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ORIGINAL RESEARCH - PRECLINICAL

Beta$_1$-Adrenergic Receptor Cleavage and Regulation by Elastase

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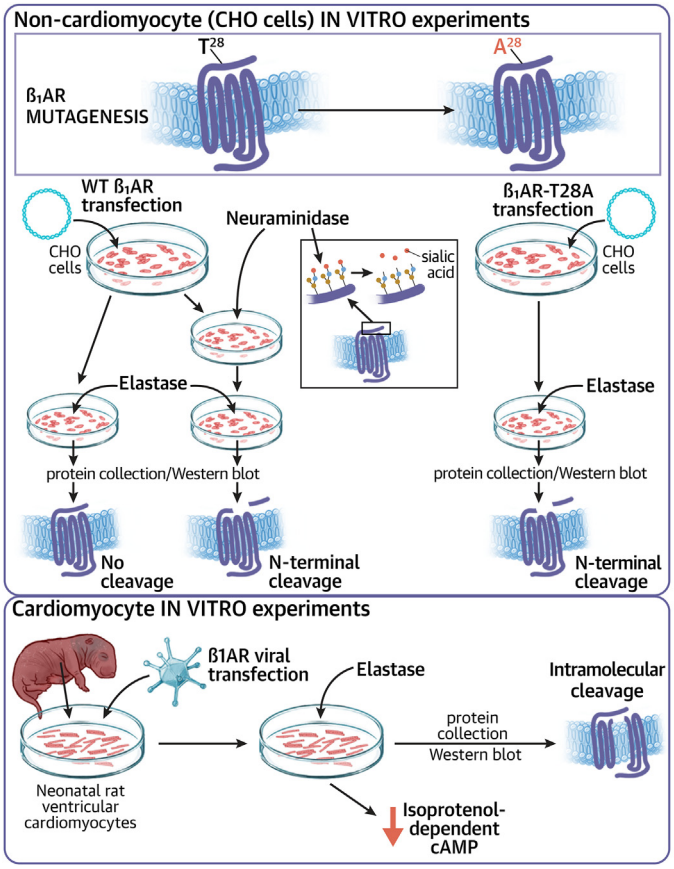
VISUAL ABSTRACT



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HIGHLIGHTS

- The β_1AR is cleaved by elastase, an inflammatory protease secreted by activated neutrophils at sites of cardiac injury or inflammation.
- β_1AR cleavage in CHO-Pro5 is at a novel N-terminal site that is protected by a sialylated O-glycan at Thr^{28}.
- β_1AR cleavage in cardiomyocytes is at the glycan-regulated N-terminal site and an intramolecular site that leads to the accumulation of signaling-incompetent fragments; elastase treatment disrupts βAR-dependent cAMP accumulation in cardiomyocytes.
- Cell-specific differences in β_1AR cleavage are attributable at least in part to cell-specific differences in their compartmentalization; β_1ARs traffic to cell surface membranes in cardiomyocytes, whereas β_1ARs are largely sequestered in an intracellular compartment in CHO-Pro5 cells.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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SUMMARY

The decrease in β_1-adrenergic receptor responsiveness in heart failure is attributed conventionally to agonist-dependent desensitization. We identify elastase-dependent β_1-adrenergic receptor cleavage as a novel proteolytic mechanism that disrupts β_1-adrenergic receptor responsiveness in the setting of tissue injury or inflammation. (J Am Coll Cardiol Basic Trans Science 2023;8:976-988)

ABBREVIATIONS AND ACRONYMS

ADAM17 = a disintegrin and metalloproteinase 17

β_1AR = beta$_1$-adrenergic receptor

cAMP = cyclic adenosine monophosphate

Iso = isoproterenol

MMP = matrix metalloproteinase

Beta$_1$-adrenergic receptors (β_1ARs) are the principal mediators of catecholamine action in cardiomyocytes. We previously identified post-translational modifications at the β_1AR extracellular N-terminus that impact on signaling responses. We showed that the β_1AR N-terminus carries O-glycan modifications at Ser^{37} and Ser^{41}, that β_1ARs accumulate as both full-length and N-terminally truncated species in cardiomyocytes and other cells types, and that maturational processing of the full-length β_1AR to an N-terminally truncated species is attributable to an O-glycan-regulated proteolytic cleavage of the β_1AR N-terminus at $R^{31}\downarrow L^{32}$ by a disintegrin and metalloproteinase 17 (ADAM17) (**Figure 1**).[1-3] We also identified an additional ADAM17-dependent N-terminal cleavage at $S^{41}\downarrow L^{42}$ that is inhibited by an O-glycan modification at S^{41}.[1] Importantly, β_1AR N-terminal truncation provides a mechanism to regulate signaling responses. Although current concepts regarding the molecular basis for βAR actions derive from literature predicated on the assumption that β_1ARs signal exclusively as full-length receptor proteins, we showed that N-terminal truncation provides a mechanism to alter the β_1AR's signaling bias to cyclic adenosine monophosphate (cAMP)/protein kinase A vs ERK pathways and only the N-terminally truncated form of the β_1AR constitutively activates AKT and confers protection against doxorubicin-dependent apoptosis in cardiomyocytes.[2,3] These studies implicate the β_1AR N-terminus as a heretofore unrecognized structural determinant of β_1AR activation.

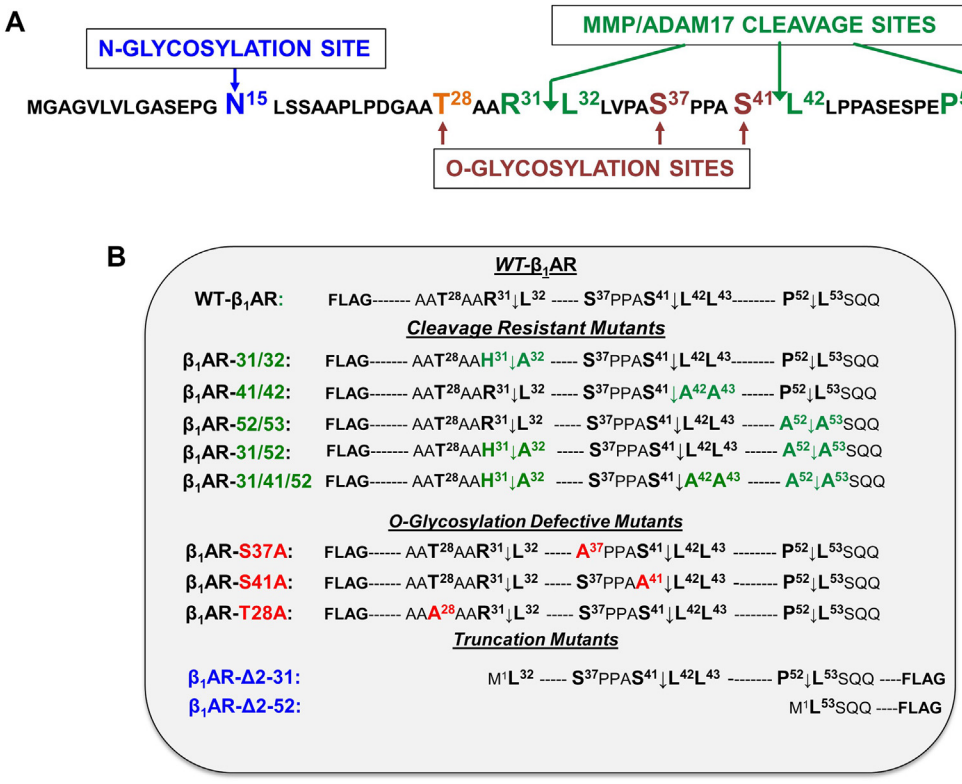
The observation that the β_1AR is a target for limited N-terminal proteolysis during the maturational processing of the receptor protein raises the question of whether full-length β_1ARs on the cell surface can be cleaved by other proteases (ie, whether β_1ARs are subject to ectodomain shedding) under certain pathophysiological conditions. Based on sequence analysis showing that the $R^{31}\downarrow L^{32}$ cleavage site conforms to a trypsin cleavage site, we recently showed that trypsin cleaves full-length β_1ARs cells at the N-terminal $R^{31}\downarrow L^{32}$ cleavage site in CHO-Pro5.[4] Cell surface β_1ARs also are targets for trypsin-dependent cleavage in cardiomyocytes. However, trypsin cleavage in cardiomyocytes is at a second site that results in the formation of ~40 kD N-terminal and ~30 kD C-terminal (presumably signaling-incompetent) fragments.[4] Although this mechanism is predicted to influence β_1AR signaling in adult cardiomyocytes isolated using standard trypsin-based protocols to isolate cardiomyocytes from the intact ventricle,[5] it would not confound the interpretation of studies on neonatal cardiomyocyte culture preparations, because studies in this preparation typically are performed 4 to 5 days after cell isolation; endogenous β_1ARs are newly synthesized during this culture interval and any β_1AR transgenes introduced into cells after cell isolation were never exposed to the trypsin treatment. Furthermore, the in vivo physiologic importance of cardiomyocyte β_1AR cleavage by trypsin (a digestive enzyme found in the gastrointestinal tract) is dubious. This study examined whether the β_1AR is a target for cleavage by other, more physiologically relevant proteases. Our studies identified β_1AR cleavage by elastase, a pathophysiologically important inflammatory protease secreted by activated neutrophils at sites of cardiac injury or inflammation. We implicate this cleavage mechanism as a heretofore unrecognized form of β_1AR processing that would serve to alter catecholamine responsiveness in the setting of clinically relevant disorders, such as viral myocarditis or myocardial infarction.

METHODS

MATERIALS. Antibodies were from the following sources: rabbit polyclonal anti-β_1AR (Cat# ab3442, raised against residues 394-408 in human β_1-ARs) was from Abcam. Mouse monoclonal anti-FLAG M2 antibody (Cat# F1804) was from Sigma-Aldrich. The Abcam rabbit polyclonal anti-β_1AR was validated in our previous publication.[3] IRDye 800CW goat anti-rabbit IgG (Cat# 925-32211) and IRDye 680RD goat

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:976–988

FIGURE 1 Schematics of Post-Translational Modifications on the β_1AR N-Terminus and the β_1AR Mutants Used in This Study



(A) The β_1AR N-terminal sequence showing a single N-linked glycosylation site at position 15 **(blue)**, the 2 previously identified O-linked glycosylation sites at S^{37} and S^{41} **(red)**, the O-linked glycosylation site newly identified in this study at T^{28} **(orange)**, and MMP/ADAM17 cleavage sites at positions 31-32, 41-42, and 52-53 **(green)**. **(B)** Schematic of the β_1AR cleavage-resistant, O-glycosylation defective, or truncation mutants used in this study. To simplify the cumbersome nomenclature, the β_1AR-R31H/L32A, β_1AR-L42A/L43A, and β_1AR-P52A/L53A constructs (which harbor single residue substitutions that disable a single cleavage site) are labeled β_1AR-31/32, β_1AR-41/42, and β_1AR-52/53, the β_1AR-R31H/L32A-P52A/L53A construct (with 2 cleavage site disabling substitutions) is labeled β_1AR-31/52, and the β_1AR-R31H/L32A-L42A/L43A-P52A/L53A construct (with disabling substitutions at all 3 cleavage sites) is labeled β_1AR-31/41/52 in the figure and elsewhere in this article. ADAM17 = a disintegrin and metalloproteinase 17; β_1AR = β_1-adrenergic receptor; MMP = matrix metalloproteinase.

anti-mouse IgG (Cat# 925-68070) secondary antibodies were from LI-COR Biosciences. Elastase from porcine pancreas (Cat# E0258), GM6001 (Cat# 364206), GI254023X (ADAM-10 inhibitor, Cat# SML0789), matrix metalloproteinase (MMP)-2/MMP-9 inhibitor I (Cat# 444241), MMP-9/MMP-13 inhibitor I (Cat# 444252), forskolin (Cat# F3917), isoproterenol (Iso) (Cat# 420355), propranolol (Cat# P0884), sotalol (Cat# S0278), and theophylline (Cat# T1633) were from Sigma-Aldrich. MMP-2/MMP-3 inhibitor I (Cat# sc-295483) and TAPI-2 (Cat# sc-205851) were from Santa Cruz Biotechnology. GW280264X (ADAM-10/ADAM-17 inhibitor, Cat# 7030) was from AOBIOUS Inc. α2-3,6,8,9 neuraminidase A (Cat #: P0722) was from New England BioLabs Inc. All other chemicals were reagent grade.

PLASMIDS AND ADENOVIRUSES. A plasmid that drives expression of the human β_1AR harboring an N-terminal FLAG-tag was from Addgene. Various single residue substituted β_1AR constructs were generated using the QuickChange II XL Site-Directed Mutagenesis Kits (Agilent) according to the manufacturer's instructions. The integrity of all constructs was confirmed by DNA sequencing (Genewiz). Adenoviral vectors that drive expression of WT or mutant forms of the β_1AR (Ad-β_1AR, Ad-β_1AR-31/52, Ad-Δ2-31-β_1AR, or Ad-Δ2-52-β_1AR; nomenclature defined in Figure 1) were prepared by Welgen Inc.

CELL CULTURE AND TRANSIENT TRANSFECTION. CHO-Pro5 cells (Cat# CRL-1781) and CHO-Lec2 cells (Cat# CRL-1736) obtained from the American Type Culture Collection were cultured in minimum

essential medium Eagle-α modification, supplemented with 5% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 100 mmol/L glutamine. Transient transfections were performed in 60-mm culture dishes with 1 μg β_1AR plasmid cDNA using the jetOPTIMUS DNA Transfection Reagent (Cat# 101000006) (Polyplus Transfection) according to the manufacturer's instructions.

NEONATAL CARDIOMYOCYTE CULTURE AND ADENOVIRAL INFECTIONS. Cardiomyocytes were isolated from the ventricles of 2-day-old Wistar rats by a trypsin dispersion technique using a differential attachment procedure to enrich for cardiomyocytes followed by irradiation as described previously.[2] Methods to infect cardiomyocytes with adenoviruses that drive expression of WT or mutant forms of the human β_1AR also are published.[2]

IMMUNOBLOTTING. Immunoblotting was performed on cell lysates according to methods described previously[2] or the manufacturer's instructions. Dilutions for primary and secondary antibodies were as follows: Abcam anti-β_1AR (ab3442) at 1:3,000 followed by IRDye 800CW goat anti-rabbit IgG at 1:5,000. Anti-FLAG M2 at 1:700 and anti-β-actin at 1:1,000 followed by IRDye 800CW or 680RD goat anti-mouse IgG at 1:5,000. Each panel in each figure represents results from a single gel exposed for a uniform duration using LI-COR Odyssey CLx imaging system (LI-COR Biosciences) for the detection of and image Studio Lite Ver 5.0 software for the quantification of protein expression. All results were replicated in at least 3 experiments on separate culture preparations.

cAMP MEASUREMENT. cAMP accumulation was measured according to standard methods as described previously.[2]

STATISTICAL ANALYSIS. Data are presented as mean ± SEM, with all results replicated in ≥3 separate experiments on different culture preparations. Analyses were performed using Prism version 9.0 (GraphPad Software) with differences between 2 groups analyzed using Student's *t*-tests and differences between >2 groups analyzed using 1- or 2-way analysis of variance followed by Bonferroni post hoc tests for multiple pairwise comparisons. Statistical significance was set at a *P* value of <0.05.

STUDY APPROVAL. All animal procedures complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Columbia University (protocol AABF2563).

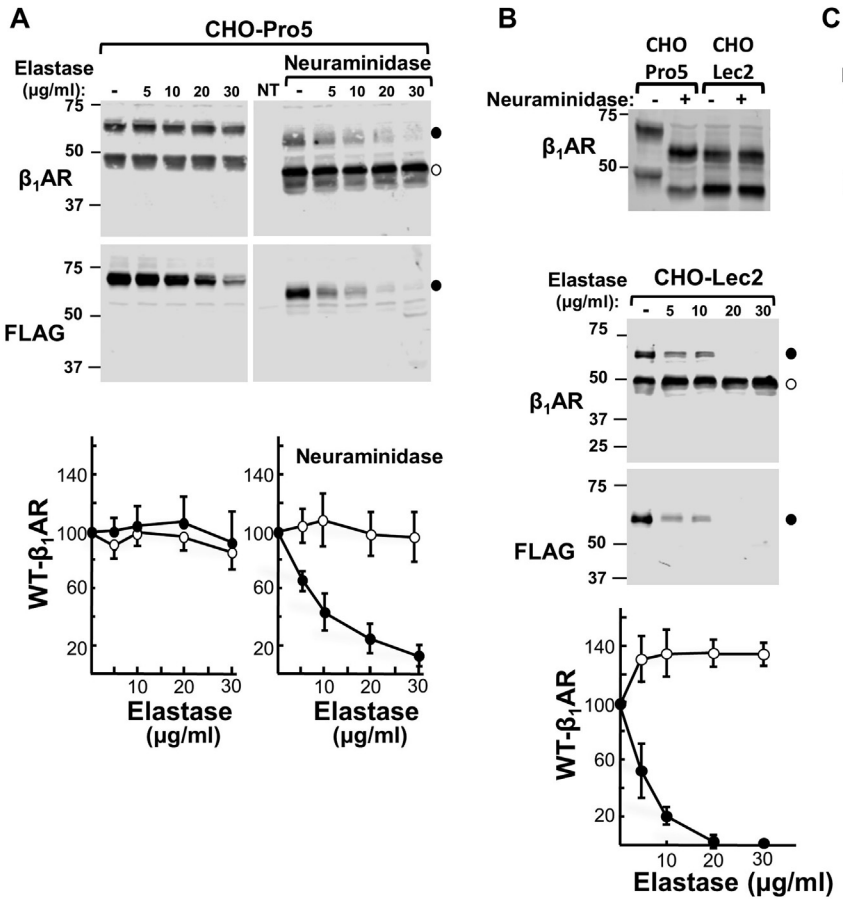
RESULTS

β_1AR CLEAVAGE BY ELASTASE IN CHO CELLS. Our recent observation that cell surface β_1ARs are cleaved by trypsin[4] provided the rationale to broaden the protease screen and examine whether β_1ARs are also cleaved by proteases with more pathophysiological relevance. Preliminary studies failed to identify a significant level of β_1AR cleavage by thrombin, cathepsin G, or elastase in CHO-Pro5 cells (**Figure 2A**, left, and data not shown). Given our previous observation that O-glycan attachments on the β_1AR N-terminus are heavily sialylated[2] and the literature showing that terminal charged sialic acid residues regulate proteolytic cleavage of certain other glycoproteins,[6-8] we repeated the protease screen in CHO-Pro5 cells treated with neuraminidase. **Figure 2A** (right) shows that full-length β_1ARs are effectively cleaved by elastase in neuraminidase-treated CHO-Pro5 cells; the N-terminally truncated form of the β_1AR is resistant to elastase cleavage. β_1AR cleavage by thrombin or cathepsin G was not detected under these conditions (data not shown).

The protease screen was performed in CHO-Lec2 cells (a derivative of the CHO-Pro5 parental line with a defect in glycoprotein sialylation[9]) as a second strategy to determine whether sialylation interferes with β_1AR cleavage by elastase. **Figure 2B** (top) validates this model, showing that β_1ARs accumulate as sialylated glycoproteins (with electrophoretic mobilities that increase upon neuraminidase treatment) in parental CHO-Pro5 cells, whereas full-length and N-terminally truncated forms of the β_1ARs accumulate in CHO-Lec2 cells as neuraminidase-insensitive species that comigrate with the neuraminidase-treated β_1AR species in the parental CHO-Pro5 cell line. **Figure 2B** (bottom) shows that the sialylation defect also renders the full-length β_1AR susceptible to cleavage by a low elastase concentration; again, the N-terminally truncated form of the β_1AR is not cleaved by elastase in CHO-Lec2 cells (**Figure 2B**, bottom) and thrombin- or cathepsin G-dependent cleavage of the β_1AR was not detected (data not shown). These studies implicate sialylation as a modification that blocks elastase-dependent cleavage of full-length β_1ARs.

The observation that elastase cleaves full-length (but not N-terminally truncated) β_1ARs suggests that cleavage maps to a site on the N-terminus. Although cleavage at an extracellular loop site is in theory possible, the observation that elastase treatment does not lead to the accumulation of β_1AR N- or C-terminal fragments in CHO-Pro5 or

FIGURE 2 Elastase Cleaves Full-Length β_1ARs in CHO-Pro5 Cells: The Role of Sialylation

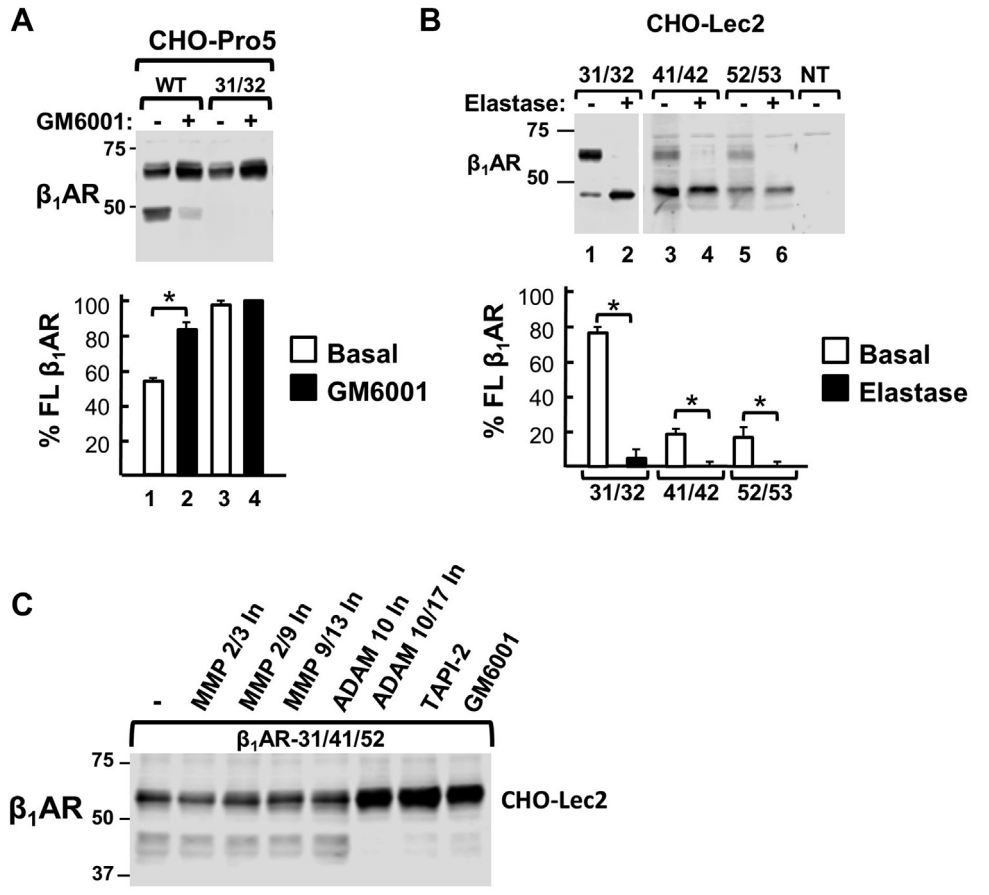


(A) WT-β_1AR expressing CHO-Pro5 cells were pretreated with vehicle or α2-3,6,8,9 neuraminidase A (20 U/mL) for 3 hours and then challenged with the indicated concentrations of elastase for 10 minutes; the reaction was stopped by the addition of 500 μM phenylmethylsulfonyl fluoride, followed by a wash with ice-cold phosphate-buffered saline and the addition of radio immunoprecipitation assay lysis buffer. Lysates were subjected to immunoblot analysis with an anti-β_1AR antibody that recognizes a C-terminal epitope and anti-FLAG that recognizes the N-terminal FLAG tag on the full-length β_1AR. Anti-FLAG and anti-β_1AR antibodies recognize the full-length human β_1AR, whereas the truncated β_1AR is detected by the anti-β_1AR antibody, but not anti-FLAG. A representative experiment is depicted on top, with results from 5 separate experiments performed on separate culture preparations quantified at the bottom. For quantification of immunoreactivity, levels of the full-length (filled circle) or truncated (open circle) forms of the β_1AR after elastase treatment were normalized to the level of the cognate species at T = 0 (which was set to 100). **(B) (Top)** Immunoblot analysis of lysates from WT-β_1AR-expressing CHO-Pro5 or CHO-Lec2 cells. α2-3,6,8,9 neuraminidase A treatment (1 U/μg protein for 1 hour) was on cell lysates. **(Bottom)** Immunoblot analysis of WT-β_1AR expressing CHO-Lec2 cells treated with the indicated concentrations of elastase for 10 minutes. A representative experiment is depicted and results from 4 separate experiments performed on separate culture preparations quantified (as described in **A**) at the **bottom**. **(C)** Immunoblot analysis on elastase-treated Δ2-31-β_1AR expressing CHO-Pro5 cells. WT = wild type. Abbreviations as in Figure 1.

CHO-Lec2 cells (Figures 2A and 2B) and that elastase does not cleave β_1AR-Δ2-31 (a β_1AR truncation mutant modeled to represent the N-terminally truncated species that accumulates during maturational processing of the receptor) (Figure 2C) supports the conclusion that elastase cleavage of the β_1AR in CHO cells is restricted to a site on the N-terminus.

ELASTASE CLEAVAGE IS AT A β_1AR N-TERMINAL SITE THAT IS DISTINCT FROM PREVIOUSLY IDENTIFIED SITES AT $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, OR $P^{52}\downarrow L^{53}$. We used a mutagenesis strategy (with the panel of constructs schematized in Figure 1) to determine whether elastase-dependent cleavage of the β_1AR N-terminus in CHO-Lec2 cells can be mapped to $R^{31}\downarrow L^{32}$ or $S^{41}\downarrow L^{42}$ (the 2 β_1AR N-terminal cleavage sites identified in a cellular

FIGURE 3 **Elastase Cleaves the Full-Length β_1AR in CHO-Lec2 Cells at a Site That Is Distinct From Previously Identified Cleavage Sites at $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, or $P^{52}\downarrow L^{53}$**



Lysates from WT-β_1AR- or β_1AR-31/32-expressing CHO-Pro5 treated with vehicle or GM6001 (10 μM for 24 hours; **A**) or from β_1AR-31/32, β_1AR-41/42, or β_1AR-52/53 cleavage-resistant mutant expressing CHO-Lec2 cells treated with vehicle or elastase (20 μg/mL for 10 minutes; **B**) were probed for anti-β_1AR immunoreactivity. A representative experiment is depicted on top, with results from 5 **(A)** or 4 **(B)** separate experiments performed on separate culture preparations quantified at the bottom. Data are shown as mean ± SEM, with the percent of full-length β_1AR (upper band) expressed as a % of total (full-length + truncated) β_1AR (*$P < 0.05$). **(C)** Immunoblot analysis of lysates from β_1AR-31/41/52 expressing CHO-Lec2 cells cultured in the presence of vehicle, MMP 2/3 inhibitor (20 μM), MMP 2/9 inhibitor (10 μM), MMP 9/13 inhibitor (20 nM), ADAM 10 inhibitor (400 nM), ADAM10/17 inhibitor (3 μM), TAPI-2 (20 μM), or GM6001 (10 μM) for 24 hours. Results were replicated in 2 separate experiments. Abbreviations as in Figure 1.

context[1]) or $P^{52}\downarrow L^{53}$ (a third predicted cleavage site based on in vitro cleavage assays with short peptides based on the β_1AR N-terminal sequence as substrate and purified MMP or ADAM family enzymes,[10] but whose importance as a target for β_1AR cleavage in cells remains uncertain[1]).

Initial studies compared the processing of cleavage-resistant β_1AR mutants in CHO-Lec2 cells with the parental CHO-Pro5 cells. Figure 3A replicates the results of previous studies showing that the maturation processing of full-length β_1ARs to an N-terminally truncated form in CHO-Pro5 cells is prevented by the MMP/ADAM17-inhibitor GM6001 or single residue substitutions that disrupt the major maturational cleavage site at $R^{31}\downarrow L^{32}$.[1] Cleavage is not prevented by single residue substitutions that prevent cleavage at $S^{41}\downarrow L^{42}$ or $P^{52}\downarrow L^{53}$ (see Zhu and Steinberg[1]). In this context, the observation that the β_1AR-31/32 mutant is expressed exclusively as a full-length protein in CHO-pro5 cells, but it is detected as both a full-length and an N-terminally truncated species in CHO-Lec2 cells (compare Figure 3A, lane 3, with Figure 3B, lane 1) was unanticipated; it exposes an additional cleavage site that contributes to

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:976–988

the maturational processing of full-length β_1ARs when sialylation is defective. Figure 3B shows that β_1AR-41/42 and β_1AR-52/53 (mutants that harbor cleavage-disabling substitutions at $S^{41}\downarrow L^{42}$ or $P^{52}\downarrow L^{53}$ but retain the $R^{31}\downarrow L^{32}$ cleavage site) are cleaved in CHO-Lec2 cells as predicted (Figure 3B, lanes 3 and 5). However, Figure 3C extends the analysis by showing that β_1AR-31/41/52 (a mutant that harbors cleavage-disabling substitutions at all 3 known cleavage sites) also is cleaved in CHO-Lec2 cells, indicating that the sialylation defect exposes a novel cleavage site that is distinct from previously identified cleavage sites at $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, or $P^{52}\downarrow L^{53}$. Figure 3C also shows that cleavage at this novel site is mediated by ADAM17; it is blocked by GM6001 (a pan-MMP inhibitor), the ADAM10/17 inhibitor and TAPI-2, but not by specific inhibitors of MMP-2/-3, MMP-2/-9, MMP-9/-13, or ADAM10.

Finally, Figure 3B shows that elastase cleaves the full-length forms of β_1AR-31/32, β_1AR-41/42, and β_1AR-52/53 in CHO-Lec2 cells, indicating that elastase cleavage is at an N-terminal site distinct from previously characterized cleavage sites at $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, or $P^{52}\downarrow L^{53}$.

THE β_1AR N-TERMINUS CARRIES AN O-GLYCAN MODIFICATION AT T^{28} THAT PREVENTS ELASTASE-DEPENDENT N-TERMINAL CLEAVAGE. The observation that elastase cleaves full-length, but not N-terminally truncated, β_1ARs and that this cleavage is blocked by a sialylated glycan suggests that cleavage is regulated by sialylation at a site unique to the full-length β_1AR (ie, not present on the N-terminally truncated species). Figure 4A shows that an Ala substitution at the previously identified N-glycosylation site (Asn^{15}) does not facilitate elastase-dependent β_1AR cleavage, and Figure 4B shows that Ala substitutions at previously identified O-glycosylation sites at Ser^{37} or Ser^{41} enhance maturational processing of the full-length β_1AR to an N-terminally truncated species (consistent with the conclusion in our previous publication[2]), but O-glycan modifications at Ser^{37} or Ser^{41} do not facilitate elastase-dependent β_1AR cleavage. This finding provided the rationale to consider other potential regulatory glycosylation sites.

In vitro studies indicate that polypeptide N-acetylgalactosaminyl transferase O-glycosylates short peptides based upon the β_1AR N-terminal sequence at Ser^{37}, Ser^{41}, Ser^{47}, Ser^{49}, and Thr^{28}. Because we showed previously that Ser^{47} and Ser^{49} do not serve as O-glycosylation site on β_1ARs in cells,[2] we considered a role for Thr^{28}, a site strategically positioned adjacent to an elastase consensus cleavage motif (ie, elastase typically cleaves scissile bonds C-terminal to small amino acid residues such as Ala, Gly, or Val) that was not considered in our previous studies that mapped O-glycosylation sites in a cellular context.[2]

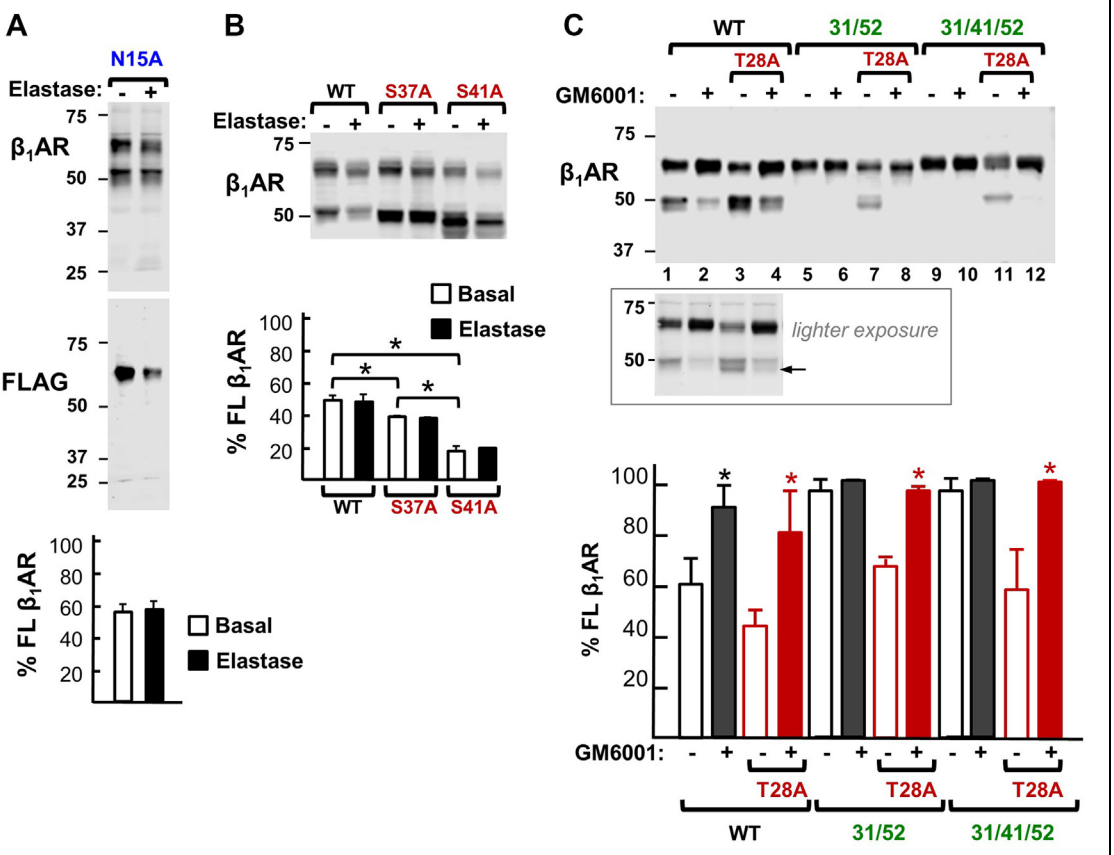
Figure 4C shows that a T28A substitution leads to the de novo appearance of a second N-terminally truncated β_1AR species that is not present in studies of the WT-β_1AR (best resolved in the lighter exposure of the gel; see the inset). This result (which mirrors results obtained in the CHO-Lec2 cells) is consistent with the notion that β_1ARs carry a sialylated O-glycan at Thr^{28} that prevents β_1AR cleavage at a site distinct from $R^{31}\downarrow L^{32}$ during maturational processing of the receptor in cells. The additional observation that the T28A substitution results in cleavage of the full-length β_1AR species when introduced into the "cleavage-resistant" β_1AR-31/52 backbone (Figure 4C, compare lanes 5 and 7) or the β_1AR-31/41/52 backbone (Figure 4C, compare lanes 9 and 11) and that this cleavage is blocked by GM6001 (Figure 4C, lanes 8 and 12) provides further evidence that a sialylated O-glycan at Thr^{28} prevents an MMP-dependent maturational cleavage of the β_1AR at a novel site that is distinct from previously identified β_1AR cleavage sites at $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, or $P^{52}\downarrow L^{53}$.

Finally, Figure 5A shows that the T28A substitution renders full-length β_1ARs elastase-sensitive in CHO-Pro5 cells, whereas β_1ARs harboring S37A or S41A substitutions are not cleaved by elastase. Figure 5B shows that the T28A substitution renders "cleavage-resistant" β_1AR-31/52 and β_1AR-31/41/52 mutants elastase-sensitive (compare lanes 6 and 8, or lanes 10 and 12).

Collectively, these results implicate a sialylated O-glycan at Thr^{28} as a post-translational modification that: 1) prevents MMP-dependent cleavage of the N-terminus at a novel site during maturational processing of the receptor in CHO-Pro5 cells; and 2) protects full-length β_1ARs from elastase-dependent cleavage. The later observation is predicted to have pathophysiologic importance, since activated neutrophils at sites of inflammation or injury release both elastase and neuraminidase (as schematized in Figure 5C).

β_1AR CLEAVAGE BY ELASTASE IN CARDIOMYOCYTES. We repeated the analysis in the more physiologically relevant cardiomyocyte context. Figure 6A (left) shows that an elastase treatment protocol that does not lead to β_1AR cleavage in CHO-Pro5 cells with intact protein O-glycosylation (see Figure 2A) results in pronounced β_1AR cleavage in cardiomyocytes. Of note, elastase treatment leads to a dose-dependent decrease in the abundance of both full-length and N-terminally truncated forms of the β_1AR in association with the accumulation of 2 β_1AR fragments; a

FIGURE 4 **An O-Glycan Attachment at T^{28} Prevents Maturational Cleavage the Full-Length β_1AR at a Site That Is Distinct From $R^{31}\downarrow L^{32}$, $S^{41}\downarrow L^{42}$, or $P^{52}\downarrow L^{53}$ in CHO-Pro5**



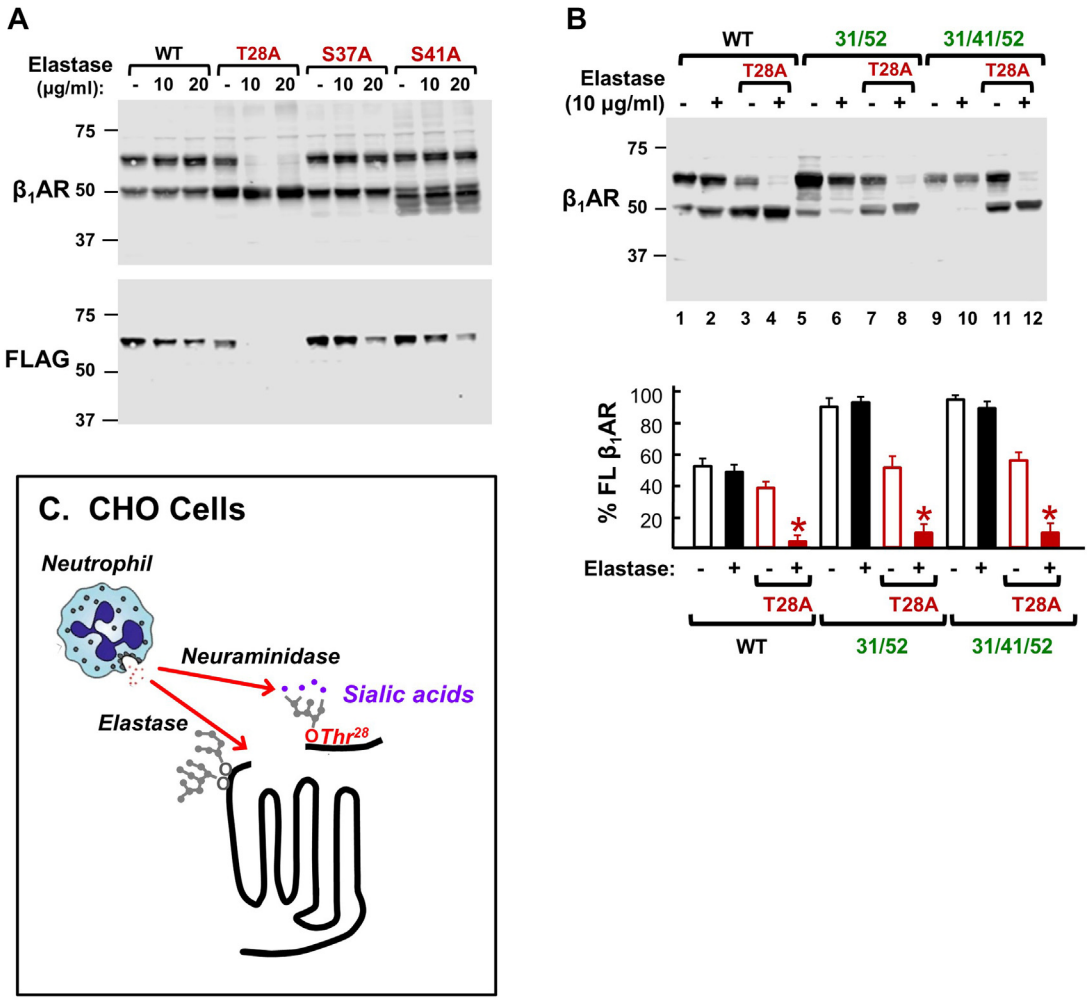
Immunoblot analysis on lysates from β_1AR-N15A-expressing CHO-Pro5 cells **(A)** or WT-β_1AR-, β_1AR-S37A-, or β_1AR-S41A-expressing CHO-Pro5 cells **(B)** cultured without or with elastase (20 μg/mL for 10 minutes). In each case, a representative experiment is depicted on top, with results from 3 separate experiments (with results expressed as described in the legend to Figure 3) quantified at the bottom (mean ± SEM, * $P < 0.05$). **(C)** CHO-Pro5 cells that express the WT-β_1AR or cleavage-resistant β_1AR-31/52 or β_1AR-31/41/52 mutant backbones, either with the WT Thr residue or an Ala substitution at position 28, were cultured in the presence of vehicle or GM6001 (10 μM) for 24 hours and then subjected to immunoblot analysis with an anti-β_1AR antibody. A representation experiment is depicted on top, with results from 3 separate experiments on separate culture preparations quantified at the bottom. Data are shown as mean ± SEM, with the percent of full-length β_1AR **(upper band)** expressed as a percent of total (full-length + truncated) β_1AR. Statistical analyses depicted in the figure are confined to comparisons between GM6001- and cognate vehicle-treated cultures for each construct (*$P < 0.05$); single residue substitutions that render the β_1AR cleavage resistant (in the β_1AR-31/52 or β_1AR-31/41/52 backbones) also result in statistically significant increases in the percent of FL-β_1AR. Abbreviations as in Figures 1 and 2.

~40-kD FLAG-tagged N-terminal fragment (detected by the anti-FLAG antibody) and a ~25-kD C-terminal fragment (detected by the anti-β_1AR antibody that recognizes a C-terminal epitope). The size of these fragments is consistent with an intramolecular cleavage in extracellular loop 2. The additional observation that elastase cleaves N-terminally truncated Δ2-31-β_1AR and Δ2-52-β_1AR mutants (Figure 6B) provides unambiguous evidence that at least one elastase cleavage site must be at an intramolecular site and not restricted to the N-terminus.

The elastase treatment was performed in parallel in neuraminidase-treated cardiomyocytes. Under these conditions, elastase-dependent β_1AR cleavage is more robust (compare results at submaximal 5-10 μg/mL elastase concentrations) and it is accompanied by the appearance of the ~25-kD C-terminal fragment. However, little to no associated accumulation of the ~40-kD FLAG-tagged N-terminal fragment was detected. This is presumed to be due to elastase's actions at a second site, namely, the O-glycan-regulated N-terminal cleavage site identified in CHO cells.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:976–988

FIGURE 5 An O-Glycan Attachment at T^{28} Prevents Elastase-Dependent Cleavage of the Full-Length β_1AR in CHO-Pro5 Cells

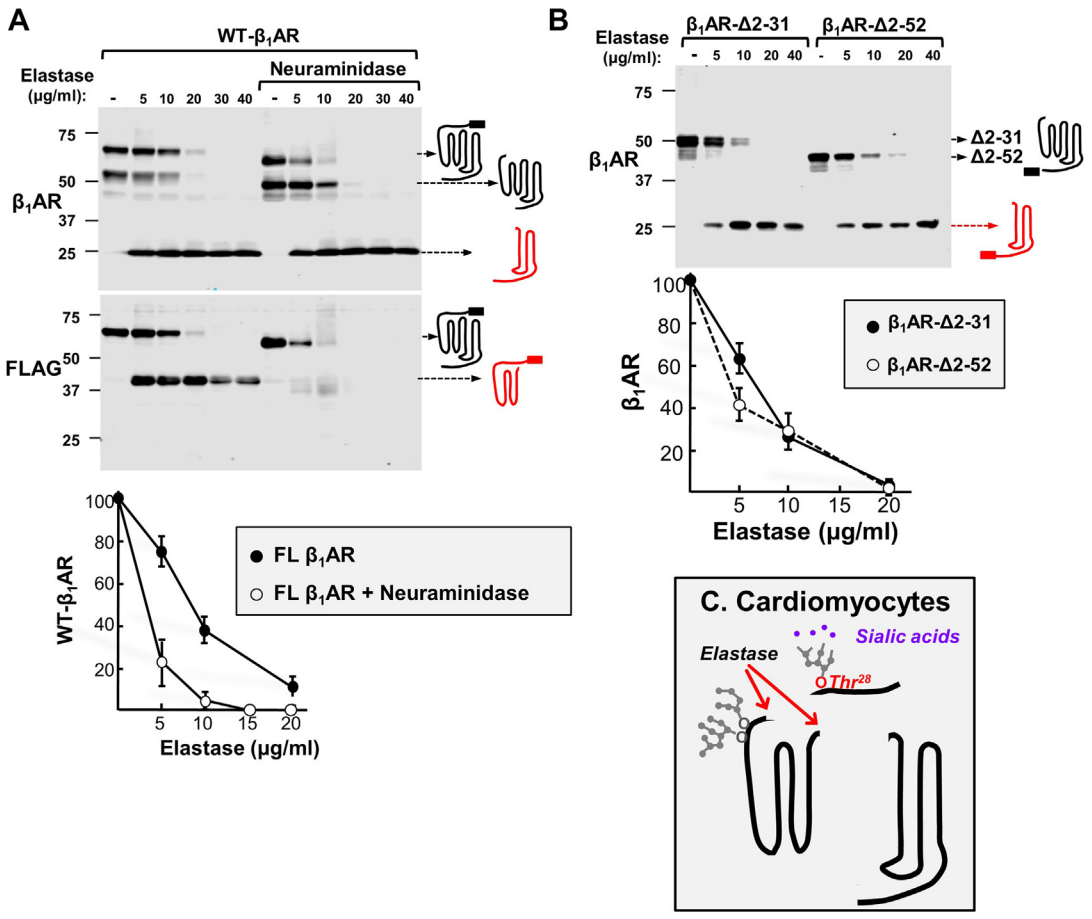


(A) WT-β_1AR-, β_1AR-T28A-, β_1AR-S37A-, or β_1AR-S41A-expressing CHO-Pro5 cells were challenged with vehicle or the indicated concentrations of elastase for 10 minutes; the reaction was stopped by the addition of 500 μM phenylmethylsulfonyl fluoride, followed by a wash with ice-cold phosphate-buffered saline and the addition of radio immunoprecipitation assay lysis buffer. Lysates were subjected to immunoblot analysis with an anti-β_1AR antibody that recognizes a C-terminal epitope and anti-FLAG that recognizes the N-terminal FLAG tag on the full-length β_1AR. **(B)** CHO-Pro5 cells that express β_1AR constructs with either the WT Thr residue or an Ala substitution at position 28 in the WT (lanes 1-4), cleavage-resistant β_1AR-31/52 (lanes 5-8), or cleavage-resistant β_1AR-31/41/52 (lanes 9-12) backbones were challenged with vehicle or elastase (10 μg/mL for 10 minutes). Reactions were stopped as described in **(A)** and lysates were subjected to immunoblot analysis with an anti-β_1AR antibody with quantification as described in Figure 3. As in Figure 4, statistical analyses depicted in this figure are confined to comparisons between elastase- and cognate vehicle-treated cultures for each construct (*$P < 0.05$); single residue substitutions that render the β_1AR cleavage resistant (in the β_1AR-31/52 or β_1AR-31/41/52 backbones) also result in statistically significant increases in the % FL-β_1AR. **(C)** Schematic of elastase-dependent β_1AR cleavage in CHO-Pro5 cells (see text). Abbreviations as in Figures 1 and 2.

Cleavage at this second site (which would be exposed by neuraminidase treatment) would remove the N-terminal FLAG-tag and result in the generation of an N-terminal fragment that is no longer FLAG-tagged and detectable in the experiment. β_1AR cleavage by elastase in cardiomyocytes is schematized in Figure 6C.

Finally, Figure 7 shows that elastase treatment leads to a defect in Iso-dependent cAMP accumulation, under conditions of no β_1AR overexpression (where elastase acts on the endogenous β_1AR) and in cardiomyocytes that heterologously overexpress the β_1AR. In each case, the elastase treatment does not alter basal or forskolin-dependent cAMP

FIGURE 6 Elastase Cleaves β_1ARs in Cardiomyocytes



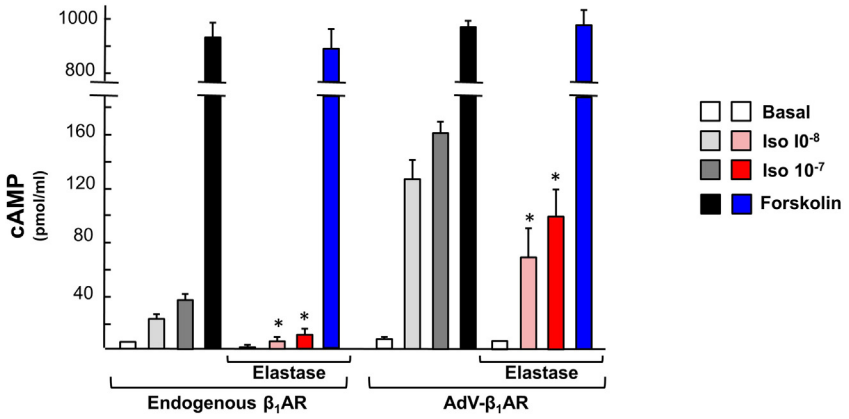
(A) WT-β_1AR-expressing cardiomyocytes pretreated with vehicle or α2-3,6,8,9 neuraminidase A (20 U/mL) for 3 hours were challenged with the indicated concentrations of elastase for 10 minutes. Lysates were then subjected to immunoblot analysis with anti-β_1AR and anti-FLAG antibodies. A representative experiment is depicted on top, with results from 4 separate experiments performed on separate culture preparations quantified at the bottom. For quantification of immunoreactivity (which is expressed as arbitrary units) levels of the full-length (FL) β_1AR species in elastase-treated cultures were normalized to the level of the FL β_1AR in the corresponding vehicle-treated culture, which was set to 100. **(B)** Immunoblot analysis on lysates of Δ2-31-β_1AR- or Δ2-52-β_1AR-expressing cardiomyocytes treated for 10 minutes with vehicle or the indicated concentrations of elastase. A representative experiment is depicted on top, with results from 3 separate experiments performed on separate culture preparations quantified (as described in **A**) at the **bottom**. **(C)** Schematic of elastase-dependent β_1AR cleavage in cardiomyocytes cells (see text). Abbreviations as in Figures 1 and 2.

accumulation, indicating that the effect of elastase is due to the cleavage of the β_1AR itself and not other downstream components in the signaling pathway. These results support the conclusion that elastase-dependent β_1AR cleavage results in the generation of signaling-incompetent β_1AR fragments.

DISTINCT β_1AR LOCALIZATION PATTERNS IN CHO CELLS AND CARDIOMYOCYTES. We used a functional approach (tracking β-agonist-dependent cAMP responses in the presence of the membrane-permeable antagonist propranolol vs the membrane-impermeable antagonist sotalol) to test the hypothesis that cell-specific differences in β_1AR cleavage by elastase can be attributed at least in part to differences in the subcellular compartmentation of β_1ARs in CHO-Pro5 cells vs cardiomyocytes (CMs). Figure 8 shows that propranolol effectively inhibits Iso-dependent cAMP accumulation in both CHO-Pro5 cells and CMs. In contrast, sotalol treatment leads to a ~50%-60% decrease in Iso-dependent cAMP accumulation in cardiomyocytes, both under conditions of no β_1AR overexpression (Figure 8, bottom left) and in

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:976–988

FIGURE 7 Elastase Treatment Leads to a Defect in β_1AR-Dependent cAMP Accumulation in Cardiomyocytes



Experiments were performed in parallel on cardiomyocyte cultures that did not overexpress the β_1AR (to track changes in cAMP accumulation resulting from elastase-dependent cleavage of the native β_1AR) and cultures that heterologously overexpress the human β_1AR. Cultures were pretreated with 5 μg/mL elastase for 10 minutes and then challenged for 5 minutes with the indicated concentrations of Iso or 10 μM forskolin. Measurements of cAMP accumulation (mean ± SEM) are from 3 separate experiments on separate culture preparations. *Significant difference in the Iso-dependent cAMP response between elastase- and corresponding vehicle-treated culture ($P < 0.05$). cAMP = cyclic adenosine monophosphate; Iso = isoproterenol. Abbreviations as in Figures 1 and 2.

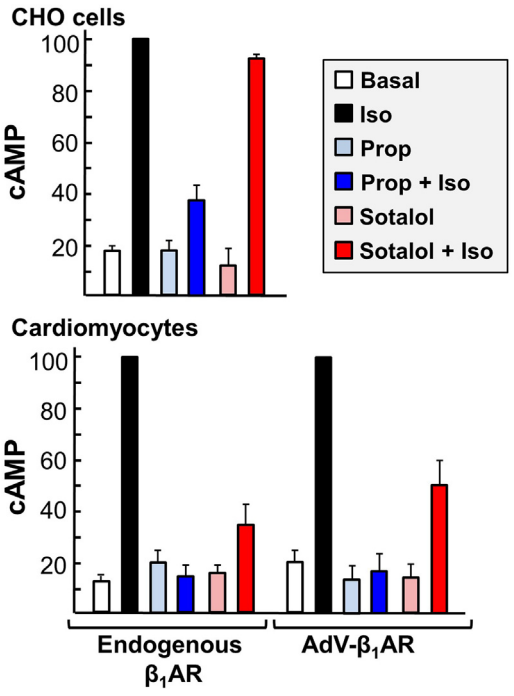
cardiomyocytes that heterologously overexpress the β_1AR (Figure 8, bottom right). In contrast, sotalol exerts only a minor inhibitory effect on the Iso-dependent cAMP response in CHO-Pro5 cells. These results indicate that β_1ARs are expressed on cell surface membranes in cardiomyocytes, whereas β_1ARs are largely sequestered in an intracellular compartment in CHO-Pro5 cells.

DISCUSSION

This study expands on our model of β_1ARs as protease-regulated receptors to show that β_1AR is cleaved by elastase. Our studies expose 2 distinct cleavage patterns. Studies in CHO-Pro5 cells identify elastase cleavage of full-length (but not N-terminally truncated) β_1ARs. We map cleavage to an N-terminal site that is distinct from the cleavage sites identified ($R^{31}\downarrow L^{32}$ or $S^{41}\downarrow L^{42}$) or predicted ($P^{52}\downarrow L^{53}$) in previous studies. We also show that cleavage at this site is prevented by sialylation of an O-linked glycan attachment at Thr^{28} (an O-glycosylation site not previously identified in a cellular context). Of note, these studies also implicate the O-glycan attachment at Thr^{28} as a post-translational modification that inhibits MMP-dependent cleavage of the β_1AR N-terminus (again, at a site distinct from the previously identified cleavage sites at $R^{31}\downarrow L^{32}$ and $S^{41}\downarrow L^{42}$)[1] during maturational processing of the receptor in cells.

The functional consequences of elastase's actions in CHO-Pro5 cells, to convert cell-surface full-length β_1ARs to an N-terminally truncated form, remains uncertain. Our previous studies established that N-terminally truncated β_1ARs remain signaling competent, but they adopt a different signaling profile. N-terminally truncated β_1ARs display altered signaling bias to cAMP and ERK pathways and they constitutively activate a G_i-AKT pathway that confers protection against doxorubicin-dependent apoptosis.[2,3] Although it is tempting to speculate that the N-terminally truncated form of the β_1AR generated on the cell surface as a result of elastase-dependent ectodomain shedding adopts an identical signaling phenotype, this conclusion would be predicated on the implicit assumption that signaling properties of a cell surface β_1AR cleaved by elastase (ie, an N-terminally truncated β_1AR confined to the cell surface) is identical to the signaling properties of the heterologously overexpressed Δ2-31-β_1AR transgene (or the N-terminally truncated form of the β_1AR that accumulates as a result of maturational processing of full-length β_1ARs in cells). In fact, recent studies indicate that β_1ARs localized to Golgi membranes activate a G_s-cAMP response that is distinct from the cAMP pathway activated by cell surface β_1ARs and that this intracellular pool of β_1ARs has privileged access to an Epac/mAKAPβ/PLCε scaffold that promotes local PI4P hydrolysis, activates a DAG/

FIGURE 8 β_1AR Partitioning Between Cell Surface and Intracellular Membranes in CHO-Pro5 Cells and Cardiomyocytes



Accumulation of cAMP in CHO-Pro5 cells and cardiomyocytes (in the case of cardiomyocytes, both without and with β_1AR overexpression) pretreated with vehicle, 10 μM propranolol, or 100 μM sotalol and then challenged with 0.1 μM Iso or 10 μM forskolin for 5 minutes. Results are normalized to the level of cAMP accumulation in vehicle pretreated cells stimulated with Iso and represent data (mean ± SEM) from 3 separate experiments performed on separate culture preparations. Abbreviations as in Figures 1 and 7.

protein kinase D pathway, and promotes CM hypertrophy.[11,12] The notion that the signaling phenotype of N-terminally truncated β_1AR species is influenced by its subcellular localization pattern is the focus of ongoing studies.

Studies in cardiomyocytes revealed a different β_1AR cleavage pattern. Here, elastase cleaves both full-length and N-terminally truncated forms of the β_1ARs at an intramolecular site tentatively mapped to extracellular loop 2. This results in the generation of signaling-incompetent β_1AR fragments that cannot support an Iso-dependent cAMP response. In an effort to define a mechanism that might explain cell-specific differences in β_1AR cleavage by elastase, we showed that β_1ARs partition between cell surface and intracellular membranes in cardiomyocytes, whereas β_1ARs are largely trapped in intracellular compartments where they are protected from extracellular proteases such as elastase in CHO-Pro5 cells. Although this cell-specific difference in β_1AR compartmentation would contribute to the distinct cleavage patterns identified in our studies, other factors might be contributory. For example, cell-specific differences in glycan modifications are quite common and might function to stabilize an N-terminal conformation that either shields or exposes an N-terminal or extracellular loop cleavage site. Studies to determine whether developmental- and/or disease-induced changes in β_1AR glycosylation or trafficking might result in different β_1AR cleavage patterns in neonatal vs adult cardiomyocytes—or in cardiomyocytes that have been induced to hypertrophy—and whether this mechanism functions to fine tune β_1AR protease-sensitivity and catecholamine responsiveness are ongoing.

The observation that cardiomyocyte β_1ARs are cleaved by elastase, a major protease released by neutrophils at sites of myocardial tissue injury and in the acute phase of viral myocarditis,[13] identifies a novel mechanism to influence cardiac catecholamine responsiveness. The additional observation that this process is influenced by sialylation is intriguing, since activated neutrophils also release a neuraminidase activity that results in local cell surface desialylation and is required for polymorphonuclear leukocytes migration to sites of cardiac inflammation.[14,15] A proteolytic cleavage event that alters β_1AR signaling responses would be highly significant because it would influence the pathogenesis of catecholamine-driven growth and/or injury responses in the setting of cardiac inflammation or myocardial infarction-induced cardiac injury.

STUDY LIMITATIONS. All of the studies in this article were performed in in vitro cell-based systems; the importance of elastin-dependent cleavage as a mechanism to regulate β_1AR signaling in vivo remains uncertain and requires further study.

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JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:976–988

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: This study builds on recent publications from our laboratory that challenge the conventional notion that the decrease in catecholamine responsiveness in the setting of heart failure or cardiac ischemia/infarction is attributable exclusively to agonist-dependent βAR desensitization or downregulation. Although β_2ARs undergo pronounced agonist-dependent desensitization, we recently showed that β_1ARs (the predominant βAR receptor subtype in cardiomyocytes) show little to no agonist-dependent desensitization in cardiomyocytes. Rather, our previous study identified oxidative stress and this study identifies proteolytic cleavage by inflammatory proteases as 2 mechanisms that decrease β_1AR expression and Iso responsiveness in cardiomyocytes. These results support a revised model that considers a role for βAR subtype-specific mechanisms in the pathogenesis of clinically important changes in cardiac catecholamine responsiveness.

TRANSLATIONAL OUTLOOK: βAR inhibitors have become mainstays in the therapy for heart failure and ischemic heart disease. The conventional notion that βAR blockers act both by antagonizing the cardiotoxic effects of elevated circulating catecholamines and by upregulating or restoring myocardial βAR expression and catecholamine responsiveness is based on the implicit assumption that β_1 and β_2ARs are controlled by similar mechanisms. But what if the mechanisms for the control of β_1AR and β_2AR responsiveness differ? This study shows that the β_1AR is a target for neutrophil elastase-dependent proteolytic cleavage and that this mechanism results in altered (if cleavage is confined to the N-terminus) or disrupted (in the case of an intramolecular cleavage) catecholamine responsiveness. These concepts (if replicated in in vivo models) would suggest novel β_1AR-targeted strategies that might be exploited for therapeutic advantage.

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