. 500µL of fluid from the “PD” tube was applied to each treatment group well that was labeled with PD123319, 24 wells in total. Cover slips were ensured to be completely submerged in their respective solutions before returning them to the incubator overnight. This was conducted for an n=2.
ICC Immunostaining

Fixing the slides
All materials were brought to room temperature before the process began. Cells were transported from the incubator to the fume hood and were rinsed gently 3x in PBS. After the third wash, the PBS was aspirated from the plate wells. Each coverslip was stained using 400µL of a 4% Paraformaldehyde (PFA), Triton-X 100, and PBS stock solution mixture in a dark fumigated environment for 10 minutes. All waste solution was aspirated and disposed of in container labeled as “toxic”. Immediately, 3-4mL of PBS was added to each coverslip well, swirled gently to rinse, and left to sit for 10 minutes. This process of rinsing with PBS was repeated two more times. After the final wash, the coverslips were left to sit in PBS at 4 degrees Celsius for the night.

Blocking
PBS was aspirated from the coverslip wells and 130µL per well of 10% goat serum (the species of the secondary antibody) was pipetted onto the coverslips and left to incubate at 37 degrees Celsius for 60 minutes.

Primary Stain
This was done in a light-protected environment. Primary antibody solution was prepared in advance, and consisted of 1% goat serum (Abcam), the designated concentration of the marker for the respective treatment group, and PBS. Measurements were based on providing 130µL of primary antibody solution per coverslip.

<table>
<thead>
<tr>
<th>Treatment Group Markers</th>
<th>Concentration</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX2</td>
<td>(1/200)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>αSMA</td>
<td>(1/100)</td>
<td>Mouse</td>
</tr>
<tr>
<td>Ki-67</td>
<td>(1/200)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>(1/100)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Vimentin</td>
<td>(1/200)</td>
<td>Mouse</td>
</tr>
<tr>
<td>Calponin</td>
<td>(1/200)</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

Seven 130µL drops (one for each coverslip treatment group) were placed into eight different clean Petri dishes (one for each treatment group and a negative control) evenly spaced out. Using forceps, coverslips were placed over the solution cell-side down and left to incubate at 4 degrees Celsius overnight. After the incubation period, coverslips were placed into 12-well plates cell-side up and left to sit in PBS for 10 minutes, then washed gently with PBS two more times.
After the final wash, the coverslips were left to sit in PBS at 4 degrees Celsius for the night.

**Secondary Stain**

For each coverslip, 130µL of secondary stain was prepared. The secondary stain consisted of 1:200 DAPI stock, 1% Goat serum, 1:200 of the appropriate secondary antibody (goat or rabbit, Abcam) Alexa Fluor 594 conjugate, and PBS. If a mouse primary antibody was used, a goat-anti-mouse IgG was used for the secondary antibody. If a rabbit primary antibody was used, a goat-anti-rabbit IgG was used for the secondary antibody. All components of the secondary stain were mixed well with a vortex and protected from light. In addition to previously treated coverslips, seven untreated coverslips from each treatment group were also stained with secondary antibody to serve as a negative control.

Coverslips were incubated in the secondary stain for 1 hour and 45 minutes in the dark. After the incubation period, coverslips were rinsed gently in PBS for 10 minutes, then the PBS was aspirated and the slips were washed 3 more times in PBS, leaving the last round of PBS sitting on the coverslips until mounting could take place.

**Mounting**

One glass slide was prepared for every coverslip, fitting two coverslips per slide. Slides were labeled with n1 or n2, the primary antibody, the date of fixing, the name of the primary handler, and the treatment group. A drop of prolong gold anti-fade reagent (Invitrogen #P36930) without any bubbles was placed onto the clean slides, then the coverslips were carefully placed cell-side down into it. All slides were laid flat in a dark environment to dry overnight, then placed in a slide box and stored in a negative 20 degrees Celsius freezer.

**Imaging**

Cells were imaged with a standard epifluorescence microscope at 20x for both DAPI and red 594. Four to five images were taken of each coverslip for both DAPI and red 594, for a total of 8-10 pictures per coverslip. Select images were compiled together for visual quantitative analysis. ImageJ was used to compile DAPI and red 594 stained images.
Results and Discussion:

**RUNX2**: Increased expression in ang-I and ang-II treatment groups in comparison to the control indicating cells became more osteogenic. In the presence of PD123319 there was less expression than in the presence of losartan indicating AT2R receptors were blocked more.

![Figure 5. Immunocytochemistry images of RUNX2 expression of the 7 treatment groups (scale bar=200µm)](image)

**αSMA**: Increased in expression for ang-I & ang-II with no inhibitors, indicating cells became more activated. In the presence of PD123319 there was more expression than in the presence of Losartan, indicating blocking the AT1R receptor had more of an effect suppressing this marker.

![Figure 6. Immunocytochemistry images of αSMA expression of the 7 treatment groups (scale bar=200µm)](image)
**Ki-67**: Increased in expression for ang-I & ang-II, indicating cells became more activated. In the presence of PD123319 there was more expression than in the presence of losartan, indicating blocking the AT1R receptor had more of an effect suppressing this marker.

![Ki-67 Immunocytochemistry Images](image)

**Figure 7. Immunocytochemistry images of Ki-67 expression of the 7 treatment groups (scale bar=200µm)**

**TGFβ1**: Increased in expression for ang-I & ang-II, indicating cells became more activated. In the presence of PD123319 & losartan there was also increased expression, indicating blocking AT1R & AT2R receptors had no effect on this marker for this particular incubation time.

![TGFβ1 Immunocytochemistry Images](image)

**Figure 8. Immunocytochemistry images of TGFβ1 expression of the 7 treatment groups (scale bar=200µm)**
**Vimentin**: Increased in expression for ang-I & ang-II, indicating cells were more quiescent VICs, which was an unexpected result. Interestingly enough, this expression was equally vibrant for both ang-I and ang-II samples, indicating a potential conversion of ang-I samples to ang-II.

![Figure 9. Immunocytochemistry images of Vimentin expression of the 7 treatment groups (scale bar=200µm)](image)

**Calponin**: Increased in expression for ang-I and especially for ang-II, indicating cells became more activated. In the presence of PD123319 and losartan there was little expression in comparison, indicating AT1R & AT2R receptors were blocked.

![Figure 10. Immunocytochemistry images of Calponin expression of the 7 treatment groups (scale bar=200µm)](image)
**Negative control:** No expression/ non-specific binding of the secondary reagent, indicating no contamination of samples.

![Immunocytochemistry images of Negative Control expression of the 7 treatment groups (scale bar=200µm)](image)

*Figure 11. Immunocytochemistry images of Negative Control expression of the 7 treatment groups (scale bar=200µm)*

**Conclusions, Limitations, and Future Directions:**

Results displayed phenotypic changes in the form of increased expression of protein signatures in VICs with ang-I and ang-II treatment groups when compared with their respective controls. Most VICs were inhibited in the presence of losartan, demonstrating that the binding of ang-II to AT1R is causing increased activation of the VICs. If ang-II is still having the same effect it does in the presence of PD123319 as it does in the presence of Losartan, then AT2R must not be the receptor responsible for the phenotypic changes. It's likely that AT2R binding isn’t making as big of an impact on the phenotype of VICs in comparison to binding to AT1R. To further verify this, future work should perform more quantitative analysis over multiple time periods.

Limitations of the procedure were not enough cells used while seeding to be able to do quantitative analysis. Cells were seeded sparsely intentionally to avoid activating them with a high density, but the density of cells should be increased when repeating this procedure. In the future, quantitative analysis will be done in more confluent samples, and western blots will be conducted to further confirm VIC phenotypic changes.
References:


proliferation and prognosis of breast cancer molecular classification subtypes. Anti-Cancer Drugs, 25(8), 950–957. doi.org/10.1097/CAD.0000000000000123.


Acknowledgements:

Special thanks Jessica Perez and Kartik Balachandran for guiding me through this process. Your encouragement and support made it all possible!

I would also like to thank the Arkansas Department of Higher Education for providing funding through the SURF grant.
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Calponin

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Calponin