Sex-Related Signaling of the Angiotensin II Pathway in Primary Aortic Smooth Muscle Cells

Arterial Stiffness

Cardiovascular disease is the **leading cause of death** worldwide, claiming **17.9 million lives** each year

Aging-associated arterial stiffness is a leading, independent **risk factor** for cardiovascular disease

Men and women exhibit differences in the **onset, severity, and pathogenesis** of arterial stiffness

This sex-associated difference likely involves **sex steroid hormones:** estrogen and testosterone

Yet, the molecular mechanisms behind this sexually dimorphic pattern are still **unknown**

Fatty Acid-Binding Proteins

Family of **ten lipid chaperones** primarily involved with the trafficking of cellular lipids

FABP3: novel biomarker for **MI, atherosclerosis, and PAD**

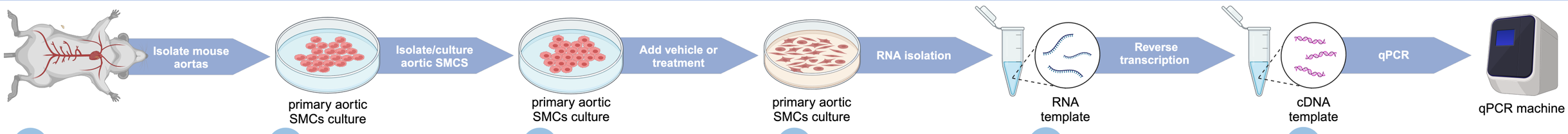
FABP4: elevated expression in women; positively correlated with **arterial stiffness**

FABPs may be a **therapeutic target of interest** for treatment of cardiovascular disease and provide key insights into the **sex-specific molecular mechanisms** behind arterial stiffness

Research Objectives

1. Uncover the **molecular mechanisms** behind how **estrogen** and **testosterone** affect the **expression of angiotensin II receptor isoforms**
2. Uncover the **molecular mechanisms** behind how **estrogen** and **testosterone** affect the **expression of markers of tissue fibrosis**
3. Computationally analyze **fatty acid-binding protein inhibitors** to investigate alternative treatment modalities for arterial stiffness

Methodology

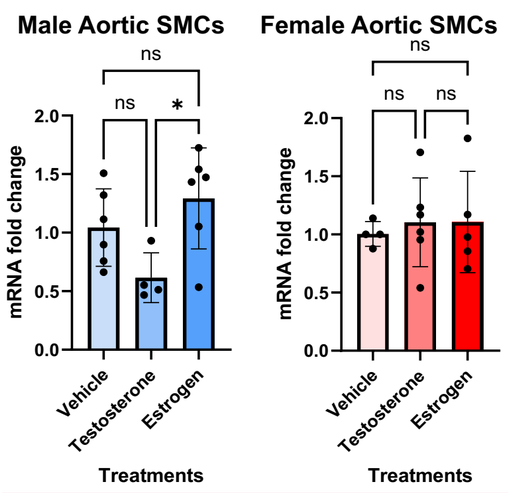


1. Aortas were isolated from male and female mice and placed into media; the adventitia was removed
2. When the cultures were 50-75% confluent, the explants were removed; the SMCs were cultured and passaged as necessary
3. Each cell culture was either administered a vehicle (ethanol), estrogen (10 nM β-estradiol), or testosterone (20 μM) treatment
4. After washing the cultures with PBS buffer, total RNA was isolated
5. RNA concentration and quality was measured; high-quality samples were reverse transcribed
6. Quantitative RT-qPCR was performed with gene-specific primers; relative gene expression calculated with the $2^{-\Delta\Delta CT}$ method

Gene Expression Targets

Expression of Angiotensin II Receptors

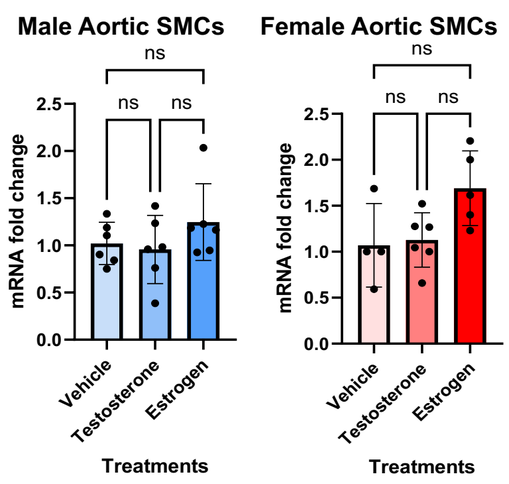
Angiotensin II Receptor 2 (AT2)



Major component of the **protective arm** of the RAAS system, **opposing** many of AT1R's functions; exhibits **anti-fibrotic phenotype**

Male cells administered **estrogen** exhibited **higher expression of AT2R** than male cells administered **testosterone (p = 0.0339)**

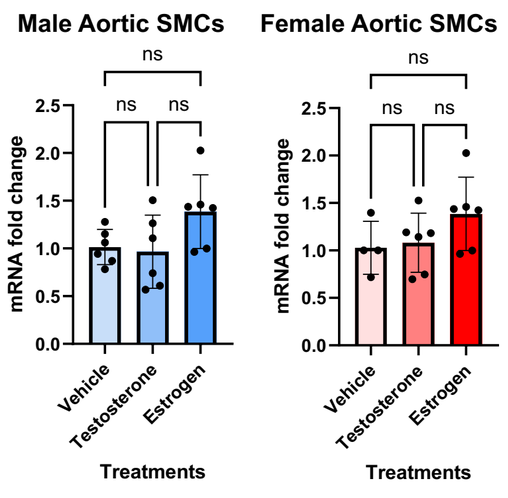
Angiotensin II Receptor 1, Subtype A (AT1A)



Closely involved with classical physiological actions of **angiotensin II**, stimulating vasoconstriction, increasing blood pressure, and activating inflammation pathways

Estrogen, testosterone, and AT1A may be **independently involved**, especially in aortic SMCs

Angiotensin II Receptor 1, Subtype B (AT1B)

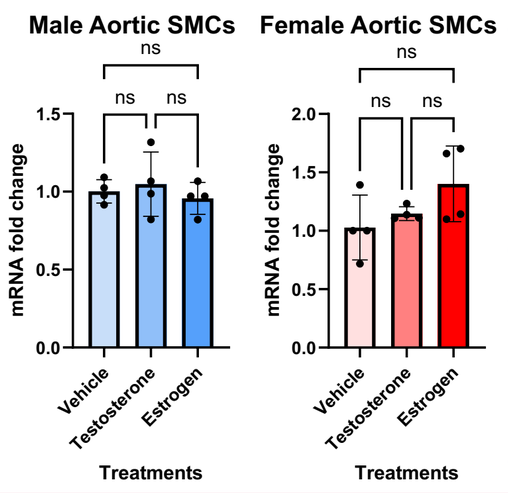


Results in increased **fibroblast proliferation,** up-regulation of **adhesion proteins**, stimulation of **pro-fibrotic cytokines,** and myofibroblast-induced **ECM synthesis**

Estrogen, testosterone, and AT1B may be **independently involved**, especially in aortic SMCs

Expression of Markers of Tissue Fibrosis

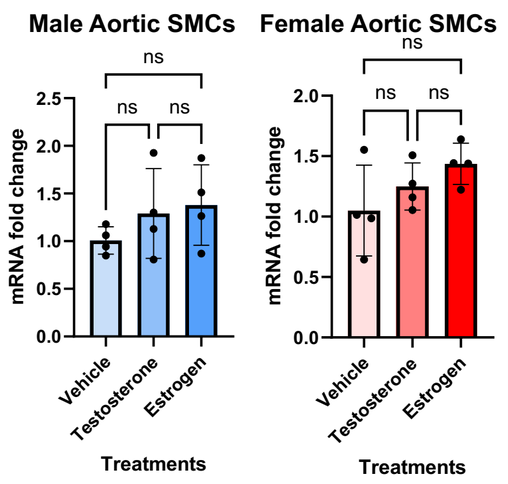
Matrix Metalloproteinase-2 (MMP-2)



Family of enzymes with **endopeptidase activity**, play key roles in **ECM remodeling**; **MMP-2** found in aging rats, localized to sites with **fragmented elastic lamellae**

Pure SMC cultures **could not simulate functional cross-talk** between SMCs and endothelial cells required in fibrosis

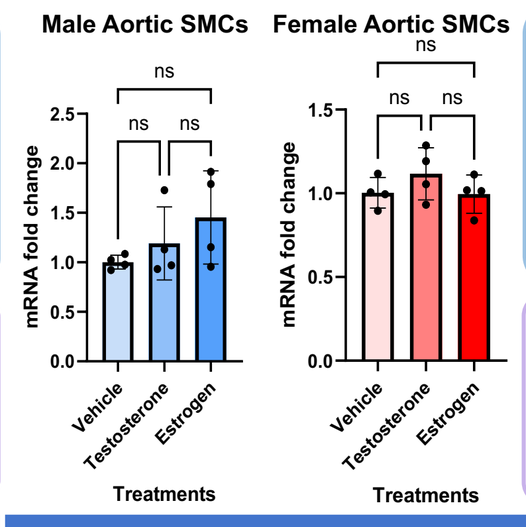
Matrix Metalloproteinase-9 (MMP-9)



Elevated MMP activity creates a **pro-inflammatory** environment, facilitating remodeling of arterial walls; **MMP-9** found in **aneurysmal aortas** with degradation of elastin and collagen

Pure SMC cultures **could not simulate functional cross-talk** between SMCs and endothelial cells required in fibrosis

Type III Collagen (COL3A1)



COL3A1 encodes for the **alpha-subunits** founds in type III collagen; a major mechanical constituent of the ECM and a key component of the **fibrotic process**

There was likely **not enough time** for fibrosis to **fully develop**, as the cultures were harvested within 24 hours

FABP Inhibitors

FABP inhibitors were computationally analyzed to determine **optimal stereochemistry**

Inhibitor of Interest: SBFI-1621

Truxilloid previously developed as a **derivative** of a hit compound that was produced in an **in silico virtual screen** of one million compounds with similar molecular footprints to oleic acid bound to FABP7

Only the ***S* enantiomer** of the original compound co-crystallizes with FABPs, indicating **stereochemical preference**

Experimentally, the two enantiomers, (+)-SBFI-1621 and (-)-SBFI-1621, exist in **nearly equal amounts**

Perform **molecular docking** to determine correlation

SBFI-1621 Ki (μM)	(-)-SBFI-1621	(+)-SBFI-1621
FABP3	>100	71.32 ± 2.40
FABP5	1.31 ± 0.16	0.77 ± 0.08
FABP7	57.62 ± 1.79	18.99 ± 1.81

Table 1: Binding affinity of (-)-SBFI-1621 and (+)-SBFI-1621, measured experimentally; *Credit: Liqun Wang, Kaczocha Lab at Stony Brook University*

Previous experimental data demonstrated that the binding affinities of **(+)-SBFI-1621** to FABP3, FABP5, and FABP7 are more **favorable**

DOCK 6.9	(S,S,S,S)-SBFI-1621 (kcal/mol)	(R,R,R,R)-SBFI-1621 (kcal/mol)	Difference (kcal/mol)
FABP3 (PDB: 4WBK)	—	—	—
FABP5 (PDB: 5UR9)	-45.706	-46.622	0.916
FABP7 (PDB: 5URA)	-46.358	-52.426	6.068

Table 2: Molecular docking of (S,S,S,S)-SBFI-1621 and (R,R,R,R)-SBFI-1621. *Calculation results confirmed by Kathryn Takemura, Ojima Lab at Stony Brook University*

Docking SBFI-1621 to FABP5 and FABP7 was **successful**

(+)-SBFI-1621 corresponds to ***(R,R,R,R)*-SBFI-1621**

The crystal structure of FABP3 could not accommodate the ligand because the original structure was bound to a ligand that **differs in its induced fit behavior**, resulting in a significantly smaller binding site

While the original hit compound only co-crystallizes in its ***S* enantiomer**, its closely-related derivative (SBFI-1621) binds mostly in its ***R* enantiomer**

The *R* enantiomer may be investigated as a novel potential **diagnostic or therapeutic** for arterial stiffness

Conclusions

Studied how **mRNA expression** of **angiotensin II receptors** and **markers of tissue fibrosis** are affected by administration of different sex steroid hormones

Male cells administered **estrogen** exhibited **higher expression of the cardioprotective AT2 receptor** than male cells administered **testosterone**

Used **molecular docking** to analyze FABP inhibitors to **identify and optimize** novel candidates for diagnostics and medications for arterial stiffness

Applications

The **AT2 receptor pathway** may be a key mechanism by which **estrogen** exerts its cardioprotective effects and by which **testosterone** exerts its deleterious effects on the cardiovascular system

Implicates **both** estrogen and testosterone's novel contributing role to the apparent sex differences in the onset and pathogenesis of aging-associated arterial stiffness and cardiovascular disease

Future Work

Explore how different dosages of estrogen and testosterone affect expression of angiotensin receptors and fibrosis markers: **critical for HRT**

Identify novel proteins, such as ERα and PARP1, and signaling pathways to understand sex-specific blood vessel dysfunction **over the entire lifespan**

Use a combination of experimental and computational studies to address the **mechanistic knowledge gap**, improving cardiovascular health in aging demographics

All images created by the student researcher unless otherwise noted