# Increased Expression of Chondroitin Sulfotransferases following AngII may Contribute to Pathophysiology Underlying Covid-19 Respiratory Failure: Impact may be Exacerbated by Decline in Arylsulfatase B Activity

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Abstract - The spike protein of SARS-CoV-2 binds to respiratory epithelium through the ACE2 receptor, an endogenous receptor for Angiotensin II (AngII). The mechanisms by which this viral infection leads to hypoxia and respiratory failure have not yet been elucidated. Interactions between the sulfated glycosaminoglycans heparin and heparan sulfate and the SARS-CoV-2 spike glycoprotein have been identified as participating in viral adherence and infectivity. In this brief report, we present data indicating that stimulation of vascular smooth muscle cells by AngII leads to increased expression of two chondroitin sulfotransferases (CHST11 and CHST15), which are required for the synthesis of the sulfated glycosaminoglycans chondroitin 4-sulfate (C4S) and chondroitin 4,6-disulfate (CSE). We suggest that increased expression of these chondroitin sulfotransferases and the ensuing production of chondroitin sulfates may contribute to viral adherence to bronchioalveolar cells and to the progression of respiratory disease in Covid-19. The enzyme Arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase), which removes 4-sulfate groups from the non-reducing end of chondroitin 4-sulfate residues, is required for degradation of C4S and CSE. In hypoxic conditions or following treatment with chloroquine, ARSB activity is reduced. Decline in ARSB can contribute to ongoing accumulation and airway obstruction by C4S and CSE. Decline in ARSB leads to increased expression of Interleukin(IL)-6 in human bronchial epithelial cells, and IL-6 is associated with cytokine storm in Covid-19. These findings indicate how chondroitin sulfates, chondroitin sulfotransferases, and chondroitin sulfatases may participate in the progression of hypoxic respiratory insufficiency in Covid-19 disease and suggest new therapeutic targets.

## Introduction

The COVID-19 pandemic presents unique challenges, as the medical and scientific communities develop new therapies, new diagnostic tests, and new insights into the mechanisms by which the SARS-CoV-2 virus acts. Studies of coronavirus-receptor interactions have implicated the sulfated glycosaminoglycans heparin and heparan sulfate in viral attachment to mammalian cells [1,2]. Recent work on the structure of the spike protein of SARS-CoV-2 has provided detail about the glycosylations of the spike protein and interactions between the spike protein and carbohydrates [3,4]. Findings suggest potential for both protein-carbohydrate and carbohydrate-carbohydrate interactions between the spike glycoprotein and cell receptors. A galectin-like fold identified in the SARS-CoV-2 spike protein suggests potential binding with chondroitin sulfates, the preferred binding partners of galectins [5]. Circular dichroism studies demonstrated similar effects of heparin and chondroitin sulfates on binding with the spike glycoprotein and on conformational change in the SARS-CoV-2 spike protein receptor binding domain (RBD) [6]. Heparin's ability to prevent infection by SARS-CoV-2 may be related to competition with chondroitin sulfates, since similar conformational changes in the SARS-CoV-2 S1 RBD follow treatment by chondroitin 4-sulfate (C4S,CSA), chondroitin 6-sulfate (C6S, CSC), dermatan sulfate (formerly CSB), and chondroitin sulfate D (chondroitin 2,6-disulfate), as well as by heparin and heparan sulfate [6].

The galectin-like fold at the N-terminal domain of the spike protein is reported in other coronaviruses to bind with carbohydrates on cell surfaces as an initial step in viral entry [8]. Insertion of amino acids at the cleavage site between the S1/S2 subunits of SARS-CoV-2 spike protein has created three potential GalNAc O-glycosylation sites [8], not previously predicted for the bat or pangolin virus [9]. Recent glycoproteomics data identify sulfation at the non-reducing end of HexNAc residues modifying amino acids of the spike protein, most notably at position 74 [10]. Together, these associations reflect the potential impact of the GalNAc moiety, which may be sulfated, on infectivity of SARS-CoV-2.

Our studies of chondroitin sulfates, chondroitin sulfatases, and chondroitin sulfotransferases demonstrate several findings that are pertinent to mechanisms of how SARS-CoV-2 infection might lead to respiratory insufficiency through interactions with sulfated GAGs [11,12,14-16,18,19]. In this brief report, data are presented showing increased expression of the chondroitin sulfotransferases CHST11 and CHST15 in human vascular smooth muscle cells following stimulation by exogenous Angiotensin (Ang) II. CHST15 (*N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase; GalNAc4S-6ST), also known as BRAG (B-cell Recombination Activating Gene), is required for the synthesis of chondroitin sulfate E (CSE; $[GlcA\text{-}GalNAc\text{-}4S,6S]_n$, in which S corresponds to sulfate) from chondroitin 4-sulfate (C4S; $[GlcA\text{-}GalNAc\text{-}4S]_n$). CSE is composed of alternating β-1,4- and β-1,3- linked D-glucuronate and D-*N*-acetylgalactosamine-4S,6S residues, and is found throughout human tissues. Increases in CHST15 or in CSE have been recognized in malignant cells and tissues and in models of tumor progression and tissue remodeling, including in pulmonary and cardiac tissues [11,20,21].

The potential effect of decline in activity of the enzyme arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase), in association with increases in CHST11 and CHST15, and the resulting increases in C4S and CSE, on Covid-19 disease is addressed. The enzyme arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase), which removes the 4-sulfate group at the non-reducing end of the chondroitin 4-sulfate chain, is required for degradation of C4S and CSE. When ARSB activity is reduced, chondroitin sulfates can accumulate and affect vital cellular functions. Several points about the biological effects of ARSB are pertinent, including: a) ARSB activation requires oxygen for post-translational modification and activation, and ARSB activity is lower in hypoxic conditions [12,13]; b) decline in ARSB is associated with increased IL-6 expression in human bronchial epithelial cells and in patients with cystic fibrosis and asthma [14]; c) decline in ARSB replicates effects of hypoxia and generation of sulfate by ARSB may be important in mitochondrial metabolism [12,15]; d) accumulation of sulfated

glycosaminoglycans when ARSB is reduced can contribute to inflammation and pulmonary pathophysiology, as in cystic fibrosis [16]; e) in patients with moderate COPD, refractoriness to oxygen therapy was associated with gene mutations regulating ARSB expression [17]; and f) changes in chondroitin 4-sulfation affect binding of the critical molecules galectin-3 and SHP2 (PTPN11; protein tyrosine phosphatase non-receptor type 11) with impact on transcriptional events and vital cell signaling [18,19]. The lysosomal enzyme galactose 6-sulfate sulfatase (GALNS) removes the 6-sulfate group at the non-reducing end of CSE, followed by removal of the 4-sulfate group at the non-reducing end by ARSB [11]. Deficiency of lysosomal acidification, as by treatment with chloroquine or hydroxychlorooquine [20], can inhibit activity of these chondroitin sulfatases and contribute to the accumulation of C4S and CSE [23]. We hypothesize that hydroxychloroquine treatment and hypoxia reduce ARSB, and that decline in ARSB contributes to cytokine storm and respiratory distress in Covid-19. Decline in ARSB activity exacerbates the impact of enhanced production of CHST11 and CHST15 due to ACE2-mediated increases in CHST11 and CHST15.

With these background considerations, we report increased expression of chondroitin sulfotransferases by the AngII-activated pathway and hypothesize how these effects might contribute to the manifestations of Covid-19 infection and suggest new approaches to reduce disease morbidity and mortality.

## Materials and Methods

Human Cell Samples

Human aortic smooth muscle cells (Lifeline Technology, Oceanside, CA, USA) were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% $CO_2$. Cells were passaged twice, then grown in multiwell culture plates to 75% confluence, and media was changed to DMEM without serum for 24 h to maintain quiescence. Cells were then treated with Angiotensin II (AngII; 1 μM x 24 h; Sigma-Aldrich, St. Louis, MO), (10 μM x 24h; Sigma-Aldrich), or their combination for 24 h, harvested and frozen for further experiments.

Circulating leukocytes cells were isolated from whole blood samples obtained from patients aged 2-17, followed at Rush University Medical Center (RUMC) for cystic fibrosis, asthma, or other conditions under a protocol approved by RUMC and the University of Illinois at Chicago (UIC) Institutional Review Boards [14]. Venous blood was collected in citrated tubes and

processed within 2 h of collection. Subjects had no acute illness at the time of blood collection. Blood samples were identified with a study number, and a registry linked patient clinical data with study identifier. Polymorphonuclear (PMN) and mononuclear (MC; monocytes and lymphocytes) white blood cells were collected separately from the whole blood samples by the Polymorphprep™ kit (AXIS-SHIELD, Oslo, Norway) [14]. Cells were stored at -80°C until further experiments.

Measurement of Arylsulfatase B Activity

ARSB activity was determined in the leukocyte samples by a fluorometric assay, following a standard protocol [11]. Protocol required 20 ml of cell homogenate, 80 ml of assay buffer (0.05 M Na-acetate buffer with 20 mmol/l barium sulfate:1.0 mol/l Na acetate, pH 5.6), and 100 ml of substrate [5 mM 4-methylumbilliferyl sulfate (MUS)] in assay buffer. Materials were combined in microplate wells, and the microplate was incubated for 30 min at 37°C. The

reaction was stopped by adding 150 ml of stop buffer (glycine–carbonate buffer, pH 10.7), and fluorescence was detected at 360 nm (excitation) and 465 nm (emission). ARSB activity was expressed as nmol/mg protein/h, and was derived from a standard curve prepared using known quantities of 4-methylumbilliferyl at pH 5.6.

Measurement of Interleukin-6 by ELISA

Interleukin (IL)-6 in the patient plasma samples was measured by the Quantikine ELISA kit for human IL-6 (R&D Systems, Minneapolis, MN), as previously [14]. IL-6 in the samples was captured into the wells of a microtiter plate pre-coated with specific anti-IL-6 monoclonal antibody, and the immobilized IL-6 was detected by a biotinylated second antibody and streptavidin-horseradish peroxidase (HRP)-conjugate with the chromogenic substrate hydrogen peroxide/ tetramethylbenzidine (TMB). Color intensity was read at 450 nm with a reference filter of 570 nm in an ELISA plate reader (FLUOstar, BMG LABTECH, Inc., Cary, NC). IL-6 concentrations were extrapolated from a standard curve plotted using known concentrations of IL-6, expressed as picograms per milliliter plasma (pg/ml).

QRT-PCR for sulfotransferases and proteoglycans

QRT-PCR was performed using established techniques and primers identified using Primer 3 software [24,25]. Primers were:

CHST11 human (NM_018143) Forward: 5’-GTTGGCAGAAGAAGCAGAGG-3’ and Reverse: 5’-GACATAGAGGAGGGCAAGGA-3’;

CHST15 human (NM_015892) Forward: 5’-ACTGAAGGGAACGAAAACTGG-3’ and Reverse: 5’-CCGTAATGGAAAGGTGATGAG-3’.

ARSB (NM_000046) Forward: 5´-AGACTTTGGCAGGGGGTAAT-3´ and Reverse: 5´-CAGCCAGTCAGAGATGTGGA-3´.

Biglycan (BC002416.2) Forward: 5’-ACTGGAGAACAGTGGCTTTGA-3’ and Reverse: 5’-ATCATCCTGATCTGGTTGTGG-3’.

CSPG4 (NM_001897) Forward: 5'-CTTCAACTACAGGGCACAAGG-3' and Reverse: 5'-AGGACATTGGTGAGGACAGG-3';

Perlecan (NM_001291860) Forward: 5'-CCTTGCTCAGAATGCACTAGG-3' and Reverse: 5'-TGATGAGTGTGTCACCTTCCA-3';

Syndecan-1 (NM_001202) Forward: 5'-CTCTGTGCCTTCGTCTTTCC-3' and Reverse: 5'-CCACCTTCCTTTGCCATTT-3';

TPST1 (NM_003596.3) Forward: 5'-GACCTCAAAGCCAACAAAACC-3' and Reverse: 5'-TCAGTAACACCAGCCTCATCC-3';

Versican (NC_0000005.10) Forward: 5'-CCACTCTGTTTTCTCCCCATT-3' and Reverse: 5'-ATCCCTTTGTGCCCTTTTTC-3'.

Relative expression was calculated by standard methods comparing treated and control cells using GAPDH as housekeeping gene.

Measurement of Total Sulfated Glycosaminoglycans and Sulfotransferase Activity

Measurement of total sulfated glycosaminoglycans (GAGs) was performed as previously described [16]. Briefly, the Blyscan™ assay kit (Biocolor Ltd, Newtownabbey, N. Ireland) was used for detection of the sulfated GAG, based on the reaction of 1,9-dimethylmethylene blue with the sulfated oligosaccharides in the GAG chains.

Sulfotransferase activity was determined using the Universal Sulfotransferase Activity kit (R&D, Minneapolis, MN), which assesses the sulfotransferase activity of all the sulfotransferases in the cells or tissue tested [26]. 3′ -Phosphoadenosine-5′ -phosphosulfate (PAPS) is the sulfate donor for the sulfotransferase reaction, and PAPS (10 µl, 1 mM), acceptor substrate (10 µl), and coupling phosphatase (3.5 µl, 100 ng/µ l) were combined with 25 µl/well of the cell suspension in the wells of a microtiter plate. For negative controls, assay buffer was substituted for the cell suspension. The mixture was incubated for 20 min at 37°C, then 30 µl of malachite green was added and the mixture gently tapped. De-ionized water (100 µl) was added to each well, color was developed, and the optical density of each well was read and compared

among the different wells. The amount of product formation was calculated using a phosphate standard curve, following subtraction of the negative control. The 3-inorganic phosphate released by the coupling phosphatase and detected by malachite green phosphate detection reagent, was proportional to the 3′-phosphoadenosine-5′-phosphate generated, thereby indicating the extent of the sulfotransferase reaction which utilized the sulfate group of PAPS.

Statistical Analysis

Data were analyzed using InStat software (GraphPad, San Diego, CA) by unpaired t-tests, two-tailed, with adjustment for equal or unequal standard deviation, or by one-way ANOVA with Tukey-Kramer post-test. For control and AngII treatments, 9-15 biological samples were tested, with technical replicates of each determination. Plasma IL-6 determinations were performed with duplicate samples, using the average of two determinations. Box-whisker plots indicate the median, 25-75% quartiles, minimum value, maximum value, and outliers. P-values $\leq$ 0.05 are considered statistically significant. *** represents $p \leq 0.001$, ** indicates $p \leq 0.01$, and * indicates $p \leq 0.05$.

## Results

AngII exposure leads to marked increase in expression of CHST11 and CHST15

Treatment of human aortic smooth muscle cells with Angiotensin (Ang) II led to marked increases in expression of CHST11 and CHST15. CHST11 increased to 3.5 times the baseline level (n=18) ($p<0.001$), and CHST15 increased to 2.5 times the baseline level (n=9) ($p<0.001$) (**Fig.1A,B**). Co-treatment with candesartan, an angiotensin(AT)-1 receptor blocker, largely, but incompletely inhibited the AngII-induced increases in expression of CHST11 and CHST15 (**Fig.1C,1D**). Consistent with the CHST increases, the total sulfated glycosaminoglycans (GAGs) were increased, and the increase was largely inhibited by ARB treatment ($p<0.001$) (**Fig.2A,2B**). The measured sulfotransferase activity was also increased ($p<0.001$) and incompletely inhibited by treatment with candesartan (**Fig.2C,2D**).

Increased expression of chondroitin sulfate associated proteoglycans

Following treatment with AngII, expression of several proteoglycans with chondroitin-sulfate attachments increased. These include increases from control values for versican, syndecan-1, biglycan, and perlecan ($p<0.001$) (**Fig.3A**). No increases were shown for chondroitin sulfate proteoglycan (CSPG)4, tyrosylprotein sulfotransferase (TPST)1, or arylsulfatase B (ARSB). Treatment with candesartan largely inhibited the increases in expression of these proteoglycans (**Fig.3B**).

Increase in IL-6 in association with Decline in ARSB

In plasma from patients with cystic fibrosis or asthma, Interleukin-6 values were significantly greater in the plasma from the patients with cystic fibrosis and in most of the patients with asthma, in contrast to the controls ($p<0.001$) (**Fig.4A**) [14]. Also, neutrophil Arylsulfatase B activity in CF was less compared to levels in neutrophils from healthy normal controls ($p<0.001$) (**Fig.4B**) [14]. An inverse relationship between ARSB and IL-6 in the subjects is apparent ($r = -0.80$) (**Fig.4C**). Previously, we reported an increase in IL-6 mRNA expression in

human bronchial epithelial cells (BEC) following ARSB silencing, and IL-6 was increased in the spent media of the cultured BEC following ARSB silencing [14].

Overall schematic showing the potential impact of increase in sulfotransferases and of decline in chondroitin sulfatases in the pathophysiology of Covid-19

An overall representation of the proposed interactions between spike protein receptor binding with the ACE2 receptor indicates increased expression of CHST11 and CHST15 (**Fig. 5**). Increases in these sulfotransferases leads to increased C4S and CSE production, which accumulate further when ARSB activity is reduced. Decline in ARSB leads to increased expression of IL-6, thereby contributing to cytokine storm and exacerbation of effects of infection with SARS-CoV-2. The enzyme N-acetylgalactosamine-6-sulfatase (Galactose 6-sulfate-sulfatase; GALNS) is required to remove the 6-sulfate from CSE, prior to removal of the 4-sulfate by ARSB. If GALNS is reduced, CSE is anticipated to accumulate.

## Discussion

In the human aortic smooth muscle cells, exposure to Angiotensin II markedly increased the mRNA expression of CHST11 and CHST15. In contrast, there was no increased expression of TPST1, a tyrosylprotein sulfotransferase. The angiotensin-receptor blocker (ARB) candesartan incompletely inhibited the increase in expression. These findings suggest that stimulation of the ACE2 receptor by SARS-CoV-2 spike protein may also stimulate expression of these chondroitin sulfotransferases and increase abundance of the chondroitin sulfates chondroitin 4-sulfate and chondroitin 4,6-disulfate. Accumulation of chondroitin sulfates in the lung is anticipated to impair airflow and oxygenation, leading to secondary decline in ARSB activity due to the requirement for oxygen for post-translational modification and activation of ARSB [13]. Decline in ARSB was shown to increase IL-6 expression in bronchial epithelial cells and to be increased in patients with cystic fibrosis and asthma [14]. Decline in ARSB has also been associated with refractoriness to oxygen therapy in patients with moderate COPD [17]. These findings indicate that increased expression of chondroitin sulfotransferases, increased abundance of chondroitin sulfates, and decline in chondroitin sulfatase activity of ARSB activity can contribute to the pathophysiology of Covid-19, with effects on cytokine storm and respiratory failure.

In the human aortic smooth muscle cells, inhibition of the AngII receptor by the angiotensin receptor blocker (ARB) candesartan largely blocked the increases in CHST11 and CHST15 expression, total sulfated glycosaminoglycans, and sulfotransferase activity. The impact of ARB or angiotensin converting enzyme inhibitor (ACEI) therapy on the expression or activity of the ACE2 in human lung cells is not clarified, and the potential impact of ARB or ACEI on the severity of Covid-19 is under intensive investigation [27-29].

Decline in ARSB was associated with increased plasma IL-6 in patients with cystic fibrosis, and increased mRNA expression and increased secretion into media of cultured human bronchial epithelial cells [14]. Since IL-6 contributes to cytokine storm in Covid-19, this finding is

particularly relevant to the mechanism whereby ARSB decline can contribute to and can accelerate Covid-19 disease. Also, genomic data indicate that mutations affecting ARSB gene expression are associated with unresponsiveness to oxygen therapy in patients with moderate chronic obstructive lung disease [17], consistent with impaired response to oxygen therapy in Covid-19.

These findings suggest that treatment with chloroquine/hydroxychloroquine might impact on clinical response to SARS-CoV-2 infection, due to inhibition of ARSB and the associated increases in chondroitin 4-sulfation. The manifestations of lower ARSB may include: impact on viral binding to respiratory tract cells in which chondroitin 4-sulfation is increased; impaired responsiveness to oxygen treatment; and enhanced IL-6 production contributing to cytokine storm. Recombinant human (rh) ARSB is used for replacement in Mucopolysaccharidosis VI (MPS VI), the inherited genetic deficiency of ARSB [30]. As we await a vaccine and/or effective antiviral treatment of Covid-19, rhARSB may be a useful, new approach to refractory hypoxia in these patients.

We are mindful of the shortcomings of the data presented and hope that other investigators will further assess how chondroitin sulfates, chondroitin sulfotransferases and chondroitin sulfatases contribute to Covid-19 pathophysiology. These investigations may lead to more effective treatment and to better understanding of how to prevent severe disease.

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**Figure Legends**

Figure 1. Increased Expression of CHST11 and CHST15 following AngII Exposure

A. Following exposure to AngII (1 μM x 24h), mRNA expression of CHST11 increased to 3.5 times the baseline level (n=18, $p<0.001$).

B. Treatment with the ACE2 blocker candesartan (10 μM x 24h) nearly completely blocked the AngII-induced increase in CHST11 mRNA expression (n=6, $p<0.001$).

C. Following exposure to AngII (1 μM x 24h), mRNA expression of CHST15 increased to 2.5 times the baseline level (n=9, $p<0.001$).

D. Treatment with the ACE2 blocker candesartan (10 μM x 24h) nearly completely blocked the AngII-induced increase in CHST15 mRNA expression (n=6, $p<0.001$).

Figure 2. Sulfotransferase Activity and Total Sulfated Glycosaminoglycans

A. Sulfotransferase activity increased to 184 ± 9% over the control level following AngII ($p<0.001$, n=9).

B. Sulfotransferase activity declined to 116 ± 7% of the control level when cells were treated with an ARB and AngII.

C. Consistent with the increases in sulfotransferase activity and the increased expression of CHST11 and CHST15, the total sulfated glycosaminoglycans increased from 18.6 ± 1.1 μg/mg protein to 24.9 ± 1.5 μg/mg protein in the vascular smooth muscle cells following treatment by AngII.

D. When treated with candesartan and AngII, the peak total sulfated glycosaminoglycans declined by 4.8 μg/mg protein to 20.1 ± 0.7 μg/mg protein, reflecting an overall increase of 1.5 μg/ml.

Figure 3. Inverse relationship between Interleukin-6 and Arylsulfatase B.

A. Plasma Interleukin (IL)-6 levels were markedly increased in cystic fibrosis (n=16) compared to the normal controls (n=11) (65.5 ± 6.3 pg/ml vs. 5.1 ± 0.7 pg/ml; $p<0.001$). Levels in patients with asthma (n=10) (17.8 ± 6.1 pg/ml) were also significantly increased.

B. ARSB activity in the circulating neutrophils was markedly reduced in the cystic fibrosis patients, compared to the normal controls (51.9 ± 5.5 vs. 75.9 ± 6.1 nmol/mg protein/h) and the patients with asthma (67.8 ± 10.2 nmol/mg protein/h).

C. There is an inverse relationship between plasma IL-6 levels and neutrophil ARSB activity (r = -0.8).

Figure 4. Impact of AngII and Blocker on Proteoglycan mRNA Expression

A. mRNA expression of several proteoglycans is increased following treatment by AngII. Increases were shown in versican, syndecan-1, biglycan, and perlecan, but not in CSPG4 or in TPST1 (not shown).

B. Treatment with candesartan reduced, but did not eliminate, increases in the proteoglycans.

Figure 5. Schematic of Overall Pathway

The overall pathway indicates that transcriptional events arise following the stimulation of the ACE2 receptor, leading to increased expression of CHST11 and CHST15. The increased sulfotransferase expression, attributable either to AngII or to spike protein receptor binding, leads to increased production of C4S and CSE. Accumulation of these sulfated glycosaminoglycans causes decline in oxygenation, leading to decline in ARSB activity, and the further accumulation of C4S and CSE and expression of IL-6, and increasing respiratory insufficiency and cytokine storm.

Figure 1. Increased Expression of CHST11 and CHST15 following AngII Exposure

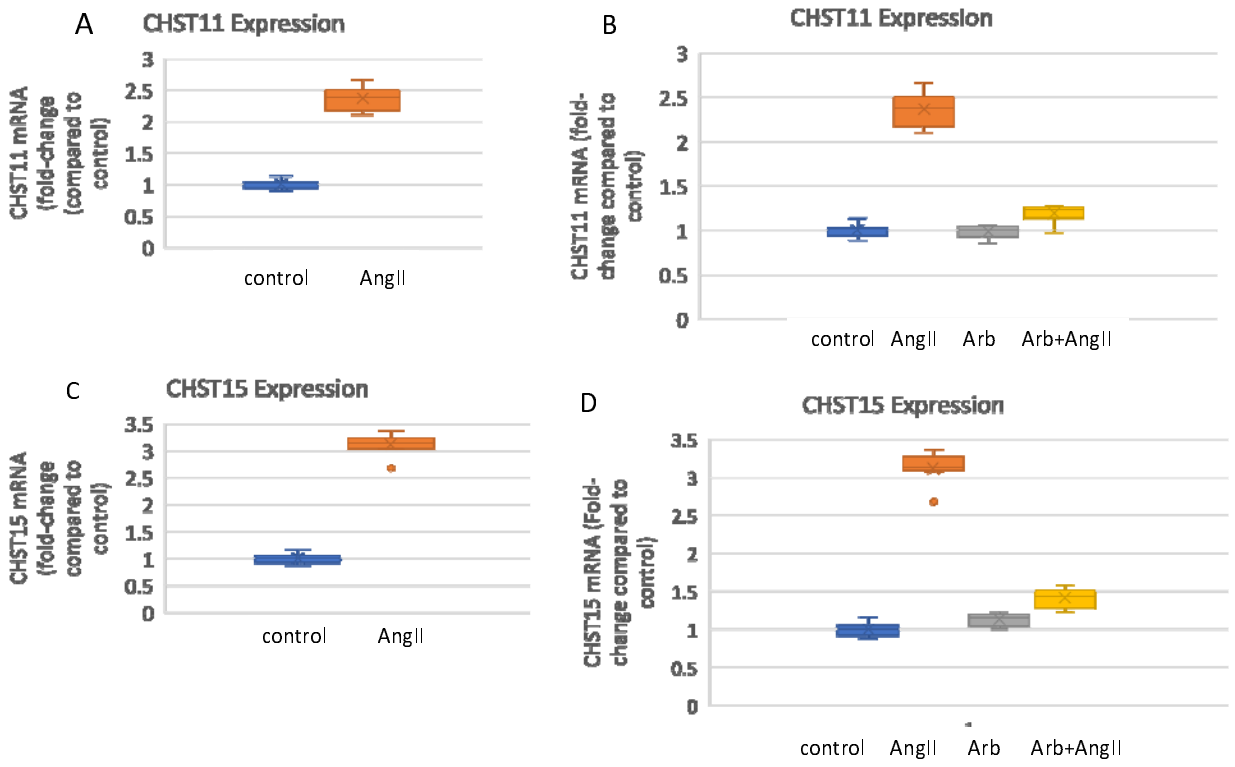

Figure 2. Sulfotransferase Activity and Total Sulfated Glycosaminoglycans

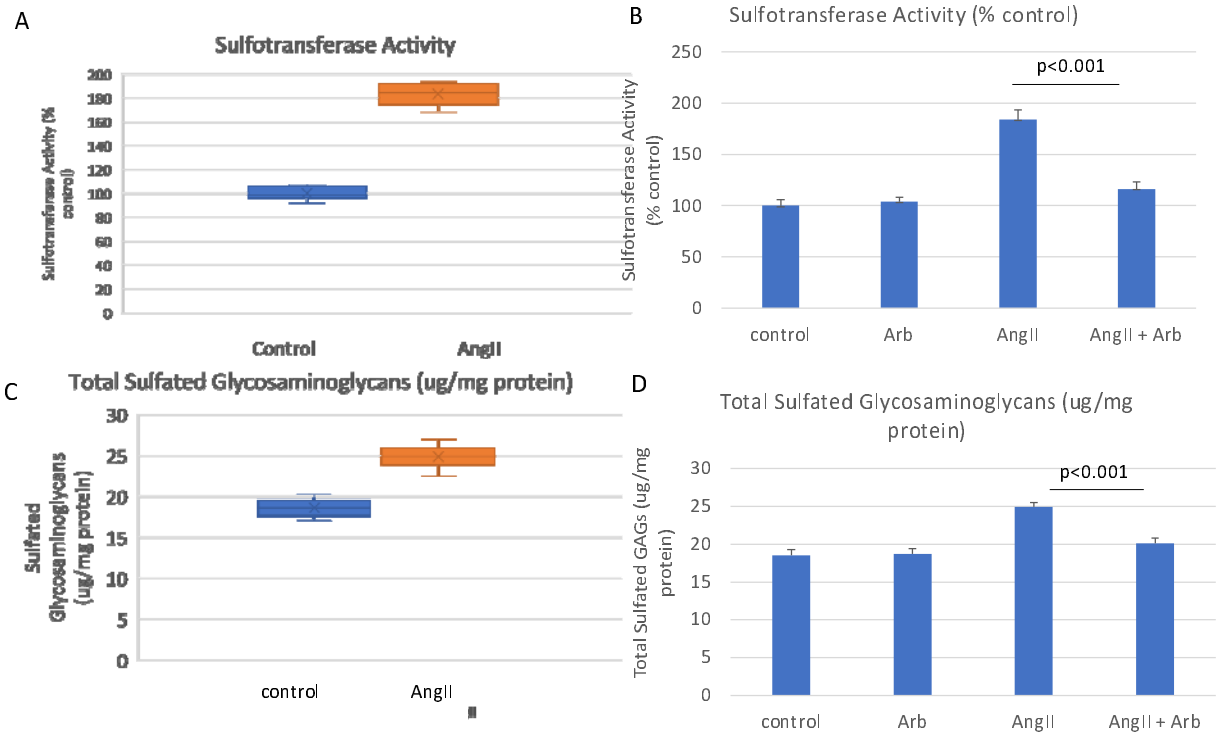

Figure 3. Inverse Relationship between Interleukin-6 and Arylsulfatase B

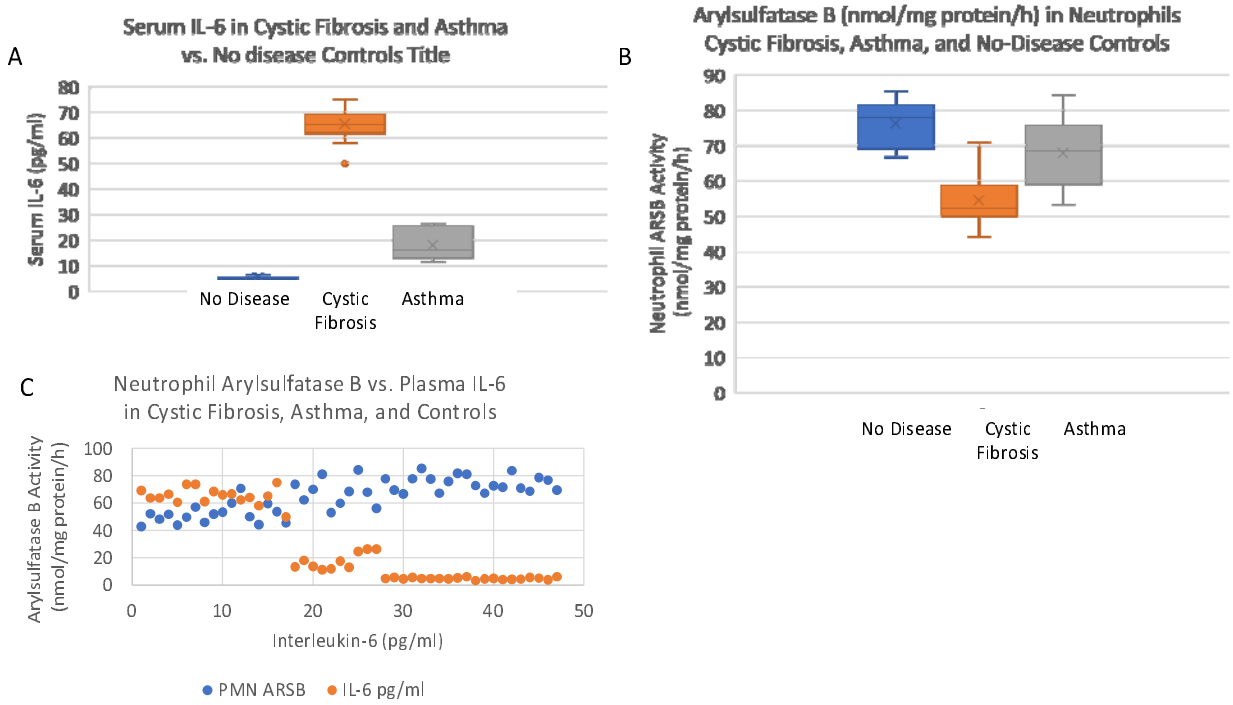

Figure 4. Impact of AngII and Blocker on Proteoglycan mRNA Expression

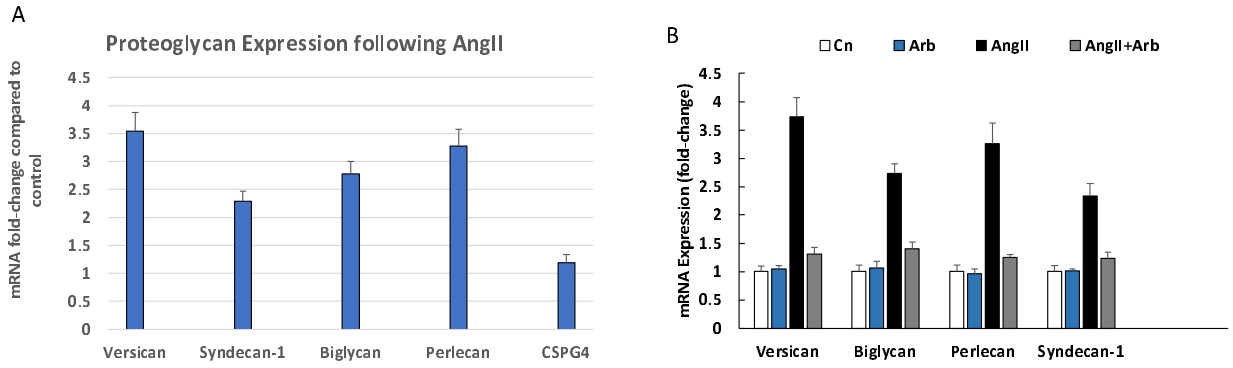
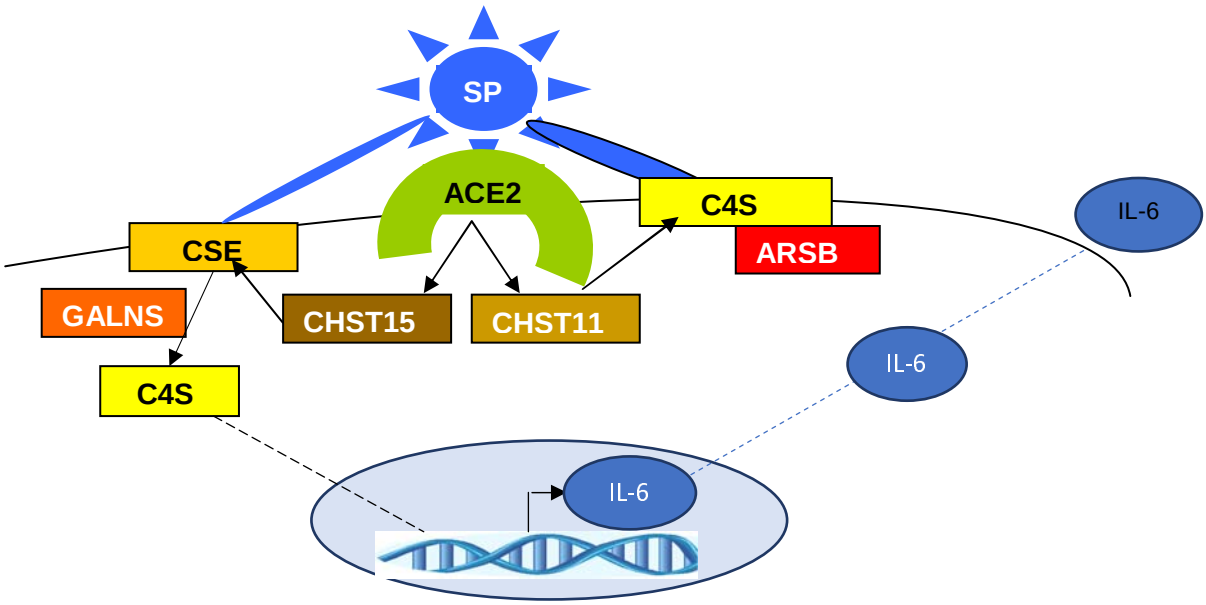
Figure 5. Schematic of Overall Pathway