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**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 non-reducing 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 regulation of the kininogen-bradykinin axis, due 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 (sGAG), 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, due to its effect on chondroitin 4-sulfation, may impact on the kininogen/bradykinin axis and, thereby, may influence blood pressure. Since 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.

Introduction

Inborn deficiency of the enzyme Arylsulfatase B (N-acetylgalactosamine-4-sulfatase; ASB; ASB) 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 expression of ASB were associated with changes in 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 in the cell membranes of colonic, bronchial, cerebrovascular, and hepatic cells [2,6,8,9].

When hyper-sulfated 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 activation of the kinin-kallikrein pathway in human plasma, resulting in profound clinical hypotension. Since 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 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 modification of the kininogen-bradykinin axis and in regulation of blood pressure. Since ASB activity is reported to vary in response to exposure to chloride and phosphate [3,16,17], as well as other ions, ASB may provide a link between bradykinin-associated mechanisms of hypertension and salt-associated mechanisms of blood pressure regulation.

Materials and Methods

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 following 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 – 1 U/ml; Sigma Chemical Co., St Louis, MO) or keratanase (1 U/ml; Sigma) for 1 hour, prior to harvesting.

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 BCATM Protein Assay Kit (Pierce, Rockford, IL). ASB activity was expressed as nmol/mg protein/hour.

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:

Sense: 5'- GGGUAUGGUCUCUAGGCAtt - 3'

Antisense: 5'- UUGCCUAGAGACCAUACCCtt - 3'

The siRNA sequences for the negative control were:

Sense: 5'- UUCUCCGAACGUGUCACGUtt - 3'

Antisense: 5'-ACGUGACACGUUCGGAGAAatt-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.

Measurement of sulfated glycosaminoglycans (sGAG)

Total sulfated glycosaminoglycans (sGAG), 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 µg/mg protein of cell lysate.

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, California), as previously described [5,6]. The specificity of the C4S antibody was reported previously [5], with a yield of pure C4S of over 90%, and with recovery of pure CSE or of C6S in a 90:10 mixture of C6S/C4S of less than 10%.

Time course of changes in cellular ASB activity and in C4S content following 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.

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. 50 μ l of samples or of standards, ranging from 10 pg to 10 ng, 25 μ l of biotinylated bradykinin tracer, and 25 μ l 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, streptavidin-horseradish peroxidase (HRP) conjugate 100 μ l, 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 2N 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 pg per mg of total cell protein

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 μ g/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). 100 μ l of NRK cell lysates, including control cell lysates, cells in which ASB was silenced, cells in which ASB was over-expressed, and cells treated with chondroitinase ABC (1 mU/ml, 10 mU/ml, 25 mU/ml, or 1 U/ml x 1 hour), were 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 μ l at a concentration of 1 μ g/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 μ l 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 μ l of hydrogen peroxide/TMB substrate, the reaction was stopped by 2N 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 μ g/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, since less of the C4S-kininogen complex would be bound to the C4S antibody. In some wells, treatment with keratanase (1 U/ml x 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 non-specific 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 % 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.

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). 100 μ l of de-proteinized 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, prior to 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 2N HCl, and color was read at 450 nm in a microplate reader (BMG).

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 1x 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°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 488 nm and 534 nm from an Ar/Kr laser. Green (HMWK) and red (C4S) fluorescence were detected through LP505 and 585 filters, scanned sequentially, and collected images were exported by Zeiss LSM Image Browser software as TIFF files for analysis and reproduction.

Statistics

Results are the mean \pm Standard Deviation (S.D.) of at least three biological determinations with two technical replicates of each. Statistical significance was determined by one-way ANOVA followed by Tukey-Kramer post-test 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, three asterisks denote $p < 0.001$; two asterisks denote $p \leq 0.01$, and one asterisk indicates $p \leq 0.05$.

Results

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, following silencing ($p < 0.001$) and overexpression ($p < 0.001$) (**Figure 1, Panel A**).

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 (**Panel B**). Following 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 (**Panel C**). Total C4S increased following ASB silencing ($p < 0.001$), and declined following ASB overexpression ($p < 0.001$), consistent with the anticipated modifications of C4S content by changes in ASB activity.

Time course of decline in ASB activity and increase in C4S following 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 (**Figure 2, Panel A**). C4S increased rapidly by 24 hours, and peaked at 2.3 times baseline. The increase was sustained for at least 2 days (**Panel B**). 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 ($p < 0.001$).

Effects of ASB expression and chondroitinase ABC treatment on bradykinin

Since we hypothesized that bradykinin release from the NRK cells was affected by changes in the C4S content, we measured bradykinin in the spent media following ASB silencing and overexpression (**Figure 3, Panel A**). Bradykinin in the spent media increased from 63 ± 5 pg/mg cell protein to 208 ± 13 pg/mg cell protein following ASB

overexpression ($p < 0.001$), but did not decline following ASB silencing. These results suggest that only increased ASB activity with resultant reduction in 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 (**Panel B**). The bradykinin content of the spent media increased from 66 ± 4 pg/mg cell protein to 229 ± 23 pg/mg cell protein ($p < 0.001$), following exposure of the living cells to chondroitinase ABC (1 U/ml x 1 hour), suggesting a profound effect of chondroitinase ABC treatment on the kininogen/bradykinin axis. Lower doses of chondroitinase ABC (1 mU/ml; 10 mU/ml, 25 mU/ml) (**Panel C**) 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 the Materials and Methods, no kininogen was demonstrated in the spent media.

C4S-associated kininogen declined following ASB overexpression and chondroitinase ABC treatment

The content of kininogen associated with C4S in NRK cell lysates following ASB silencing and overexpression was assessed by a plate assay in which the wells were coated with C4S antibody. Following 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 (**Figure 4, Panel A**). In contrast, following 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 following treatment with chondroitinase ABC (**Panel B**). Marked decline in

the cellular kininogen content was demonstrated following chondroitinase ABC (1 U/ml x 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 (**Panel C**), the C4S-associated kininogen progressively declined with exposure to increasing concentrations of chondroitinase ABC (from 1 mU/ml to 25 mU/ml x 1 hour). No further increase in the effect occurred with a higher dose of chondroitinase ABC (1 U/ml x 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.

Co-localization of C4S and kininogen

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

Discussion

The study findings demonstrate increases in total cellular sGAG and C4S following silencing of ASB and declines in total cellular sGAG and C4S following 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 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, suggesting 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 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 over-sulfated 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 further elucidate 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 work, increases in C4S disaccharides were demonstrated, following decline in ASB activity [6,8], as expected by the established mechanism of action of ASB [20,21].

Since chloride, as well as sulfate, sulfite, and phosphate, are 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 inter-relationship 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.

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Figure Legends**Figure 1. ASB activity, sGAG, and C4S in rat kidney epithelial cells.**

Panel A. In the NRK epithelial cells, ASB activity at baseline was 163 ± 3 nmol/mg protein/hour. Activity increased significantly following overexpression (to 245 ± 18 nmol/mg protein/hour) and decreased significantly following silencing by siRNA (to 29 ± 1 nmol/mg protein/hour) at 24 hours. Differences in activity following silencing and overexpression are statistically significant ($p < 0.001$; $n = 3$). One-way ANOVA with Tukey-Kramer post-test 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]

Panel B. Inversely, the total sGAG increased following ASB silencing for 24 hours (from 10.2 ± 0.2 to 18.1 ± 1.7 $\mu\text{g}/\text{mg}$ protein; $p < 0.001$; $n = 3$) and decreased following ASB overexpression for 24 hours (to 5.5 ± 0.5 $\mu\text{g}/\text{mg}$ protein; $p < 0.001$; $n = 3$). [sGAG = sulfated glycosaminoglycans]

Panel C. Changes in chondroitin-4-sulfate (C4S) content largely accounted for the changes in total sGAG following ASB silencing for 24 hours (from 5.5 ± 0.4 to 12.6 ± 1.2 $\mu\text{g}/\text{mg}$ protein; $p < 0.001$, $n = 3$) or ASB overexpression for 24 hours (to 1.0 ± 0.1 $\mu\text{g}/\text{mg}$ protein; $p < 0.001$, $n = 3$) at 24 hours.

Figure 2. Time course of decline in ASB activity and of associated increase in C4S following ASB silencing.

Panel A. ASB was silenced by siRNA, and ASB activity was measured at 24, 48, 72, and 96 hours following silencing. Maximum decline in ASB activity occurred by 24 hours (to 24 ± 1 nmol/mg protein/hr from baseline 165 ± 16 nmol/mg protein/hr) 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 ($p < 0.001$, $n = 3$).

Panel B. C4S rose to its maximum ($13.3 \pm 0.2 \mu\text{g}/\text{mg}$ protein) by 48 hours and remained at this level at 72 hours, but began to return to baseline by 96 hours ($10.0 \pm 0.2 \mu\text{g}/\text{mg}$ protein). C4S rise followed the ASB decline. All increases in C4S are statistically significant ($p < 0.001$, $n=3$).

Figure 3. Bradykinin content in spent media increased following ASB overexpression or chondroitinase ABC treatment.

Panel 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.

Panel B. When living NRK cells in cell culture were treated with chondroitinase ABC (1 U/ml x 1 hour) and then harvested and the spent media 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 x 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]

Panel C. Increased bradykinin in the spent media was evident following treatment of the NRK cells with increasing concentrations of chondroitinase ABC (1 mU/ml - 25 mU/ml x 1 hour). Little further increase occurred between concentrations of 25 mU/ml and 1 U/ml.

Figure 4. Chondroitin 4-sulfate (C4S) associated-kininogen declined following ASB overexpression or chondroitinase ABC treatment.

Panel A. Kininogen associated with C4S was measured in the NRK cell lysates following ASB silencing or overexpression x 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.

Panel B. The NRK cell lysate was treated with chondroitinase ABC (1 U/ml x 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 following chondroitinase ABC treatment ($p < 0.001$, $n = 3$). Competition with free C4S (10 $\mu\text{g/ml}$) further reduced the C4S-bound kininogen, to $9 \pm 0.5\%$ of baseline ($p < 0.001$; $n = 3$).

Panel C. Increasing concentrations of chondroitinase ABC (from 1 mU/ml – 25 mU/ml x 1 hour) produced progressive declines in the C4S-bound kininogen. Little difference was found between exposure to chondroitinase ABC 25 mU/ml and 1U/ml x 1 hour.

Figure 5. Confocal microscopy co-localizes C4S and kininogen in NRK cells.

Fluorescent immunostaining demonstrates co-localization of C4S and kininogen.

Panel A shows kininogen in green.

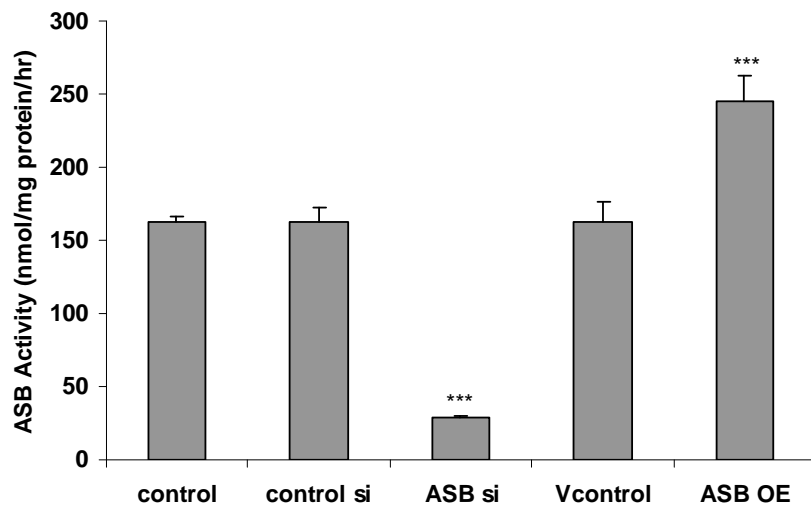
Panel B shows C4S in red.

Panel C shows DAPI (blue)-stained nuclei.

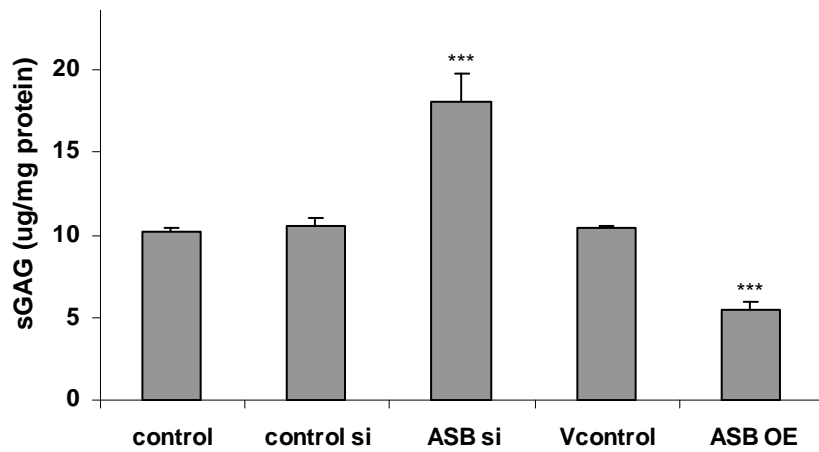
Panel D demonstrates merged yellow staining where kininogen and C4S have co-localized in the cytoplasm of the NRK cells.

Figure 1.

A.



B.



C.

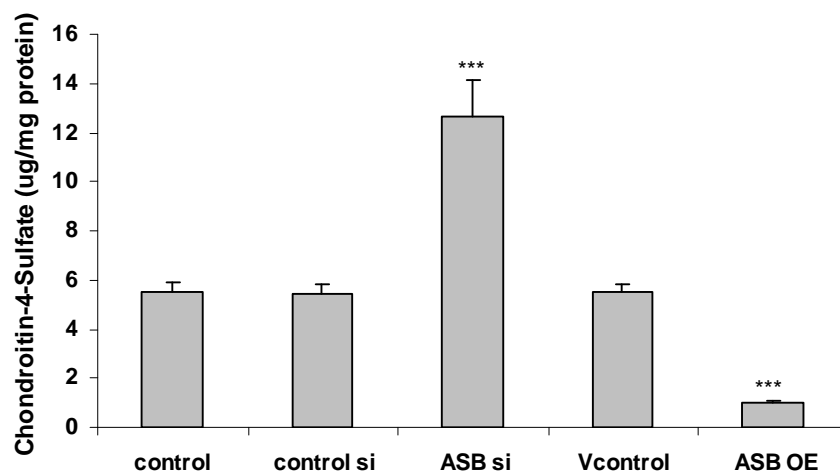


Figure 2

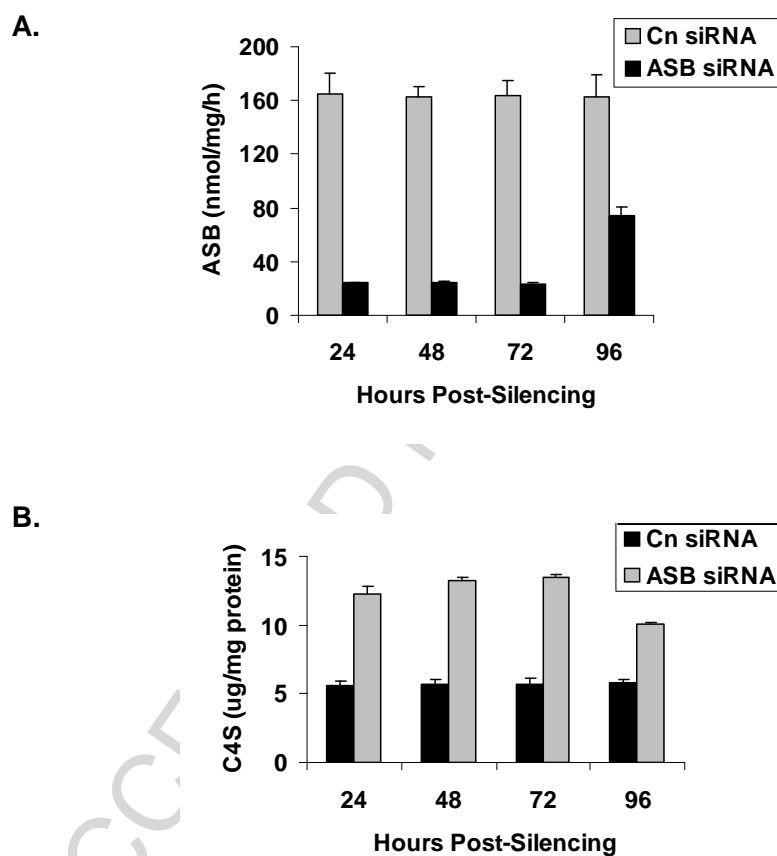


Figure 3.

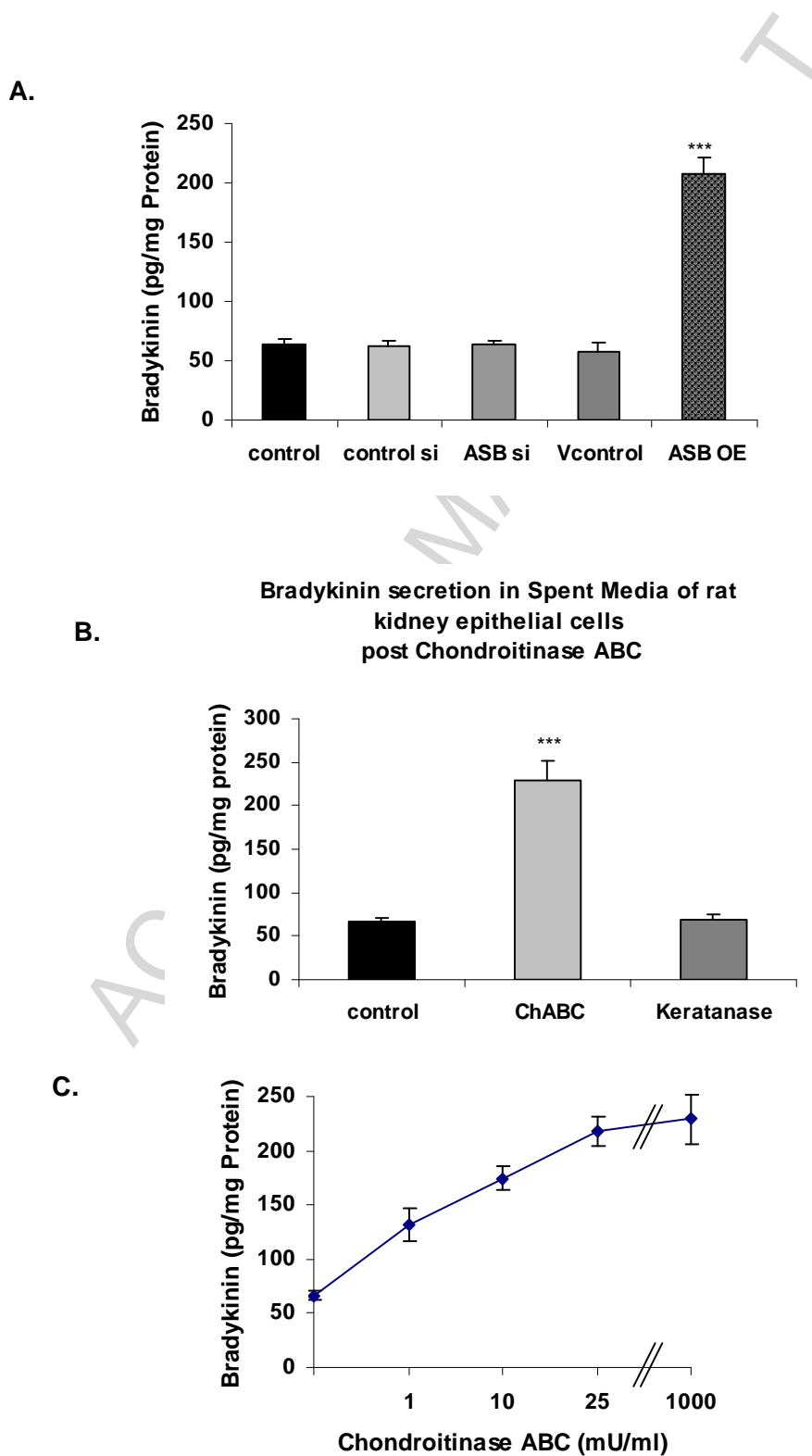


Figure 4.

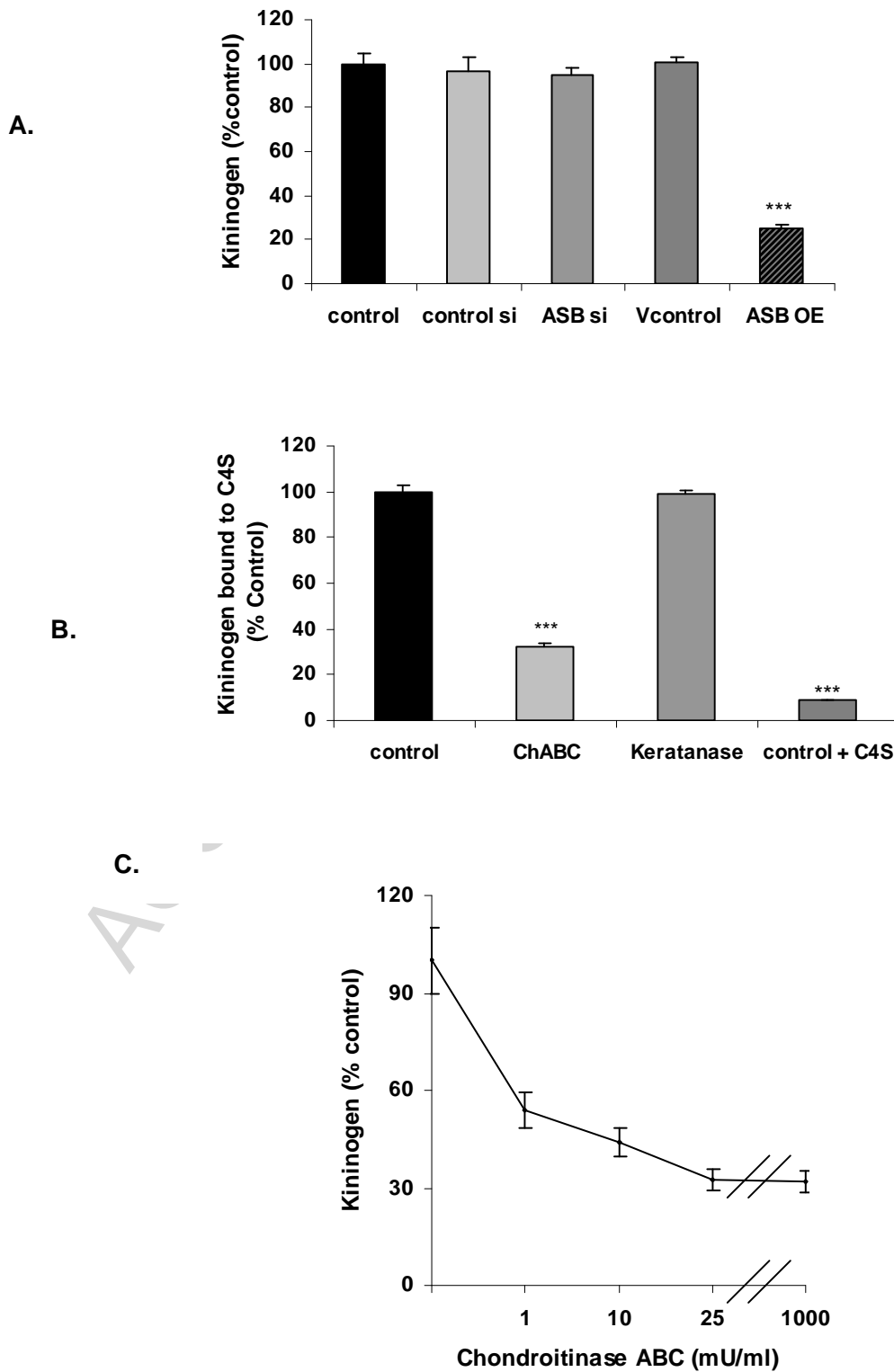
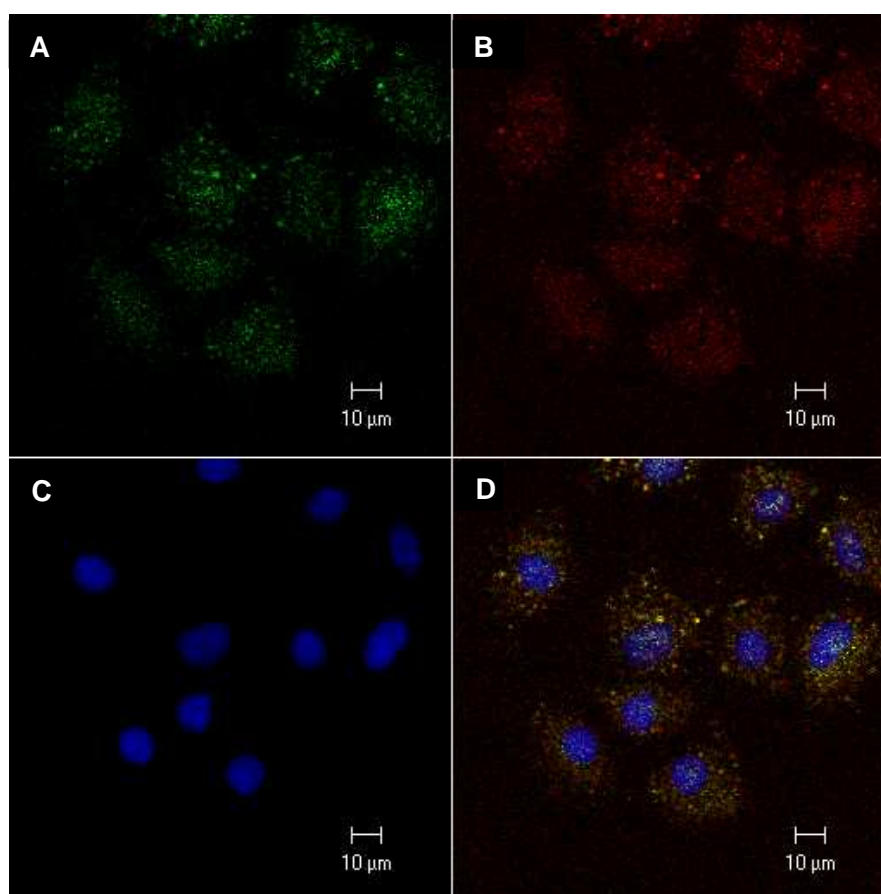


Figure 5.



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