Angiotensin II Upregulates the Expression of an Oxysterol Producing Enzyme: Cholesterol-25-Hydroxylase in Vascular Smooth Muscle Cells

PhD thesis

Kinga Bernadett Kovács

Division of Molecular Medicine
Semmelweis University

Supervisor: András Balla, PhD

Official reviewers: Róza Zákány, PhD
Szabolcs Sipeki, PhD

Head of the Complex Examination Committee:
Szabolcs Várbíró MD, PhD, Med., Habil.

Members of the Final Examination Committee:
Éva Ruisanchez, MD, PhD
Anita Alexa, PhD

Budapest
2024

1. Introduction

Cardiovascular diseases (CVD) present considerable health problems and remain the most common cause of death. Amongst the risk factors we find the disruption of physiological regulatory processes.

Angiotensin II (AngII), a major endocrine mediator of vascular function has been found to be an important mediator of atherogenic processes, contributing to vascular dysfunction. Through the type 1 AngII receptor (AT1R) AngII is able to activate diverse signaling pathways, leading to reactive oxygen species (ROS) generation, and growth factor receptor transactivation. By virtue of its multifaceted signaling AngII promotes gene expression changes in vascular smooth muscle cells (VSMC). In order to understand the long term effects of AngII the gene expression changes exerted by the hormone must be explored.

Oxysterols, the oxygenated products of cholesterol, have also been found to possess deleterious effects on vascular functions. 25-hydroxycholesterol (25-HC), a widely studied oxysterol in the field of immunology shares common atherogenic functions with AngII, such as the promotion of endothelial dysfunction and inflammation. The 25-HC is primarily produced by the enzyme cholesterol-25-hydroxylase (CH25H), whose expression is prominent in immune cells in response to Toll-like receptor stimulus. The p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) have been shown to promote *Ch25h* expression. Macrophages and oxidized low density lipoproteins have been found to be sources of 25-HC in the vessel wall.

Despite the effects of 25-HC on VSMCs, the relationship between *Ch25h* and these cells has not yet been investigated. The commonalities of pathological actions exhibited by both AngII and 25-HC make it intriguing to explore the relationship between these two factors in the context of VSMC functions.

2. Objectives

This study aimed to characterize AngII-induced *Ch25h* expression in primary rat VSMCs isolated from the thoracic aorta. The objectives can be divided into two groups:

2.1. Investigation of AngII-induced *Ch25h* expression in VSMCs and underlying signaling pathways:

- Determination of the *Ch25h* messenger ribonucleic acid (mRNA) level changes in response to AngII stimulus and assessment of the time kinetics of the gene expression change.
- Defining the role of AT1R-related $G_{q/11}$ or β-arrestin signaling.
- Investigating and comparing the role of MAP kinase proteins in *Ch25h* and dual-specificity MAPK phosphatase (*Dusp*) gene expression.
- Investigating the phosphorylation status of p38 MAPK and signal transducer and activator of transcription-1 (STAT1).
- Investigating the role of Nox activity.

2.2. Examination of CH25H activity in VSMCs:

- Verifying the subcellular localization of the CH25H enzyme.
- Assessing 25-HC levels in the supernatant of AngII-stimulated VSMC cultures.

3. Methods

3.1. Isolation of primary rat VSMCs

The primary rat VSMCs used in our experiments originated from male Wistar rats (Charles River Laboratories-Semmelweis University, Budapest). Our laboratory follows the guidelines established by the Guide for the Care and Use of Laboratory Animals (NIH, 8th edition, 2011) and national legal and institutional guidelines for animal care. This study was approved by the Animal Care Committee of Semmelweis University, Budapest, and by Hungarian authorities (No. 001/2139–4/2012). All procedures followed legal and institutional guidelines for animal care.

The VSMCs were isolated from the thoracic aorta of animals aged between 40-50 days, weighing between 170-250g. The isolation followed the explant method. The thoracic aorta was excised – tunica adventitia removed -, then cut into pieces of circa 1mm followed by collagenase treatment for 25 minutes. Aorta pieces were laid onto culture plates, allowing VSMC migration onto the plate surface. Tissue was removed once VSMCs were observed around aorta-rings. VSMCs were further passaged, cells used in the following experiments were in their second or third passages. Homogeneity of VSMCs was assessed with smooth muscle alpha-actin immunocytochemical labeling. Cells were visualized using confocal laser-scanning microscopy.

3.2. Stimulus and inhibitor treatment of VSMCs

Prior to experiments VSMCs were serum deprived overnight, using serum-free Dulbecco's modified Eagle media (DMEM) supplemented with 0.1% bovine serum albumin (BSA). During the experiments, VSMCs remained in serum-free DMEM and all reagents used to treat and stimulate the cells were also diluted in serum-free DMEM.

When investigating the time kinetics of *Ch25h* mRNA expression, we stimulated VSMCs with 100 nM AngII for 1, 2,

3, 4, 5 and 6 hours. Control group received no stimulus. When investigating the effects of β-arrestin-biased AT1R agonist, TRV120023 (TRV3), VSMCs were stimulated with 3 μM TRV3 for 1 hour.

We investigated the effect of AngII on 25-HC concentration. In these experiments VSMCs were stimulated with 1 μM AngII for 2, 4, 8, 16, and 24 hours or not stimulated. We prepared duplicate samples during the course of the experiments. Following the hormone stimulus, the supernatants of VSMCs were collected and subjected to Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) measurement.

In order to investigate the actions of various proteins in signaling events leading to gene expression changes, we employed AT1R antagonist and inhibitors. Control groups received dimethyl sulfoxide (DMSO) vehicle pretreatment. Other groups were pretreated with 10 μM candesartan, 50 μM SB202190, 20 μM PD98059, 5 μM DPI, 1 μM JNK-IN-8 (IN-8) or 1 μM YM-254890. Pretreatment lasted for 30 minutes. Then we stimulated cells with 100 nM AngII or vehicle for 1 hour in case of *Ch25h* mRNA detection and for 2 hours in case of *Dusp* mRNA detection. Samples were then prepared for RNA extraction.

3.3. RNA and cDNA preparation

Total RNA was isolated using the RNeasy Plus Mini kit from Qiagen, according to manufacturer's instructions. The RNA samples were then subjected to cDNA preparation. Reverse transcription was carried out with the RevertAid Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA samples were then diluted with 80 μl RNase-free water and stored at -20°C.

3.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR reactions were prepared using the SYBR Green Kit (LightCycler 480 SYBR Green I Master; Roche) according to the manufacturer's instructions. For the assessment of gene

expression of target genes, we employed relative quantification, where the mRNA level of target genes was compared to that of a reference gene. As a reference, we used the mRNA level of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The measurements were carried out with the LightCycler 480 instrument (Roche). The cycle threshold (Ct) was calculated with the second derivative method using LightCycler 480 Software.

3.5. Western blot

Serum deprived VSMCs were pretreated with 50 μM SB202190 or 1 μM YM-254890 for 30 minutes. VSMCs were then stimulated with 100 nM AngII for 10 minutes or 20 minutes. VSMCs were lysed with 100 μl 2X Laemmli sample buffer and subjected to electrophoresis. Phospho-p38 MAPK (Thr180/Tyr182) protein was labeled in samples that were stimulated with AngII for 10 minutes, whereas phospho-STAT1 (Ser727) was labeled in samples stimulated with AngII for 20 minutes. Horseradish peroxidase (HRP) conjugated secondary antibody against rabbit IgG was used to label both primary antibodies.

3.6. Transfection of A7R5 cell line and primary rat VSMCs

A7R5 cells between their third and tenth passages were plated onto μ-Slide 8 well plate (Ibidi) in a 1×10^5 cells/well density. The three separate DNA construct combinations used were as follows: Cerulean-CH25H + mRFP-SAC1; Cerulean-CH25H + mRFP-TGN38; Cerulean-CH25H + mRFP-Giantin. A7R5 cells were cotransfected with one of the DNA construct combinations using the Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. The primary rat VSMCs were plated onto μ-Slide 8 well plate in a 1×10^5 cells/well density. VSMCs were cotransfected with Cerulean-CH25H and mRFP-SAC1 constructs, using the FuGENE 6 transfection reagent (Promega) according to manufacturer's instructions. To detect fluorescent signals of the

expressed fluorescent fusion proteins in transfected rat primary VSMCs and A7R5 cells, we utilized confocal laser-scanning microscopy (Zeiss LSM 710).

3.6. Liquid Chromatography–Tandem Mass Spectrometry

LC-MS/MS was performed in the Research Center for Natural Sciences. Briefly, proteins were precipitated in the supernatant (300 µl) of AngII-stimulated VSMCs with methanol (900 µl). Following vortex and centrifugation of the samples a volume of 100 µl was subjected to LC-MS/MS on a Sciex 6500QTrap mass spectrometer coupled with an Agilent 1100 HPLC system.

3.7. Statistical analysis

GraphPad Prism 9.1.2 software was used to perform statistical analysis and to plot graphs. The qRT-PCR and 25-HC concentration data were analyzed using the multiple linear regression procedure with a 95% confidence interval. To analyze data of experiments employing TRV3 stimulus, we applied the unpaired *t*-test, which was suitable for comparison of the control and the stimulated groups.

4. Results

4.1. *Ch25h* gene expression is upregulated in response to AngII stimulus in primary rat VSMCs

In order to determine the time kinetics of AngII-induced gene expression change we stimulated VSMCs with 100 nM AngII for various time spans. We found that *Ch25h* mRNA levels increased in response to the AngII stimulus. The peak of *Ch25h* mRNA levels was observed in the group that was stimulated for 1 hour. At this time-point, AngII induced a more than fifty-fold increase compared to the baseline *Ch25h* mRNA levels.

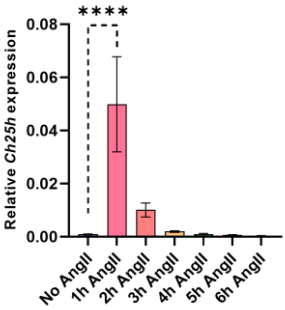

Figure 1. AngII induces the upregulation of *Ch25h* in rat VSMC. *Ch25h* mRNA levels are shown relative to *Gapdh* mRNA levels. The figure depicts mean values ± SEM of n = 5 independent experiments. Data were analyzed using multiple linear regression, **** $p < 0.0001$.

4.2. AT1R and $G_{q/11}$ activity is responsible for AngII-induced *Ch25h* upregulation in primary rat VSMC

VSMCs were pretreated with 10 μM candesartan, AT1R antagonist, for 30 minutes prior to 1 hour of AngII (100 nM) stimulus. We found that the candesartan pretreatment completely abolished the AngII-induced *Ch25h* upregulation.

To assess the role of $G_{q/11,}$ its inhibitor the YM-254890 was applied at a concentration of 1 μM, as described previously. YM-254890 pretreatment rendered AngII unable to induce *Ch25h* upregulation. We also found that the TRV3 stimulus was insufficient to induce *Ch25h* upregulation in VSMCs.

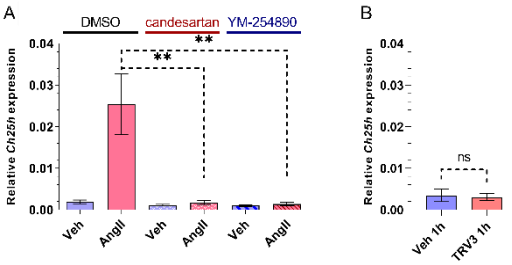

Figure 2. AT1R- and $G_{q/11}$-dependent *Ch25h* gene expression in rat VSMCs in response to AngII. *Ch25h* mRNA levels are shown relative to *Gapdh* mRNA levels. (A) The figure depicts mean values ± SEM of n = 4–5 independent experiments. Data were analyzed using multiple linear regression, ** $p < 0.01$. (B) The figure depicts mean values ± SEM of n = 3 independent experiments. Data were analyzed using unpaired *t*-test, ns: not significant.

4.3. Role of the MAP kinase family proteins in AngII-induced gene expression changes in primary rat VSMCs

Literature data indicates that MAPK proteins can affect *Ch25h* and *Dusp* expression. In order to find out how individual MAPKs influence these AngII-induced gene expression changes, we employed several inhibitors. We utilized the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (20 μM) for the elimination of ERK1/2 activity, SB202190 (50 μM) for p38 MAPK activity inhibition, and IN-8 (1 μM) to inhibit JNKs. Inhibitor pretreatment was followed by AngII (100 nM) stimulus.

We found that lack of ERK1/2 activity caused a slight decrease of *Ch25h* mRNA levels in the AngII-stimulated group, however it significantly inhibited the AngII-induced *Dusp6* upregulation.

Our results showed that the inhibition of p38 MAPK completely blocked the AngII-induced *Ch25h* upregulation. Similarly, AngII-induced *Dusp5* and *Dusp6* upregulation was significantly decreased as a result of p38 MAPK inhibition. We found that neither the lack of ERK1/2 activity nor the lack of p38 MAPK activity affected *Dusp10* upregulation.

The inhibition of JNK did not alter AngII-induced *Ch25h* upregulation.

4.3.1. Effect of pharmacological inhibitors on the phosphorylation of p38 MAPK and STAT1

As p38 MAPK was shown to be significant in AngII-induced *Ch25h* upregulation, we assessed its phosphorylation status, thus its activity, in response to inhibitors which were effective in abolishing *Ch25h* expression. Furthermore, we investigated the phosphorylation of the p38 MAPK substrate, the signal transducer and activator of transcription (STAT1), which transcription factor was shown to be promoting *Ch25h* expression.

Our Western blot data showed that AngII increases phosphorylation of both p38 MAPK and STAT1. However, inhibition of $G_{q/11}$ reduced phosphorylation of both proteins. We also found that the inhibition of p38 MAPK caused the decrease of phosphorylated p38 MAPK and STAT1 levels.

4.4. The inhibition of Nox activation does not impact AngII-induced *Ch25h* upregulation in primary rat VSMCs

We hypothesized that Nox proteins on account of their role in reactive oxygen species production may affect *Ch25h* expression. To investigate this possibility, we utilized diphenyleneiodonium chloride (DPI), a compound which inhibits a wide range of Nox proteins.

Our results showed that DPI did not affect AngII-induced upregulation of *Ch25h*.

4.5. CH25H protein localizes to the endoplasmic reticulum in the A7R5 rat VSMC cell line and in primary rat VSMCs

The subcellular localization of CH25H protein was investigated in transfected primer rat VSMCs and A7R5 VSMC cell line. We designed and created a DNA construct encoding Cerulean-labeled CH25H fusion protein (Cerulean-CH25H). Additionally, we used various mRFP-labeled organelle marker fusion protein encoding DNA constructs: ER marker; phosphatidylinositol-3-phosphatase (SAC1) (mRFP-SAC1),

Golgi apparatus membrane marker; (mRFP-Giantin), trans-Golgi network membrane marker (mRFP-TGN38).

Confocal images showed that the Cerulean-CH25H and mRFP-SAC1 proteins presented strong colocalization in the case of A7R5 and primary VSMCs. However, neither mRFP-Giantin nor mRFP-TGN38 signals showed strong colocalization with the Cerulean-CH25H signal in the transfected A7R5 cells. Based on our data, the CH25H protein localizes to the ER of VSMCs.

4.6. AngII-stimulated primary rat VSMCs release 25-HC

In order to investigate 25-HC levels in the supernatant of VSMCs, hours-long AngII stimuli were employed. A concentration of 1 μM AngII was used and stimuli of VSMC cultures lasted for 2, 4, 8, 16, and 24 hours. 25-HC levels in the various supernatant samples were assessed with LC-MS/MS. We identified a progressive increase of 25-HC concentration up until the 4-hour time point, where an average of 8.2 ng/ml was reached. After the 16-hour time point, 25-HC levels in the VSMC supernatants return to approximately the baseline levels. We found that 25-HC concentrations were significantly increased in the supernatant of the group stimulated with AngII for 4 hours compared to that of the non-stimulated group.

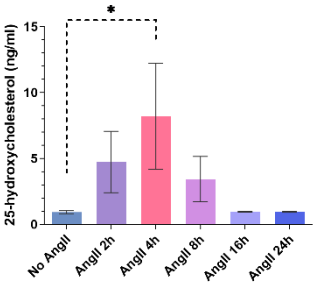

Figure 3. AngII induces 25-HC production by VSMCs. The 25-HC concentration in the supernatants of AngII-stimulated VSMCs was measured with LC-MS/MS. Average 25-HC concentration values are shown as mean ± SEM based on n = 3 independent experiments. Statistical analysis was carried out using multiple linear regression, * $p < 0.05$.

5. Conclusions

The aims of this study were the identification of the underlying signaling mechanisms that lead to *Ch25h* expression in VSMCs and the investigation of CH25H subcellular localization and action. The major conclusions of this thesis, as per respective objectives, are the following **(Figure 4)**:

5.1. Investigation of AngII-induced *Ch25h* expression in VSMCs and underlying signaling pathways:

- AngII induces the upregulation of *Ch25h* in primary rat VSMCs.
- AngII-induced *Ch25h* upregulation is dependent on AT1R and $G_{q/11}$ activity.
- The p38 MAPK is essential for the AngII-induced *Ch25h* upregulation, whereas ERK1/2 activity has no significant effect on the expression of *Ch25h*.
- The AngII-induced upregulation of *Dusp* isoforms is affected by p38 MAPK and ERK1/2. p38 MAPK is significant in the AngII-induced expression of *Dusp5* and *Dusp6*, whereas ERK1/2 plays a significant role in *Dusp6* expression.
- AngII stimulus of VSMCs causes phosphorylation of p38 MAPK and STAT1 transcription factor. The p38 MAPK activity is necessary for STAT1 phosphorylation on the Ser727 residue.
- Nox functions have no effect on AngII-induced *Ch25h* upregulation in VSMCs.

5.2. Examination of CH25H activity in VSMCs:

- The CH25H enzyme localizes to the ER in VSMC cell lines.
- AngII promotes the production of 25-HC by VSMCs, demonstrating the catalytic activity of the upregulated CH25H in VSMCs.

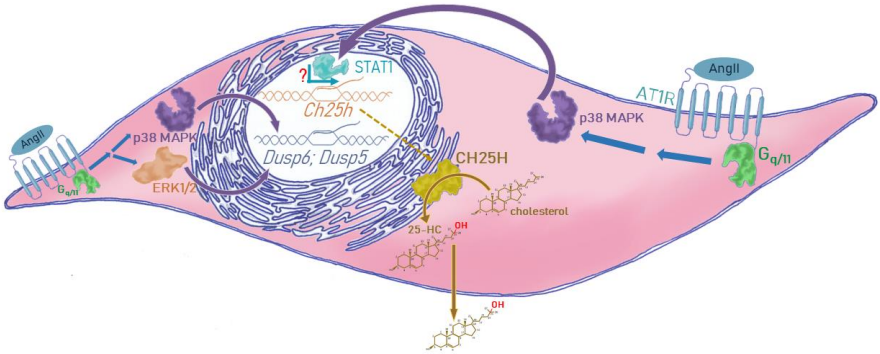

Figure 4. Scheme of AngII-induced signaling that leads to *Dusp* and *Ch25h* expression and 25-HC release. (Own figure based on the conclusions of chapter 5.)

6. Bibliography of the candidate's publications

Publications related to the thesis:

I. **Kovács, Kinga Bernadett** ; Szalai, Laura ; Szabó, Pál ; Gém, Janka Borbála ; Barsi, Szilvia ; Szalai, Bence ; Perey-Simon, Bernadett ; Turu, Gábor ; Tóth, András Dávid ; Várnai, Péter ; Hunyady, László ; Balla, András. An Unexpected Enzyme in Vascular Smooth Muscle Cells: Angiotensin II Upregulates Cholesterol-25-Hydroxylase Gene Expression. International Journal of Molecular Sciences, 2023, 24.4: 3968. **IF(2023): 4.9**

II. Gém, Janka Borbála ; **Kovács, Kinga Bernadett*** ; Szalai, Laura ; Szakadáti, Gyöngyi ; Porkoláb, Edit ; Szalai, Bence ; Turu, Gábor ; Tóth, András Dávid ; Szekeres, Mária ; Hunyady, László ; Balla, András. Characterization of Type 1 Angiotensin II Receptor Activation Induced Dual-Specificity MAPK Phosphatase Gene Expression Changes in Rat Vascular Smooth Muscle Cells. Cells, 2021, 10.12:3538. **IF(2021): 7.666**

Publications not related to the thesis:

I. Szalai, Laura ; Sziráki, András* ; Erdélyi, László Sándor ; **Kovács, Kinga Bernadett** ; Tóth, Miklós ; Tóth, András Dávid ; Turu, Gábor ; Bonnet, Dominique ; Mouillac, Bernard ; Hunyady, László ; Balla, András. Functional Rescue of a Nephrogenic Diabetes Insipidus Causing Mutation in the V2 Vasopressin Receptor by Specific Antagonist and Agonist Pharmacochaperones. Frontiers in Pharmacology, 2022, 13: 811836. **IF(2022): 5,6**

II. Bugyi, Fanni ; Tóth, Gábor ; **Kovács, Kinga Bernadett** ; Drahos, László ; Turiák, Lilla. Comparison of solid-phase extraction methods for efficient purification of phosphopeptides with low sample amounts. Journal of Chromatography A, 2022, 1685: 463597. **IF(2022): 4.1**

III. Vass, Zsolt ; Shenker-Horváth, Kinga ; Bányai, Bálint ; Vető, Kinga Nóra ; Török, Viktória ; Gém, Janka Borbála ; Nádasy, György L. ; **Kovács, Kinga Bernadett** ; Horváth, Eszter Mária ; Jakus, Zoltán ; Hunyady, László ; Szekeres, Mária ; Dörnyei, Gabriella. Investigating the Role of Cannabinoid Type 1 Receptors in Vascular Function and Remodeling in a Hypercholesterolemic Mouse Model with Low-Density Lipoprotein–Cannabinoid Type 1 Receptor Double Knockout Animals. International Journal of Molecular Sciences, 2024, 25.17:9537. **IF(2023): 4.9**

∑**IF:** 27.166