



Arylsulfatase B regulates interaction of chondroitin-4-sulfate and kininogen in renal epithelial cells

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ABSTRACT

The enzyme arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase; ASB; ARSB), which removes 4-sulfate groups from the nonreducing end of chondroitin-4-sulfate (C4S; CSA) and dermatan sulfate, has cellular effects, beyond those associated with the lysosomal storage disease mucopolysaccharidosis VI. Previously, reduced ASB activity was reported in cystic fibrosis patients and in malignant human mammary epithelial cell lines in tissue culture compared to normal cells. ASB silencing and overexpression were associated with alterations in syndecan-1 and decorin expression in MCF-7 cells and in IL-8 secretion in human bronchial epithelial cells. In this report, we present the role of ASB in the regulation of the kininogen–bradykinin axis owing to its effect on chondroitin-4-sulfation and the interaction of C4S with kininogen. Silencing or overexpression of ASB in normal rat kidney epithelial cells in tissue culture modified the content of total sulfated glycosaminoglycans (sGAGs), C4S, kininogen, and bradykinin in spent media and cell lysates. Treatment of the cultured cells with chondroitinase ABC also increased the secretion of bradykinin into the spent media and reduced the C4S-associated kininogen. When ASB was overexpressed, the cellular kininogen that associated with C4S declined, suggesting a vital role for chondroitin-4-sulfation in regulating the kininogen–C4S interaction. These findings suggest that ASB, owing to its effect on chondroitin-4-sulfation, may impact on the kininogen–bradykinin axis and, thereby, may influence blood pressure.

Because ASB activity is influenced by several ions, including chloride and phosphate, ASB activity may provide a link between salt responsiveness and the bradykinin-associated mechanism of blood pressure regulation.

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1. Introduction

Inborn deficiency of the enzyme arylsulfatase B (*N*-acetylgalactosamine-4-sulfatase; ASB; ARSB) is the cause of the lysosomal storage disease mucopolysaccharidosis (MPS) VI, also known as Maroteaux–Lamy syndrome. Recent evidence has demonstrated deficiency of ASB in association with other cellular pathophysiology, including reduced activity in malignant mammary cell lines, in metastatic colonic epithelial cells, in cystic fibrosis cells, and in patients with cystic fibrosis [1–4]. Changes in the expression of ASB were associated with changes in the expression of syndecan-1 and decorin in MCF-7 cells, in IL-8 secretion and neutrophil migration in bronchial epithelial cells, and in total sulfated glycosaminoglycan and chondroitin-4-sulfate content in cultured epithelial cells [2,5,6]. Also, manipulations of chondroitin-4-sulfation have altered axonal regeneration in spinal cord lesions in mice [7]. Importantly, ASB appears to be localized

not only in the lysosomes but also in the cell membranes of colonic, bronchial, cerebrovascular, and hepatic cells [2,6,8,9].

When hypersulfated chondroitin sulfate was substituted for heparin in solutions used for dialysis patients, increased patient morbidity and mortality resulted [10–13]. These disastrous clinical outcomes were attributable to the activation of the kinin–kallikrein pathway in human plasma, resulting in profound clinical hypotension. Because increased sulfation of chondroitin was identified as the cause of the dialysis-associated hypotension, we hypothesized that variation in sulfation of naturally present chondroitin might affect blood pressure regulation. Also, high-molecular weight kininogen was previously reported to interact with heparin and chondroitin sulfate in endothelial cells [14,15].

The experiments presented in this report address a mechanistic association between ASB and hypertension, using as our model of cultured normal rat kidney epithelial cells. The study findings demonstrate that increased ASB activity produces decline in cellular C4S and C4S-associated high-molecular weight kininogen and increase of bradykinin in the spent media, thereby suggesting a role for ASB in the modification of the kininogen–bradykinin axis and in the regulation of blood pressure. Because ASB activity is reported to vary in response to exposure to chloride and phosphate [3,16,17], as

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well as other ions, ASB may provide a link between bradykinin-associated mechanisms of hypertension and salt-associated mechanisms of blood pressure regulation.

2. Materials and methods

2.1. Cell culture of rat kidney epithelial cells

Normal rat kidney epithelial cells (NRK-E52; ATCC, Manassas, VA) were grown in DMEM with 10% FBS, at 37 °C, 5% CO₂, and 95% humidity. When cells reached 65%–70% confluence, ASB silencing or overexpression was performed (see below), and cells were harvested by scraping.

Spent media from untreated, mock-treated, or treated NRK cells and NRK cells were collected after 24, 48, 72, or 96 hours of incubation. The samples were frozen at –20 °C immediately, pending further analysis. In some of the experiments, the NRK cells in culture were treated with chondroitinase ABC (1 mU/ml to 1 U/ml; Sigma Chemical Co., St Louis, MO) or keratanase (1 U/ml; Sigma) for 1 hour before harvesting.

2.2. Arylsulfatase B (ASB; N-acetylgalactosamine-4-sulfatase) activity assay

ASB measurements were performed using a fluorometric assay with the synthetic substrate 4-methylumbelliferyl sulfate (4-MUS), by a standard method [1,3]. Protein content of the cell homogenate was measured using BCA™ Protein Assay Kit (Pierce, Rockford, IL). ASB activity was expressed as nanomole per milligram of protein per hour.

2.3. Silencing and overexpression of ASB

Small interfering RNA (siRNA) to silence ASB was obtained commercially (Qiagen, Valencia, CA) and tested for specific effect on reduction of ASB activity, as reported previously [2,5,6]. For ASB (NM_000046) silencing, the siRNA sequences were as follows:

Sense: 5'- GGGUAUGGUCUCUAGGCAtt - 3'

Antisense: 5'- UUGCCUAGAGACCAUACCCtt - 3'

The siRNA sequences for the negative control were as follows:

Sense: 5'- UUCUCCGAACGUGUCACGUtt - 3'

Antisense: 5'- ACGUGACACGUUCGGAGAAtt - 3'

ASB and control plasmids in pCMV6-XL4 vector were obtained (Origene) and overexpressed in the NRK cells by transient transfection using 2 µg of the plasmid and Lipofectamine™ 2000 (Invitrogen, CA), as previously reported [2,5]. Controls included untransfected cells and cells transfected with vector only. Cells were incubated for the stated times in humidified, 37 °C, 5% CO₂ environment, and then harvested 24 hours after the initiation of silencing or overexpression, except in the time course experiment.

2.4. Measurement of sulfated glycosaminoglycans (sGAG)

Total sulfated glycosaminoglycans (sGAGs), including chondroitin-4-sulfate (C4S), chondroitin-6-sulfate, keratan sulfate, dermatan sulfate, heparan sulfate, and heparin, were measured in cell lysates by sulfated GAG assay (Blyscan™, Biocolor Ltd, Newtownabbey, Northern Ireland), as previously reported [2,5,6]. In this assay, the cationic dye 1,9-dimethylmethylene blue reacts with the sulfated GAG to produce a dye–GAG complex. The ratio of the GAG dye-binding capability to the C4S sulfation level is reported as 1.0:1.0 for C4S from bovine trachea by this assay. Concentration is expressed as micrograms per milligram protein of cell lysate.

2.5. Immunoprecipitation of cell lysates by chondroitin-4-sulfate (C4S) antibody

Cell lysates were prepared from NRK cells that had been treated with siRNA for ASB or ASB plasmid and the appropriate controls for silencing and overexpression and immunoprecipitated with C4S antibody (4D1; Santa Cruz Biotechnology, Santa Cruz, CA, or Abnova, Walnut, CA), as previously described [5,6]. The specificity of the C4S antibody was reported previously [5], with a yield of pure C4S of more than 90%, and with recovery of pure CSE or of C6S in a 90:10 mixture of C6S/C4S of less than 10%.

2.6. Time course of changes in cellular ASB activity and in C4S content after knockdown by siRNA

NRK cells were grown to 60% confluence in 12-well plates and treated with ASB siRNA as described above. After 24, 48, 72, and 96 hours, cells were harvested and measurements of ASB activity and C4S content were performed, as described above.

2.7. Measurement of bradykinin in the spent media

Bradykinin in the spent media was determined by competitive EIA (BACHEM, Torrance, CA). In this assay, color development was inversely proportional to the bradykinin content of the samples. Fifty microliters of samples or of standards, ranging from 10 pg to 10 ng, 25 µl of biotinylated bradykinin tracer, and 25 µl of rabbit polyclonal antisera to bradykinin were added into the wells of a 96-well plate, which were coated with a goat anti-rabbit IgG. Samples were incubated for 2 hours at room temperature and washed three times. Then, 100 µl of streptavidin–horseradish peroxidase (HRP) conjugate was added to each well for 1 hour at room temperature. Color was developed by adding hydrogen peroxide/3,3',5,5'-tetramethylbenzidine (TMB) substrate. The reaction was stopped by 2 N HCl, and the color was read at 450 nm in a plate reader (FLUOstar, BMG Labtech, Cary, NC). The concentrations of bradykinin in the samples were extrapolated from the standard curve and expressed as picograms per milligram of total cell protein.

2.8. Assay of cellular kininogen bound to C4S

A 96-well ELISA plate was coated overnight at room temperature with specific antibody to C4S at a concentration of 4 µg/ml. After coating, the wells were washed three times with wash buffer (PBS with Tween-20 0.05%) and blocked for 1 hour at room temperature with blocking buffer (1% BSA in PBS). One hundred microliters of NRK cell lysates, including control cell lysates, cells in which ASB was silenced, cells in which ASB was overexpressed, and cells treated with chondroitinase ABC (1 mU/ml, 10 mU/ml, 25 mU/ml, or 1 U/ml × 1 hour), was added to the coated wells and incubated for 2 hours at room temperature. Wells were washed three times and high-molecular weight kininogen (HMWK) antibody (rabbit polyclonal IgG, 100 µl at a concentration of 1 µg/ml; H-70, SCBT) was added, and preparations were incubated for 2 hours at room temperature. This antibody recognizes the epitope from AA:261–330 of HMWK. Next, goat anti-rabbit IgG–HRP (100 µl at a concentration of 1:1000) was added to each well, and the plate was then incubated for 1 hour at room temperature. Color was developed by adding 100 µl of hydrogen peroxide/TMB substrate, the reaction was stopped by 2 N HCl, and the color was read at 450 nm in a microplate reader (BMG).

The specificity of the assay was determined by several control experiments. In some control wells, exogenous C4S (Blyscan) at a concentration of 10 µg/ml was added as a competitive, control assay. In these wells, the exogenous C4S competed with cellular C4S for the C4S antibody coated onto the wells, consistent with the specificity of the antibody–antigen reaction. In these wells, reduced binding of the

anti-kininogen antibody was expected because less of the C4S–kininogen complex would be bound to the C4S antibody. In some wells, treatment with keratanase (1 U/ml \times 1 hour) was used as a control enzymatic digestion that, unlike chondroitinase ABC, was not expected to hydrolyze bonds of C4S and to not change the amount of kininogen bound to C4S. To exclude nonspecific binding of kininogen antibody to the cell lysates, normal rabbit serum was added to some wells instead of kininogen antibody.

The OD data for cellular kininogen bound to C4S are presented as percent control, in which control is the cell lysate that was not exposed to chondroitinase ABC, to silencing or overexpression of ASB, to keratanase, or to exogenous C4S.

2.9. Assay for kininogen in NRK spent media

A 96-well ELISA plate was coated overnight at room temperature with an antibody to high molecular weight kininogen (HMWK; H-70, SCBT) at a concentration of 4 μ g/ml. After coating, the cells were washed three times with wash buffer (PBS with Tween-20 0.05%) and blocked for 1 hour at room temperature with blocking buffer (1% BSA in PBS). One hundred microliters of deproteinized spent media with no added serum was added to the coated wells, and preparations were incubated for 2 hours, then washed three times, prior to adding 100 μ l of a second kininogen antibody (M-20, SCBT), at a concentration of 1 μ g/ml and incubating for 2 hours at room temperature. Neither of the kininogen antibodies was directed at the bradykinin nonapeptide (AA:381–389 AA). Preparations were washed three times, and 100 μ l of donkey anti-goat IgG–HRP at 1:1000 dilution was added before incubation for 1 hour at room temperature. Color was developed by adding 100 μ l of hydrogen peroxide/TMB substrate; the reaction was stopped by 2 N HCl, and color was read at 450 nm in a microplate reader (BMG).

2.10. Confocal microscopy

For confocal microscopy, NRK cells were grown in 4-chamber tissue culture slides for 24 hours, and staining methods that have been previously described were performed [2,6,8]. Cells were fixed in 2% paraformaldehyde for 90 minutes, washed once in 1 \times PBS containing 1 mM calcium chloride (pH 7.4), and permeabilized with 0.08% saponin. Slides were washed again with PBS, blocked in 5% normal goat serum, and incubated overnight with high-molecular weight kininogen (HMWK) rabbit polyclonal antibody (H-70, SCBT; 1:25) and C4S antibody (4D1; SCBT; 1:25) at 4 $^{\circ}$ C. Slides were washed and stained with goat–anti-rabbit Alexa Fluor[®] IgG 568 (1:100; Invitrogen) and Alexa Fluor[®] 488 (1:100; Invitrogen), and coverslipped using DAPI-mounting medium (Vectashield[®]; Vector Laboratories, Inc., Burlingame, CA) for nuclear staining. Stained cells were observed with a Zeiss LSM 510 laser scanning confocal microscope (excitation of 488 nm and 534 nm from an Ar/Kr laser). Green (HMWK) and red (C4S) fluorescence were detected through LP505 and 585 filters and scanned sequentially, and collected images were exported by Zeiss LSM Image Browser software as TIFF files for analysis and reproduction.

2.11. Statistics

Results are the mean \pm SD of at least three biological determinations with two technical replicates of each. Statistical significance was determined by one-way ANOVA followed by Tukey–Kramer posttest to correct for multiple comparisons using InStat3 software (GraphPad, San Diego, CA), unless stated otherwise. When experiments were repeated more than three times, the figures present the mean of three representative, independent experiments and their technical replicates. A p value of <0.05 is considered statistically significant. In the figures, *** $p < 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

3. Results

3.1. ASB activity, sGAG, and C4S in rat kidney epithelial cells

The enzyme arylsulfatase B (ASB) was silenced and overexpressed in normal rat kidney epithelial cells (NRK) in cell culture, and cells, with appropriate controls, were harvested after 24 hours. ASB activity changed significantly from baseline after silencing ($p < 0.001$) and overexpression ($p < 0.001$) (Fig. 1A).

The total cellular sGAGs, including chondroitin-4-sulfate (C4S), chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, heparin, and heparan sulfate, were also measured in these same preparations

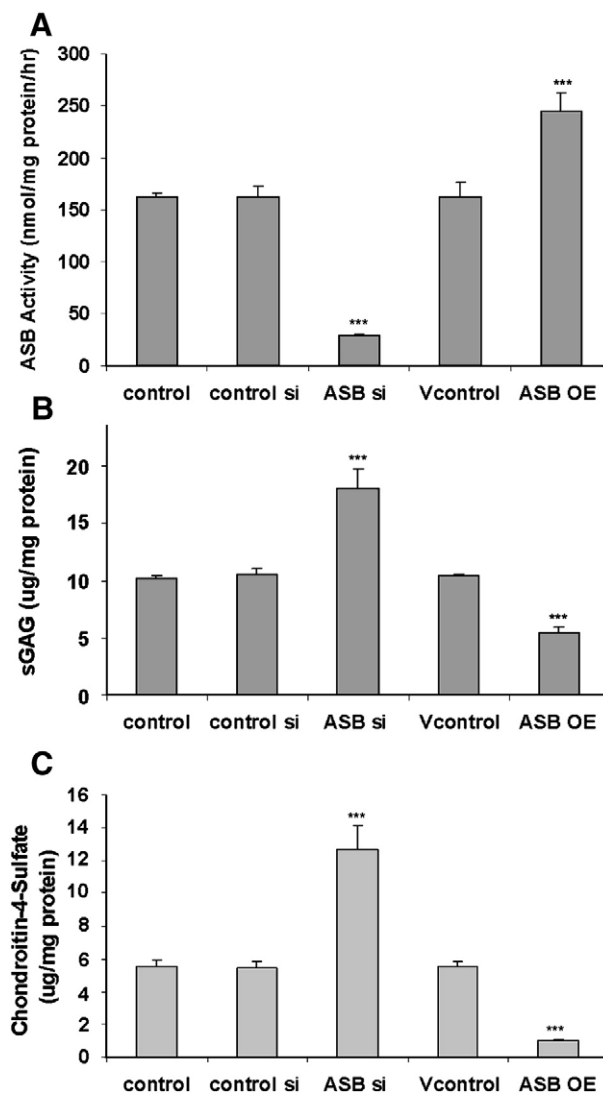


Fig. 1. ASB activity, sGAG, and C4S in rat kidney epithelial cells. (A) In the NRK epithelial cells, ASB activity at baseline was 163 ± 3 nmol/mg protein/hour. Activity increased significantly after overexpression (to 245 ± 18 nmol/mg protein/hour) and decreased significantly after silencing by siRNA (to 29 ± 1 nmol/mg protein/hour) at 24 hours. Differences in activity after silencing and overexpression are statistically significant ($p < 0.001$, $n = 3$). One-way ANOVA with Tukey–Kramer posttest was used for determinations of statistical significance, unless stated otherwise. control si = control siRNA; ASB si = arylsulfatase B siRNA; Vcontrol = vector control for overexpression; ASB OE = arylsulfatase B overexpression. (B) Inversely, the total sGAG increased after ASB silencing for 24 hours (from 10.2 ± 0.2 to 18.1 ± 1.7 μ g/mg protein; $p < 0.001$, $n = 3$) and decreased after ASB overexpression for 24 hours (to 5.5 ± 0.5 μ g/mg protein; $p < 0.001$, $n = 3$). sGAG = sulfated glycosaminoglycans. (C) Changes in chondroitin-4-sulfate (C4S) content largely accounted for the changes in total sGAG after ASB silencing for 24 hours (from 5.5 ± 0.4 to 12.6 ± 1.2 μ g/mg protein; $p < 0.001$, $n = 3$) or ASB overexpression for 24 hours (to 1.0 ± 0.1 μ g/mg protein; $p < 0.001$, $n = 3$) at 24 hours.

(Fig. 1B). After both ASB silencing and overexpression, significant changes in sGAG content occurred.

Next, control and treated NRK cell lysates were immunoprecipitated with C4S antibody, and the sGAG was measured (Fig. 1C). Total C4S increased after ASB silencing ($p < 0.001$) and declined after ASB overexpression ($p < 0.001$), consistent with the anticipated modifications of C4S content by changes in ASB activity.

3.2. Time course of decline in ASB activity and increase in C4S after ASB siRNA

ASB activity declined maximally by 24 hours and remained at 15% of baseline for 72 hours, then rose somewhat to 45% of baseline activity by 96 hours (Fig. 2A). C4S increased rapidly by 24 hours and peaked at 2.3 times the baseline. The increase was sustained for at least 2 days (Fig. 2B). By 96 hours, C4S content declined somewhat to 1.7 times the baseline value, consistent with the rise in ASB activity. All changes from baseline values were statistically significant ($p < 0.001$).

3.3. Effects of ASB expression and chondroitinase ABC treatment on bradykinin

Because we hypothesized that bradykinin release from the NRK cells was affected by changes in the C4S content, we measured bradykinin in the spent media after ASB silencing and overexpression (Fig. 3A). Bradykinin in the spent media increased from 63 ± 5 pg/mg cell protein to 208 ± 13 pg/mg cell protein after ASB overexpression ($p < 0.001$) but did not decline after ASB silencing. These results suggest that only increased ASB activity with resultant reduction in

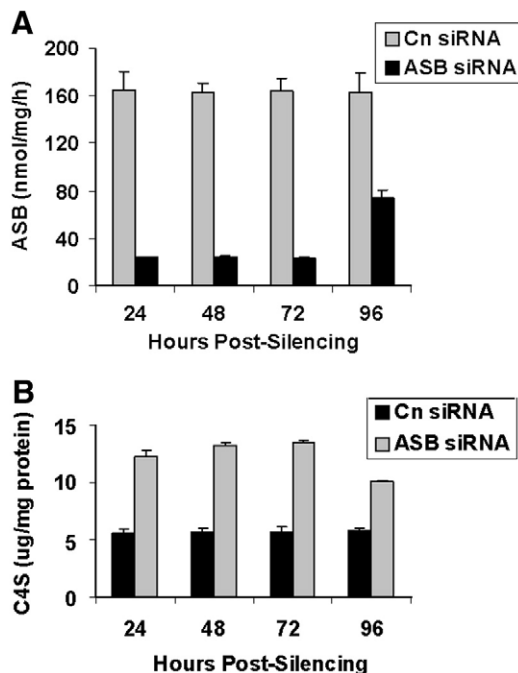


Fig. 2. Time course of decline in ASB activity and of associated increase in C4S after ASB silencing. (A) ASB was silenced by siRNA, and ASB activity was measured at 24, 48, 72, and 96 hours after silencing. Maximum decline in ASB activity occurred by 24 hours (to 24 ± 1 nmol/mg protein/hour from baseline 165 ± 16 nmol/mg protein/hour) and persisted for 72 hours at 15% of the baseline activity level. By 96 hours, ASB activity had started to rise and was 45% of baseline. All declines were statistically significant ($p < 0.001$, $n = 3$). (B) C4S rose to its maximum (13.3 ± 0.2 μ g/mg protein) by 48 hours and remained at this level at 72 hours but began to return to baseline by 96 hours (10.0 ± 0.2 μ g/mg protein). C4S rise followed the ASB decline. All increases in C4S are statistically significant ($p < 0.001$, $n = 3$).

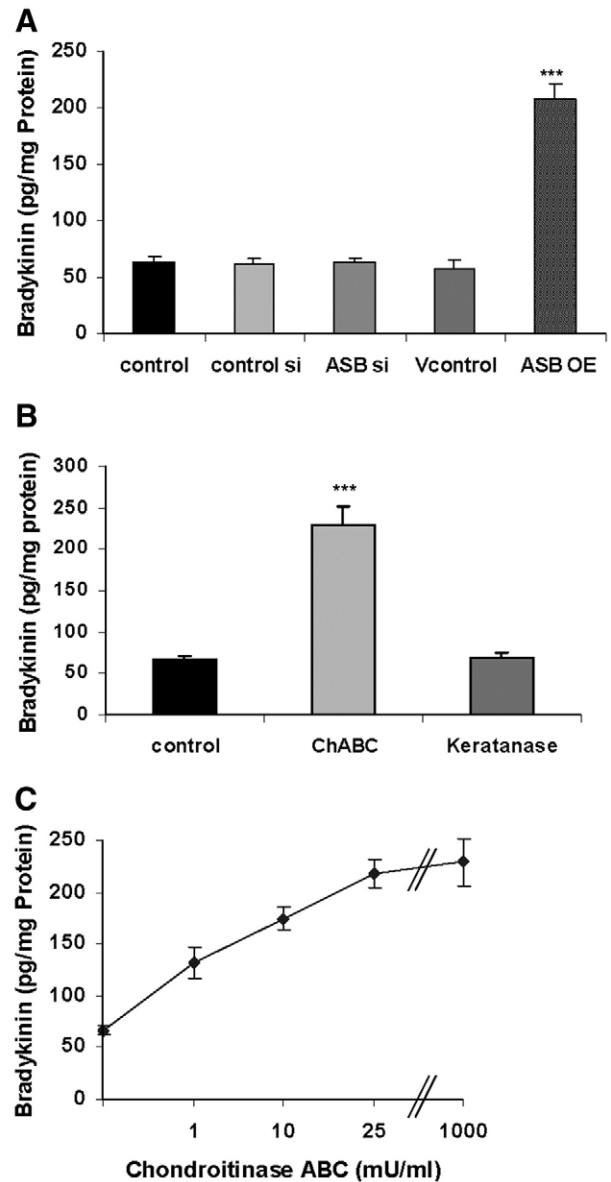


Fig. 3. Bradykinin content in spent media increased after ASB overexpression or chondroitinase ABC treatment. (A) Baseline bradykinin content released into the spent media was 63.0 ± 5.0 pg/mg cell protein. Overexpression of ASB for 24 hours produced a significant increase, to 207.6 ± 13.1 pg/mg cell protein ($p < 0.001$). When ASB was silenced for 24 hours in the NRK cells, no change in bradykinin in the spent media was observed. (B) When living NRK cells in cell culture were treated with chondroitinase ABC (1 U/ml \times 1 hour) and then harvested and the spent media was collected, the content of bradykinin in the spent media increased significantly ($p < 0.001$, $n = 3$), compared to media from untreated cells. Treatment with keratanase (1 U/ml \times 1 hour), which hydrolyzes bonds in keratan sulfate, had no effect. Results are consistent with cellular C4S inhibiting the release of bradykinin (or a bradykinin precursor) into the spent media. ChABC = chondroitinase ABC. (C) Increased bradykinin in the spent media was evident after treatment of the NRK cells with increasing concentrations of chondroitinase ABC (from 1 to 25 mU/ml \times 1 hour). Little further increase occurred between concentrations of 25 mU/ml and 1 U/ml.

chondroitin-4-sulfation had an impact on bradykinin release from the NRK cells.

To address further the underlying mechanism by which ASB and C4S might affect bradykinin content in the spent media, NRK cell preparations were treated with chondroitinase ABC (Fig. 3B). The bradykinin content of the spent media increased from 66 ± 4 pg/mg cell protein to 229 ± 23 pg/mg cell protein ($p < 0.001$) after exposure of the living cells to chondroitinase ABC (1 U/ml \times 1 hour), suggesting a profound effect of chondroitinase ABC treatment on

the kininogen–bradykinin axis. Lower doses of chondroitinase ABC (1, 10, or 25 mU/ml) (Fig. 3C) produced smaller, but significant increases of bradykinin in the spent media ($p < 0.001$).

When the spent media were assayed for kininogen by a sandwich assay that utilized two different antibodies to kininogen, neither of which included the bradykinin nonapeptide in their epitope, as described in Section 2, no kininogen was demonstrated in the spent media.

3.4. C4S-associated kininogen declined after ASB overexpression and chondroitinase ABC treatment

The content of kininogen associated with C4S in NRK cell lysates after ASB silencing and overexpression was assessed by a plate assay in which the wells were coated with C4S antibody. After ASB silencing, no change in the C4S-associated kininogen content was detected, consistent with the lack of impact of ASB silencing on the bradykinin content in the spent media (Fig. 4A). In contrast, after ASB overexpression, the cellular kininogen content declined significantly ($p < 0.001$), consistent with the observed increase in bradykinin in the spent media.

The content of kininogen associated with C4S in the NRK cell lysates was also determined after treatment with chondroitinase ABC (Fig. 4B). Marked decline in the cellular kininogen content was demonstrated after chondroitinase ABC (1 U/ml \times 1 hour; $p < 0.001$), in contrast to no effect of keratanase treatment. When exogenous C4S competed with the C4S in the NRK cell lysate for binding to the C4S antibody coating in the plate assay, NRK cell lysate binding declined to 9% of the baseline value ($p < 0.001$).

In the cell lysates treated with increasing concentrations of chondroitinase ABC (Fig. 4C), the C4S-associated kininogen progressively declined with exposure to increasing concentrations of chondroitinase ABC (from 1 to 25 mU/ml \times 1 hour). No further increase in the effect occurred with a higher dose of chondroitinase ABC (1 U/ml \times 1 hour). The similarity between the effects of treatment with chondroitinase ABC and ASB overexpression on cellular kininogen content demonstrates that ASB has a significant impact on the integrity of the cellular C4S.

3.5. Colocalization of C4S and kininogen

Confocal microscopy was performed on NRK cells grown for 24 hours in chamber slides. As presented in Fig. 5, immunofluorescent staining indicates merging of the images of kininogen (green, A) and C4S (red, B), demonstrating their colocalization (yellow, D). These results are consistent with other study findings that demonstrate the association of kininogen with C4S in the NRK cells.

4. Discussion

The study findings demonstrate increases in total cellular sGAG and C4S after silencing of ASB and declines in total cellular sGAG and C4S after overexpression of ASB in normal rat kidney epithelial (NRK) cells. In association with declines in cellular C4S when the ASB activity was increased by overexpression, bradykinin in the spent media increased and cellular kininogen declined. These findings are consistent with the hypothesis that changes in chondroitin-4-sulfation that follow changes in ASB activity may have a role in regulation of the kininogen–bradykinin axis.

The Rat Genome Database maps several Quantitative Trait Loci (QTLs) in the vicinity of ASB (Chromosome 2, from 24067560 to 24223821) that have been associated with blood pressure [18]. These include the following QTLs: 115, 243, 18, 36, 270, and 240. Also, several genes related to kidney mass (QTLs: 27, 28, 24, and 23) and cardiac mass (QTLs: 41, 42, 39, and 38) map to this ASB region, sug-

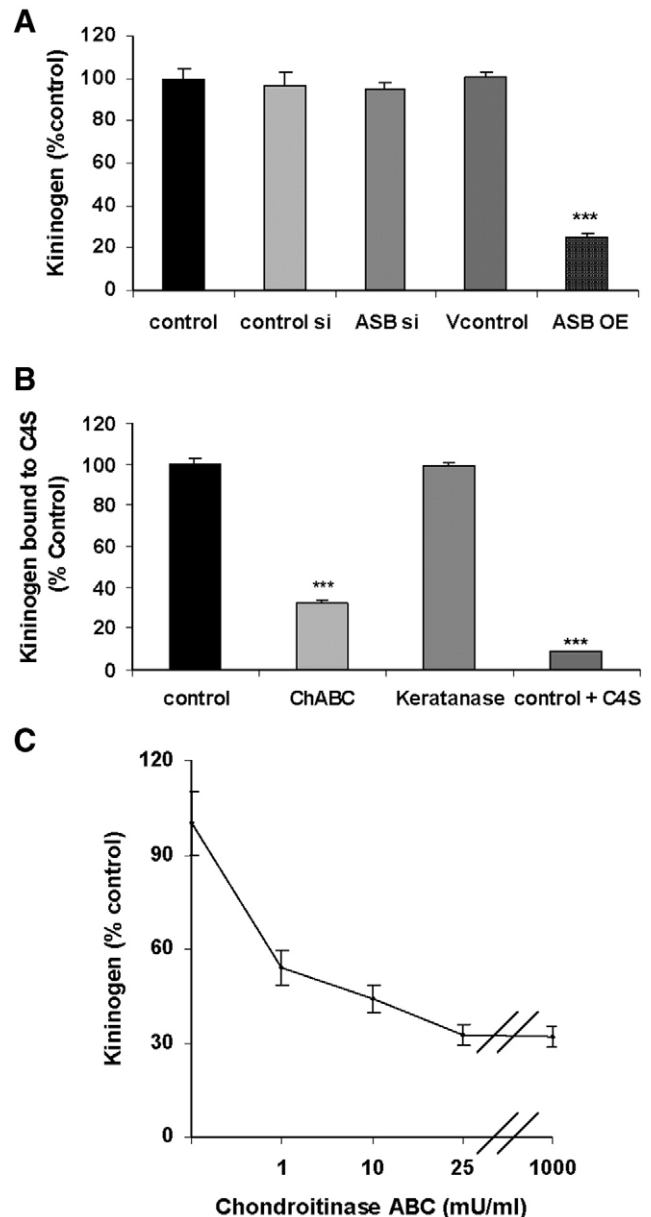


Fig. 4. Chondroitin 4-sulfate (C4S) associated-kininogen declined after ASB overexpression or chondroitinase ABC treatment. (A) Kininogen associated with C4S was measured in the NRK cell lysates after ASB silencing or overexpression \times 24 hours. When ASB was overexpressed for 24 hours, kininogen associated with C4S in the cell preparation declined significantly, to $25 \pm 2\%$ of baseline ($p < 0.001$, $n = 3$). In contrast, but consistent with the lack of impact of ASB silencing on bradykinin in the spent media, ASB silencing had no effect on the content of C4S-associated kininogen. (B) The NRK cell lysate was treated with chondroitinase ABC (1 U/ml \times 1 hour) and assayed for kininogen that was associated with cellular C4S. The cellular C4S was captured by binding to C4S antibody, which coated the bottom of wells of a microtiter plate. The C4S-associated kininogen declined to $32 \pm 2\%$ of baseline after chondroitinase ABC treatment ($p < 0.001$, $n = 3$). Competition with free C4S (10 μ g/ml) further reduced the C4S-bound kininogen, to $9 \pm 0.5\%$ of baseline ($p < 0.001$, $n = 3$). (C) Increasing concentrations of chondroitinase ABC (from 1 to 25 mU/ml \times 1 hour) produced progressive declines in the C4S-bound kininogen. Little difference was found between exposure to chondroitinase ABC 25 mU/ml and 1 U/ml \times 1 hour.

gesting relevant relationships to renal and cardiac pathophysiology, as well as to blood pressure in the rat genome.

Mechanisms of hypertension are complex, and the regulation of blood pressure requires the interplay of multiple vital substrates and receptors. The experiments in this report present evidence that suggests that the enzyme ASB, by modification of cellular chondroitin-4-sulfation, may exert a profound effect on blood pressure by

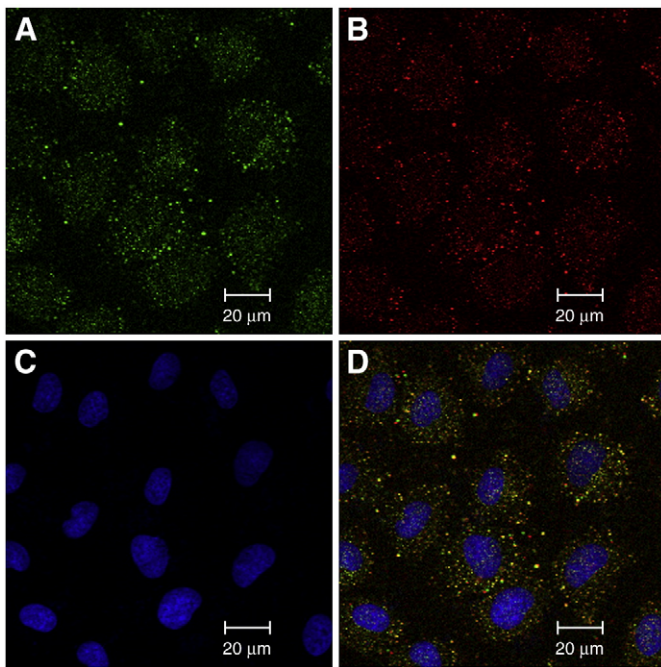


Fig. 5. Confocal microscopy colocalizes C4S and kininogen in NRK cells. Fluorescent immunostaining demonstrates colocalization of C4S and kininogen. (A) Kininogen in green. (B) C4S in red. (C) DAPI (blue)-stained nuclei. (D) Merged yellow staining where kininogen and C4S have colocalized in the cytoplasm of the NRK cells.

influencing the interaction between C4S and kininogen, thereby affecting the production/release of bradykinin. The specific molecular interactions between C4S and kininogen, although critical to understanding the production of bradykinin from kininogen, are beyond the scope addressed in this report.

The production of systemic hypotension, when oversulfated chondroitin sulfate was substituted for heparin during hemodialysis [10–13], provided the basis for further consideration of the effects of chondroitin-4-sulfation and ASB activity on bradykinin availability. Recent Biacore experiments, designed to elucidate further the interaction between sGAG and the kininogen–bradykinin axis, confirm an association between oversulfated chondroitin sulfate, heparin, plasma kallikrein, high-molecular weight kininogen (HMWK), and factor XII [19]. Increased production of bradykinin was hypothesized to arise from the favorable geometric orientation in the circulation of bradykinin, kininogen, and kallikrein, in association with sGAG and factor XII.

Although biotinylated-C4S did not produce similar results in the Biacore experiments as heparin and oversulfated chondroitin sulfate, additional factors, including the proteins to which C4S is bound *in vivo* and other differences in the cellular microenvironment *in vitro*, as well as the chemical interaction required for biotin binding, may explain how the Biacore result differs from the findings in the experiments that we have performed, which suggest the importance of the C4S–kininogen interaction.

The association between kininogen and C4S appears to be greater when ASB activity is lower and may be attributable to the presence of the 4-sulfate groups of C4S. In other works, increases in C4S disaccharides were demonstrated, after decline in ASB activity [6,8], as expected by the established mechanism of action of ASB [20,21].

Because chloride, as well as sulfate, sulfite, and phosphate, is reported to reduce ASB activity [3,16,17], it is possible that increased chloride due to high salt intake may contribute to reduction of ASB

activity *in vivo*. Reduced ASB activity might then lead to increased chondroitin-4-sulfation, increased binding of kininogen, and reduced bradykinin release. These interactions suggest a mechanism for an interrelationship between salt and bradykinin in the regulation of blood pressure. This innovative approach into mechanisms of hypertension may help to establish effective, individualized regimens for blood pressure control.

References

- [1] S. Bhattacharyya, J.K. Tobacman, Steroid sulfatase, arylsulfatases A and B, galactose 6-sulfatase, and iduronate sulfatase in mammary cells and effects of sulfated and non-sulfated estrogens on sulfatase activity, *J. Steroid Biochem. Mol. Biol.* 103 (2007) 20–34.
- [2] S. Bhattacharyya, J.K. Tobacman, Arylsulfatase B regulates colonic epithelial cell migration by effects on MMP9 expression and RhoA activation, *Clin. Exp. Metastasis* 26 (6) (2009) 535–545.
- [3] S. Bhattacharyya, J.K. Tobacman, Increased arylsulfatase B activity in cystic fibrosis cells following correction of CFTR, *Clin. Chim. Acta* 380 (2007) 122–127.
- [4] G.B. Ferrero, S. Pagliardini, A. Veljkovic, F. Porta, C. Bena, I. Tardivo, G. Restagno, M.C. Silengo, E. Bignamini, *In vivo* specific reduction of arylsulfatase B enzymatic activity in children with cystic fibrosis, *Mol. Gen. Metab.* 94 (2008) 139.
- [5] S. Bhattacharyya, K. Kotlo, S. Shukla, R.S. Danziger, J.K. Tobacman, Distinct effects of *N*-acetylgalactosamine-4-sulfatase and galactose-6-sulfatase expression on chondroitin sulfate, *J. Biol. Chem.* 283 (2008) 9523–9530.
- [6] S. Bhattacharyya, K. Solakyildirim, Z. Zhang, R.J. Linhardt, J.K. Tobacman, Cell-bound IL-8 increases in bronchial epithelial cells following arylsulfatase B silencing, *Am. J. Respir. Cell. Mol. Biol.* 42 (1) (2010) 51–61.
- [7] H. Wang, Y. Katagiri, T.E. McCann, E. Unsworth, P. Goldsmith, Z.X. Yu, F. Tan, L. Santiago, E.M. Mills, Y. Wang, A.J. Symes, H.M. Geller, Chondroitin-4-sulfation negatively regulates axonal guidance and growth, *J. Cell Sci.* 121 (2008) 3083–3091.
- [8] S. Bhattacharyya, K. Solakyildirim, Z. Zhang, R.J. Linhardt, J.K. Tobacman, Chloroquine reduces arylsulfatase B activity and increases chondroitin 4-sulfate: implications for mechanisms of action and resistance, *Malaria J.* 8 (1) (Dec 17 2009) 303 [Electronic publication ahead of print].
- [9] K. Mitsunaga-Nakatsubo, S. Kusunoki, H. Kawakami, K. Akasaka, Y. Akimoto, Cell-surface arylsulfatase A and B on sinusoidal endothelial cells, hepatocytes, and Kupffer cells in mammalian livers, *Med. Mol. Morphol.* 42 (2009) 63–69.
- [10] D.B. Blossom, A.J. Kallen, P.R. Patel, A. Elward, L. Robinson, G. Gao, R. Langer, K.M. Perkins, J.L. Jaeger, K.M. Kurkjian, M. Jones, S.F. Schillie, N. Shehab, D. Ketterer, G. Venkataraman, T.K. Kishimoto, Z. Shriver, A.W. McMahon, K.F. Austen, S. Kozlowski, A. Srinivasan, G. Turabelidze, C.V. Gould, M.J. Arduino, R. Sasisekharan, Outbreak of adverse reactions associated with contaminated heparin, *N. Engl. J. Med.* 359 (2008) 2674–2684.
- [11] T.K. Kishimoto, K. Viswanathan, T. Ganguly, S. Elankumaran, S. Smith, K. Pelzer, J.C. Lansing, N. Sriranganathan, G. Zhao, Z. Galcheva-Gargova, A. Al-Hakim, G.S. Bailey, B. Fraser, S. Roy, T. Rogers-Cotrone, L. Buhse, M. Whary, J. Fox, M. Nasr, G.J. Dal Pan, Z. Shriver, R.S. Langer, G. Venkataraman, K.F. Austen, J. Woodcock, R. Sasisekharan, Contaminated heparin associated with adverse clinical events and activation of the contact system, *N. Engl. J. Med.* 358 (2008) 257–2467.
- [12] M. Guerrini, D. Beccati, Z. Shriver, A. Naggi, K. Viswanathan, A. Bisio, I. Capila, J.C. Lansing, S. Guglieri, B. Fraser, A. Al-Hakim, N.S. Gunay, Z. Zhang, L. Robinson, L. Bushe, M. Nasr, J. Woodcock, R. Langer, G. Venkataraman, R.J. Linhardt, B. Casu, G. Torri, R. Sasisekharan, Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events, *Nat. Biotechnol.* 26 (2008) 669–675.
- [13] S. Bairston, J. McKee, M. Nordhaus, R. Johnson, Identification of a simple and sensitive microplate method for the detection of oversulfated chondroitin sulfate in heparin products, *Anal. Biochem.* 388 (2009) 317–321.
- [14] T. Renné, K. Schuh, W. Müller-Esterl, Local bradykinin formation is controlled by glycosaminoglycans, *J. Immunol.* 175 (2005) 3377–3385.
- [15] A.J. Gozzo, V.A. Nunes, A.K. Carmona, H.B. Nader, C.P. von Dietrich, V.L.F. Silveira, K. Shimamoto, N. Ura, M.U. Sampaio, C.A.M. Sampaio, M.S. Araújo, Glycosaminoglycans affect the action of human plasma kallikrein on kininogen hydrolysis and inflammation, *Int. Immunopharmacol.* 2 (2002) 1861–1865.
- [16] B. Wójcicki, Lysosomal arylsulfatases A and B from horse blood leukocytes: purification and physico-chemical properties, *Biol. Cell* 57 (1986) 147–152.
- [17] G.J. Rao, M.E. Christie, Inhibition of rabbit liver arylsulfatase B by phosphate esters, *Biochim. Biophys. Acta* 788 (1) (1984) 58–61.
- [18] <http://rgd.mcw.edu/plf/plfRGD/8/11/2009>. Rat Genome Database.
- [19] B. Li, J. Suwan, J.G. Martin, F. Zhang, Z. Zhang, D. Hoppensteadt, M. Clark, J. Fareed, R.J. Linhardt, Oversulfated chondroitin sulfate interaction with heparin-binding proteins: new insights into adverse reactions from contaminated heparins, *Biochem. Pharmacol.* 78 (2009) 292–300.
- [20] C.S. Bond, P.R. Clements, S.J. Ashby, C.A. Collyer, S.J. Harrop, J.J. Hopwood, J.M. Guss, Structure of a human lysosomal sulfatase, Structure 5 (1997) 277–289.
- [21] G. Lukatela, N. Krauss, K.K. Theis, T. Selmer, V. Gieselmann, K. von Figura, W. Saenger, Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis, *Biochemistry* 37 (1998) 3654–3664.