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Original Article

Aldo-keto reductase family 1 member B8 is secreted via non-classical pathway

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Abstract: Mouse aldo-keto reductase family 1 member B8 (AKR1B8) has the highest similarity to human aldo-keto reductase family 1 member B10 (AKR1B10), a secretory protein through lysosomes-mediated non-classical secretory pathway. To identify whether AKR1B8 is secreted through the same pathway, we carried out this study. Self-developed sandwich ELISA and western blot were used to detect AKR1B8 in cells and culture medium of CT-26 murine colon carcinoma cells. AKR1B8 releases in an independent manner to Brefeldin A, an inhibitor of ER-to-Golgi classical secretion pathway. Several factors, which are involved in the non-classical secretion pathway, such as temperature, ATP and calcium ion, regulated AKR1B8 secretion from mouse colorectal cancer cells CT-26. Lysosomotropic NH_4Cl increased AKR1B8 secretion, and AKR1B8 was located in isolated lysosomes. Therefore, AKR1B8 is a new secretory protein through the lysosomes-mediated non-classical pathway.

Keywords: Aldose reductase, aldose reductase family 1 member B8, AKR1B8, secretion, non-classical pathway

Introduction

Aldo-keto reductase (AKR) superfamily is composed of more than 100 proteins that are structurally and/or functionally conserved in the hierarchy of organisms, from bacteria to humans [1-5]; these proteins are widely implicated in carbonyl detoxification, pro-carcinogen activation, lipid metabolism, and cell carcinogenesis and cancer therapy [6-11]. In mice, there are three AKR1B homologs, i.e., aldo-keto reductase family 1 member B3 (AKR1B3, also referred as mouse aldose reductase, mAR), aldo-keto reductase family 1 member B7 (AKR1B7, also named as mouse vas deferens protein, MVDP), and aldo-keto reductase family 1 member B8 (AKR1B8, also called as mouse fibroblast growth factor-1 regulated protein, FR-1) [12-14]. Mouse AKR1B8 reduces aldehydes and their glutathione conjugates and regulates fatty acid synthesis by associating murine acetyl-CoA carboxylase-α [15].

In the AKRs family, AKR1B7 and AKR1C, also named as dihydrodiol dehydrogenase (DDH), are secretory proteins [16-18]. Recently, AKR-1B10, also known as aldose reductase like-1 (ARL-1), has been shown to be secreted from cancer cells through lysosome-mediated non-classical pathway [19]. AKR1B8 has the highest similarity to human aldo-keto reductase family 1 member B10 (AKR1B10) in terms of amino acid sequence, three dimensional structure, substrate spectra and specificity, and tissue distribution. AKR1B8 shows the highest identity to AKR1B10 (82%) and they appear from a same evolutionary node [15], therefore murine AKR1B8 is the ortholog of human AKR1B10. It was reported that AKR1B10 is secreted through lysosomes-mediated non-classical secretory

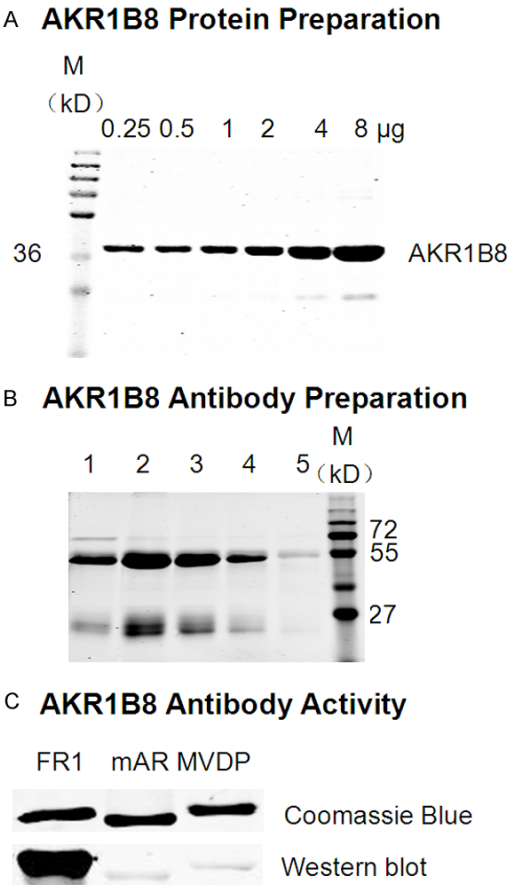

Figure 1. Preparations of AKR1B8 protein and antibody. A. Purification of AKR1B8 protein with different concentrations was analyzed by Coomassie blue staining. B. Purification of rabbit anti-AKR1B8 antibody was analyzed by Coomassie blue staining. C. Specificity of AKR1B8 antibody to AKR1B8 (FR1) and not to mAR and MVDP was analyzed by western blot.

pathway. Therefore, it is suggested that AKR1B8 may also be a secretory protein. In this study we identified whether AKR1B8 secretes from CT-26 cell line, a murine colon adenocarcinoma cell line derived from BALB/C (H-2d) mice.

Proteins are secreted via either classical (ER/Golgi-dependent) or nonclassical (ER/Golgi-independent) secretion pathways. In the classical pathway, secretory proteins typically contain an N-terminal signal peptide that directs the protein into endoplasmic reticulum (ER) and secretes through the Golgi complex [20-22]. In contrast, the nonclassical protein export features the lack of the conventional signal peptide at N-terminus and resistance to the ER/Golgi-dependent protein secretion inhibitor brefeldin A. Proteins secreted via the nonclassical pathway usually have a low molecular weight at 12-45 kDa [23, 24]; and several mechanisms are described for their secretion including a lysosome-mediated secretory pathway, plasma membrane exocytosis, exosome release and export using a specific membrane transporter [25]. Among these, the lysosome-mediated pathway is the most common and well investigated, and many proteins are secreted by this pathway, such as interleukin-1 beta (IL-1β), heat shock protein 70 (HSP70), ferritin, high-mobility group protein 1 (HMGB1) and AKR1B10 [19, 22, 23, 26, 27]. In this study we explored whether AKR1B8 is secreted through a lysosomes-mediated nonclassical pathway.

Materials and methods

Cell cultures

CT-26 murine colon carcinoma cells were cultured as suggested by American Type Culture Collection (Manassas, VA), in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA) at 37°C, 5% CO_2. The medium was replaced with fresh serum-free medium. 30 min later, the fresh medium was collected, and centrifuged at 800 × g, 4°C for 10 min to remove cells and debris, and then subjected to sandwich ELISA and western blot analysis.

Preparation of AKR1B8 recombinant protein and antibodies

AKR1B8 recombinant protein was prepared as described previously [28]. Briefly, the plasmid pQE-80 with His-tagged AKR1B8 cDNA was transfected into *E. coli* bacteria host M15. When OD_{A600} of culturing medium is about 0.8, the bacteria were induced by 2 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The bacteria were harvested and lysed by sonication. After centrifugation to remove debris, the supernatant was mixed with 50% slurry of nickel-nitrilotriacetic acid resin. The slurry was then loaded into a column and eluted with 0.5 M imidazole wash buffer. Elutions containing the recombinant protein were dialyzed, and stored at -20°C.

Western blot

80 μl medium or 30 μg of cell lysate were boiled in 5 × SDS sample loading buffer at 95°C for 5 min and then separated on 12% SDS-PAGE.

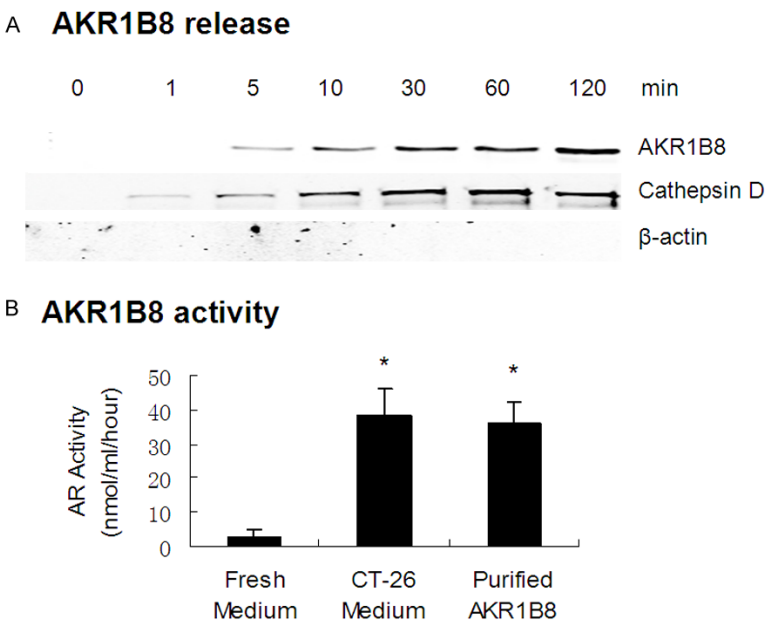

Figure 2. AKR1B8 release from CT-26 cells. A. AKR1B8 secreted from CT-26 cells in medium was detected by western blot. B. AKR1B8 activity. Cells (1×10^7) as indicated were seeded into six-cm dishes overnight and then fed with 2 ml of serum-free medium for 30 min. Medium was collected and subjected to an enzyme activity assay as described in the Materials and methods section. $^*p < 0.01$ vs Fresh Medium.

Separated proteins were blotted onto nitrocellulose membranes at 260 mA for 150 min using a Bio-Rad Mini-Protean II transfer apparatus (Bio-Rad Laboratories, CA). Blots were blocked at room temperature with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h. Rabbit anti-AKR1B8 (generated in our laboratory), goat anti-Vimentin (Cell Signaling, Inc), and rabbit anti-β-actin (Sigma-Aldrich Inc., MO) antibodies were probed and detected as previously described [29].

AKR1B8 enzymatic activity

Previous studies have demonstrated the enzymatic activity of mouse AKR1B members [2, 30, 31]. However, enzyme activity of AKR1B8 in medium had not detected before. 1 x 10^6 CT-26 Cells in a 60 mm-dish were incubated overnight in medium containing 10% FBS. After being immediately washed once with PBS, the cells were fed with 2 ml of serum-free medium for 30 min. The medium was collected, centrifuged at 600 × g for 10 min to remove cells and debris, concentrated 5-fold with a dialysis column (Millipore, CA), and then subjected to enzyme activity assays in 500 µl reaction mixtures containing 20 mM DL-glyceraldehyde, 135 mM sodium phosphate (pH 7.0), 0.2 mM NADPH, 50 mM KCl, and 200 µl concentrated medium. Reactions were conducted at 35°C for 30 min. Oxidized NADPH was measured at OD_{340} to indicate enzymatic activity. 100 ng of purified AKR1B8 recombinant protein was used as a positive control, and fresh serum-free medium was a blank control. Enzymatic activity is expressed as: nmol (oxidized NADPH)/ml medium/hour.

Signal peptide prediction

The amino acid sequence of AKR1B8 was input into the signal peptide prediction software SignalP 4.0 (www.cbs.dtu.dk/services/SignalP/). The organism group was set as Eukaryotes, and analysis methods of Neural networks and Hidden Markov models were chosen. A standard output format was produced.

Lysosome isolation and proteinase K protection

Lysosomes were isolated as described previously [32]. Briefly, 2×10^7 CT-26 cells were washed three times with PBS, resuspended in 2 ml of PBS containing 10 µg/ml leupeptin and 0.5 mM PMSF and disrupted using a Dounce homogenizer. Debris and nuclei were discharged at 1200 g for 10 min, followed by 50000 g for 30 min at 4°C. Supernatants (about 2 ml) were collected; pellets (lysosomes) were washed three times with PBS and suspended in 200 µl of PBS. For protection assays, pellets and supernatants (50 µl of each) were exposed on ice to 0.1 mg/ml proteinase K for 30 min, and then subjected to detecting AKR1B8 and Cathepsin D by western blot.

Statistic analysis

All the experiments were repeated three times. Data indicate a means ± SD of three independent experiments. Statistic analysis was performed using Student's *t*-test or Chi-square

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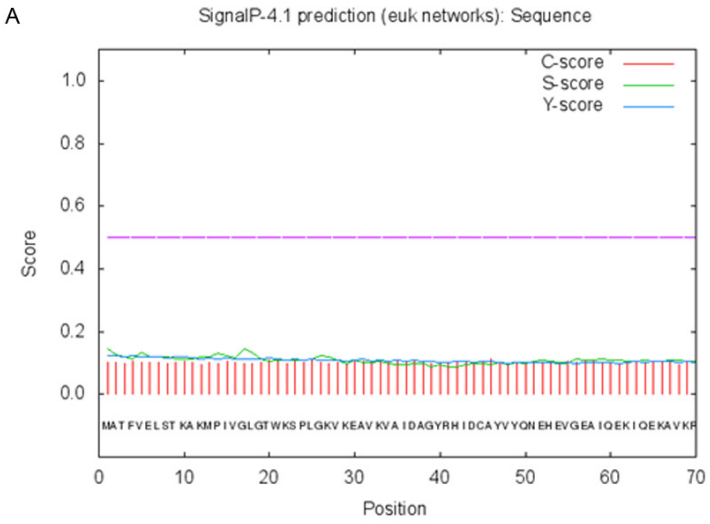


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  max. Y    20       0.117
  max. S     1       0.145
  mean S     1-19    0.123
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B

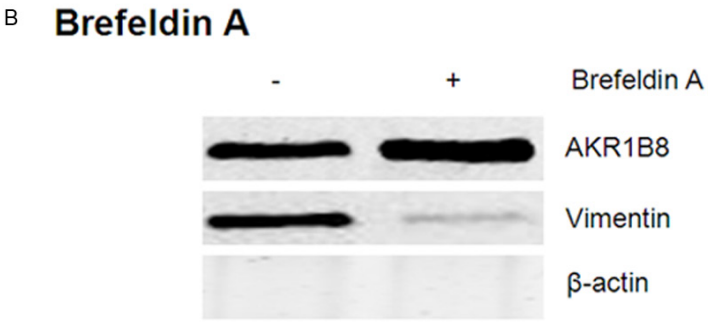

Figure 3. AKR1B8 secretes via a non-classical pathway. (A) Signal-peptide analyses in AKR1B8. Amino acid sequence of full-length mouse AKR1B8 was inputted into the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), and neural networks (NN) and hidden Markov models (HMM) were run. Both SignalP-NN (A) and SignalP-HMM (B) results showed no signal peptide in the AKR1B8 sequence. (B) Brefeldin (A) cells (2.5×10^5) were cultured in six-well plates overnight and then expose to the ER-to-Golgi protein transport inhibitor brefeldin A at 10 μg/ml in fresh serum-free medium for 8 h. Medium was collected for Western blotting. Vimentin was detected by Western blotting as a positive control.

tests, as appropriate, with INSTAT statistical analysis package (Graph Pad Software, CA), for statistical significance at $p < 0.05$.

Results

Preparations of AKR1B8 antigen and antibody

The purity of the AKR1B8 recombinant protein was analyzed by SDS-PAGE (**Figure 1A**). The AKR1B8 protein was used to produce goat and rabbit anti-AKR1B8 antibodies, and the specificity of rabbit anti-AKR1B8 was detected by western blot (**Figure 1B**).

AKR1B8 is secreted into culture medium

AKR1B8 was detected in the medium of CT-26 cells by western blot (**Figure 2A**). To exclude the possibility that AKR1B8 in the medium was derived from dead cells, the cultured cells were washed twice with cold PBS, and fresh serum-free medium was applied for 30 min. After collection, the medium was centrifuged at 600 × g for 5 min, 800 × g for 5 min and 1200 × g for 5 min sequentially to remove dead cells, debris and nuclei. β-actin, a non-secretory protein, was not detectable in the medium (**Figure 2A**). AKR1B8 concentration in medium increased with the prolonging time, and its peak reached at 60 min and then a high concentration plateau appeared.

AKR1B8 is a reductase with activity to aldehydes [15]. To understand the functionality of the secreted AKR1B8, we tested its enzyme activity to DL-glyceraldehyde, and data showed that the AKR1B8 in the medium was enzymatically active with purified AKR1B8 as positive control (**Figure 2B**).

AKR1B8 is secreted by a non-classical protein secretion pathway

Soluble proteins are secreted by either classical or nonclassical pathways [22]. In the classical protein secretion pathway, a secretory protein is translocated by an N-terminal signal

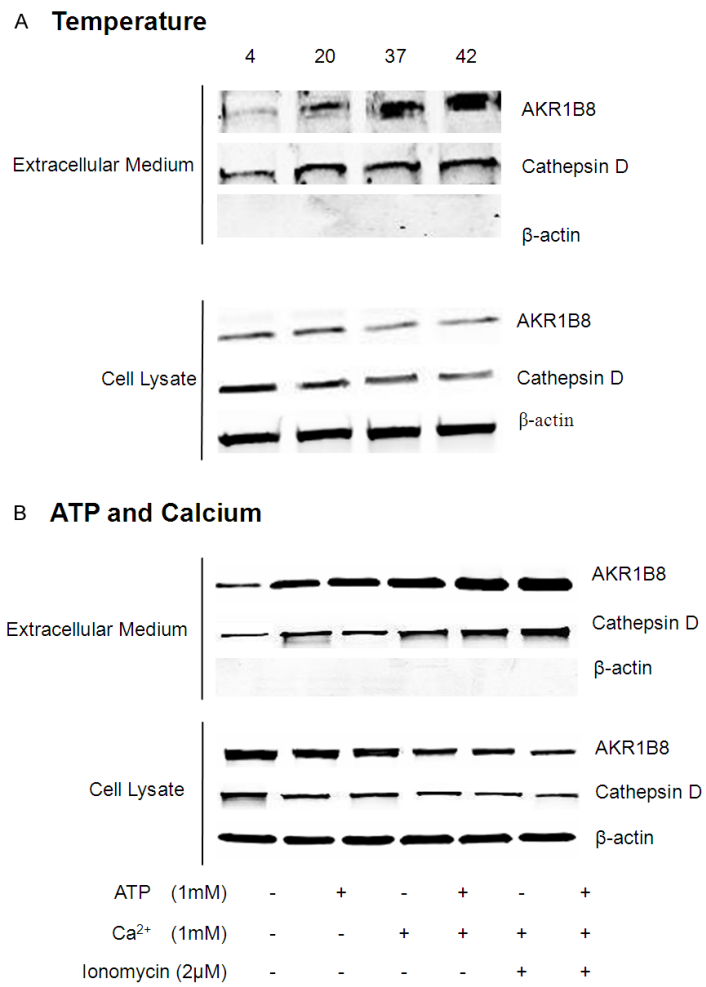

Figure 4. AKR1B8 secretion is affected by nonclassical secretory pathway-related factors. A. Temperature. cells (2.5×10^5) were seeded into six-well plates overnight and then exposed to the temperature (°C) indicated for 30 min. The cells were fed with fresh serum-free medium at the same temperature for 30 min, and medium was collected for Western blotting as described in the Materials and methods. B. ATP and Calcium. Cells (2.5×10^5) were incubated in six-well plates overnight and then exposed to 1 mM Ca^{2+} ions or 2 μM Ionomycin in serum-free medium for 2 h. At 30 min before harvest, ATP (1 mM) was added or not, and the medium was subjected to Western blot analysis.

peptide into endoplasmic reticulum (ER) after the beginning of protein synthesis and then secreted via Golgi complex. This secretion is affected by an inhibitor of transport from ER to Golgi. To identify the AKR1B8 secretory pathway, we analyzed its amino acid sequence using SignalP 3.0, and found that AKR1B8 does not contain any signal peptide (probability = 0.000; **Figure 3A**). Exposing CT-26 cells to 10 μg/ml brefeldin A, a classical secretion pathway inhibitor that blocks protein transport from ER to Golgi, did not block but slightly stimulated AKR1B8 secretion. As a positive control, brefeldin A efficiently blocked the secretion of Vimentin through the classical ER/Golgi pathway [33] (**Figure 3B**). These data suggest that AKR1B8 is secreted through a nonclassical protein secretion pathway.

AKR1B8's secretion is affected by nonclassical secretory pathway-related factors

We observed the effect of small molecules and factors on AKR1B8 secretion that influence nonclassical protein secretion pathway, such as temperature, ATP, and Ca^{2+} [23, 34]. Data showed that in CT-26 cells, AKR1B8's secretion was blocked at 4°C, but stimulated at 42°C (**Figure 4A**), demonstrating a temperature-dependent relationship. Similarly, ATP stimulated AKR1B8's secretion [35, 36] (**Figure 4B**). Ca^{2+} plays a critical role in merger between subcellular compartments and plasma membranes, promoting vesicle transportation and exocytosis [37]. This study demonstrated that AKR1B8's secretion was significantly stimulated by Ca^{2+} ions (100 μM) and Ionomycin (2 μM), a transporter of Ca^{2+} ions [38] (**Figure 4B**).

Lysosomes are involved in AKR1B8 secretion

Secretory lysosome-mediated protein secretion is a common and well-known non-classical pathway [19, 26] and was examined for AKR1B8 secretion. Ammonium chloride (NH_4Cl), a lysosomotropic drug increasing in lysosomal pH to result in ΔpH decrease between cytosol and vesicle lumen, promotes lysosome exocytosis [36, 39, 40]. To study whether AKR1B8-containing vesicles behave as secretory lysosomes and compared the effects of lysosomotropic drugs on Cathepsin D and AKR1B8 release, we cultured CT-26 cells were cultured overnight with complete RPMI 1640 medium at

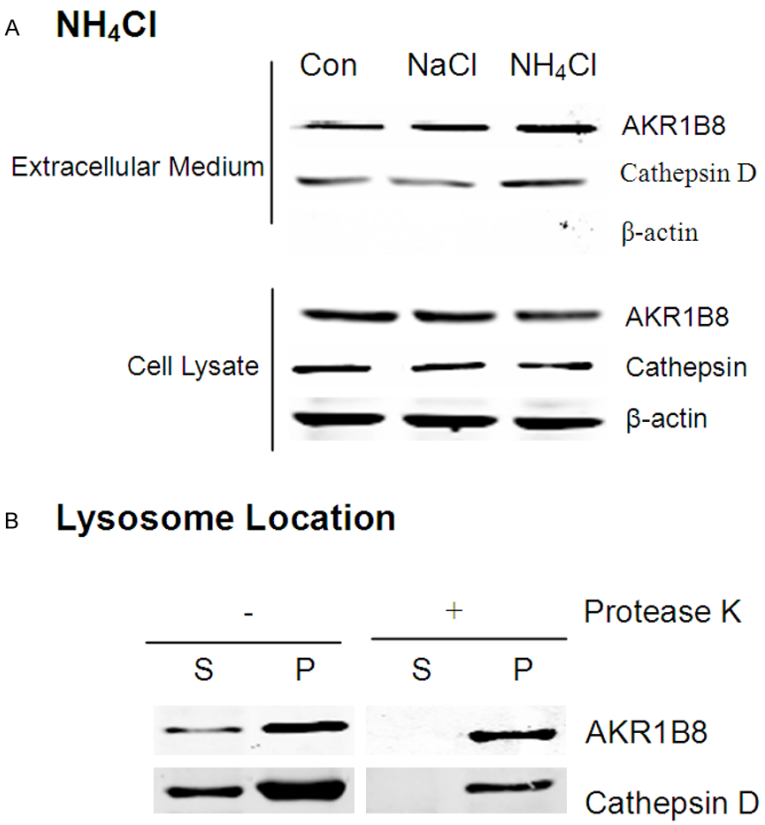

Figure 5. Lysosomes are involved in AKR1B8 secretion. A. AKR1B8 secretions were enhanced by NH_4Cl treatment. B. AKR1B8 was present inside the isolated lysosomes by western blotting analysis.

the density of 1.5 × 10^5 cells per well into 12-well plates. The mediums were replaced with serum-free mediums with 50 mM NH_4Cl, and 30 min later, the culture supernatants were collected and subjected to ELISA and Western blot. As shown in **Figure 5A**, AKR1B8 and cathepsin D secretions were enhanced by NH_4Cl treatment. Lysosomes were isolated by ultracentrifugation. Western blotting showed that AKR1B8 was present inside the lysosomes, as indicated by Cathepsin D, and was protected from proteinase K (0.0125 mg/ml) digestion (**Figure 5B**).

Discussion

With more than 100 members, AKR proteins represent a superfamily. This study showed AKR1B8, the ortholog of secretory human AKR1B10, is also a secretory protein of this superfamily. AKR1B8 is secreted through a nonclassical pathway, as evidenced by lines of experimental data: 1) no signal peptide in amino acid sequence of AKR1B8; 2) no effect of brefeldin A on AKR1B8 secretion; and 3) Positive effect of temperature, ATP, and Ca^{2+} on AKR1B8 secretion [23, 34]. ATP activates G protein-coupled P2 receptors (P2Y and P2X receptors), triggers Ca^{2+} mobilization [41, 42], and provides energy for the nonclassical secretion. This study revealed that AKR1B8 secretion is influenced by these factors.

Several pathways are involved in non-classical protein secretion, and a protein may export via multiple non-classical secretory pathways [43]. IL-1β releases via (i) exocytosis of IL-1β-containing secretory lysosomes [44], (ii) release of IL-1β from shed plasma membrane microvesicles [45], (iii) fusion of multivesicular bodies with the plasma membrane and subsequent release of IL-1β-containing exosomes, and (iv) export of IL-1β through the plasma membrane using specific membrane transporters [46]. Multiple studies have indicated that the plasma membrane-mediated non-classical secretory pathway is a Ca^{2+}-dependent process [37, 47, 48]. In this study, AKR1B8 secretion was regulated by Ca^{2+}, so plasma membrane-mediated vesicle transportation and exocytosis might be a mechanism of AKR1B8 secretion. The detailed mechanism of AKR1B8 release will be further explored in a future study. The lysosome-mediated AKR1B8 was further confirmed with lysosome-specific protein markers. Cathepsin D, a luminal protein of lysosomes, was detected together with AKR1B8 in the medium of CT-26 cells. Non-secretory β-actin was not detected either, excluding the protein sources from dead cells. Taken together, these results suggest that AKR1B8 is secreted through lysosomal exocytosis.

In this study, we found that AKR1B8 in medium has the same molecular weight as in cytosol and retains enzymatic activity. Therefore, the

secreted AKR1B8 is functionally normal. Several house-keeping reductases are secretory, such as thioredoxin reductase (TrxR), MVDP, DDH, AKR1B10 [49, 50]. AKR1B8 is an important host-protection reductase, detoxifying electrophilic carbonyls and their glutathione conjugates at physiological levels [51]. AKR1B8 is secreted as an enzymatic active protein, suggesting its potential role as a carbonyl scavenger in cell spaces or far distant organs, particularly in inflammatory tissues with enhanced oxidative stress and lipid peroxidation [52].

In summary, this study identified AKR1B8 as a novel secretory protein through a lysosomes-mediated nonclassical pathway. Secreted AKR1B8 may also play an important role in reducing carbonyl compound and protecting cells from oxidative stress damage. A better understanding of AKR1B8 release mechanisms is of significance to understanding the pathophysiological roles of AKR proteins, and the secretory feature of AKR1B8 may indicate its potential significance for study of AKR1B10 in human diseases.

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Disclosure of conflict of interest

The authors indicated no potential conflicts of interest.

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