Extracellular vesicles regulate purinergic signaling and epithelial sodium channel expression in renal collecting duct cells

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Abstract
Purinergic signaling regulates several renal physiological and pathophysiological processes. Extracellular vesicles (EVs) are nanoparticles released by most cell types, which, in non-renal tissues, modulate purinergic signaling. The aim of this study was to investigate the effect of EVs from renal proximal tubule (HK2) and collecting duct cells (HCD) on intra- and intersegment modulation of extracellular ATP levels, the underlying molecular mechanisms, and the impact on the expression of the alpha subunit of the epithelial sodium channel (αENaC). HK2 cells were exposed to HK2 EVs, while HCD cells were exposed to HK2 and HCD EVs. Extracellular ATP levels and αENaC expression were measured by chemiluminescence and qRT-PCR, respectively. ATPases in EV populations were identified by mass spectrometry. The effect of aldosterone was assessed using EVs from aldosterone-treated cells and urinary EVs (uEVs) from primary aldosteronism (PA) patients. HK2 EVs downregulated ectonucleoside-triphosphate-diphosphohydrolase-1 (ENTPD1) expression, increased extracellular ATP and downregulated αENaC expression in HCD cells. ENTPD1 downregulation could be attributed to increased miR-205-3p and miR-505 levels. Conversely, HCD EVs decreased extracellular ATP levels and upregulated αENaC expression in HCD cells. ENTPD1 downregulation could be attributed to increased miR-205-3p and miR-505 levels. Conversely, HCD EVs decreased extracellular ATP levels and upregulated αENaC expression in HCD cells, probably due to enrichment of 14-3-3 isoforms with ATPase activity. Pretreatment of donor cells with aldosterone or exposure to uEVs from PA patients enhanced the effects on extracellular ATP and αENaC expression. We demonstrated inter- and intrasegment modulation of renal purinergic signaling by EVs. Our findings postulate EVs as carriers of information along the renal tubules,

Abbreviations: AQP2, aquaporin 2; ARR, aldosterone to plasma renin activity ratio; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; ENaC, epithelial sodium channel; ENTPD, ectonucleoside-triphosphate-diphosphohydrolase; EVs, extracellular vesicles; HCD, human renal collecting duct cells; HK2, human renal proximal tubule cells; PA, primary aldosteronism; PANX1, pannexin-1; uEVs, urinary extracellular vesicles. Eric R. Barros Lamus, Valentina Carotti, Joost G. J. Hoenderop and Juan P. Rigalli are equal contribution.
ATP hydrolysis by ecto-ATPases. Among the latter, membranes from the afferent and efferent arterioles. Extracellular as well as in the glomeruli, endothelial, and smooth muscle expressed throughout the different segments of the nephron.

Purinergic signaling constitutes a key regulatory mechanism in several renal physiological and pathophysiological processes. For instance, extracellular nucleotides have been described to modulate reabsorption of ions, water, and other solutes (eg, urea) from the pro-urine. Hereby, the epithelial sodium channel (ENaC) constitutes a major purinergic target. Purinergic signaling results from the binding of extracellular ATP and its hydrolysis products to purinergic receptors of the P1 (ie, adenosine receptors) and P2 (ie, nucleotide receptors) families. In the kidney, purinergic receptors are expressed throughout the different segments of the nephron as well as in the glomeruli, endothelial, and smooth muscle cells from the afferent and efferent arterioles. Extracellular ATP levels result from the balance between ATP release by channels such as pannexin 1 (PANX1) and connexin 30 and ATP hydrolysis by ecto-ATPases. Among the latter, members like the ectonucleotidase 1 (ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1, NTPDase 2) catalyze the hydrolysis of ATP to ADP, which also represents a P2 agonist. On the contrary, the ectonucleotidase 1 (ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1, NTPDase 1, CD39) catalyzes the hydrolysis of ATP to AMP and results in an attenuation of P2-mediated signaling. Although the role of ENTPD1 in the pathogenesis of diseases such as pulmonary hypertension and Crohn’s disease is well acknowledged, the regulatory mechanisms of ecto-ATPases, as well as of ATP secreting channels in the kidney have been less investigated.

Extracellular vesicles (EVs) are a group of membrane-enclosed nanosized particles released by several types of cells mainly consisting of exosomes and microvesicles. EVs carry proteins, RNA (eg, microRNAs, mRNAs), signaling lipids, metabolites, and carbohydrates within their cargo, which ultimately determine the biological effect of a given set of EVs. In the kidney, research has focused mainly on urinary EVs (uEVs), released by the epithelial cells from the renal tubules and the urinary tract, and their potential as a source of biomarkers for renal disorders. However, more interest has been placed recently in the role of EVs in the regulation of renal physiological and pathophysiological processes (overview recently reviewed in Rigalli et al). Noteworthy, the intrinsic structure of EVs, where the cargo is protected from proteases and RNAses by a lipid bilayer, may provide an efficient way for the intercellular shuttle of molecules and signals between different segments of the nephron in a chemically aggressive environment such as the pro-urine. This way, cells located in distal segments of the nephron may be able to receive signals from cells in the more proximal segments. For instance, Jella et al reported the inhibition of ENaC in collecting duct cells exposed to EVs derived from proximal tubule cells. In another study, the transfer of functional aquaporin 2 (AQP2) by EVs within different collecting duct cells has been described. Interestingly, AQP2 activity in the target cells was higher when donor cells (ie, EV-releasing cells) had been previously exposed to the vasopressin analogue desmopressin. Moreover, proteomics and next-generation sequencing studies performed on uEVs confirmed the presence of more than 1,000 proteins and 500 miRNA and pre-miRNA molecules with different biological activities. Similarly, a proteomics analysis of EVs isolated from cortical collecting duct cells identified the presence of more than 100 proteins. This extremely diverse cargo confers EVs an immense potential to mediate other, yet unknown, renal physiological processes, in particular those involving the remote exchange of information between cells.

Modulation of purinergic signaling by EVs has already been described, although not yet in the kidney. For instance, EVs isolated from pleural fluid from mesothelioma patients exhibited ENTPD1 activity mediating the hydrolysis of extracellular ATP to AMP, which was further hydrolyzed to adenosine by CD73, also present in the vesicles. Finally, adenosine binding to A2A receptors suppressed T-cell activation and thus the antitumoral response. In another study, exosomes released by head and neck squamous cell carcinoma cells upregulated ENTPD1 expression in T-regulatory lymphocytes, also showing the potential of EVs to regulate purinergic signaling. Considering this, we hypothesize that EVs secreted by renal tubular epithelial cells modulate extracellular ATP levels and, therefore, purinergic signaling.

The aim of this study was to investigate the effect of EVs from renal proximal tubule and collecting duct cells on extracellular ATP levels, the underlying molecular mechanisms and the downstream impact on the expression of SCNN1A (ie, subunit alpha of the epithelial sodium channel, αENaC), a well-known purinergic target.
2 | MATERIALS AND METHODS

2.1 | Materials

Dynasore, POM1, and suramin were from Santa Cruz Biotechnology (Dallas, TX, USA). Apyrase and γ-S-ATP were from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade quality or higher.

2.2 | Cell culture

Human proximal tubule epithelial cells (HK2, ATCC®CRL-2190™, passage number 15-30) kindly donated by Dr Rody San Martín (UACh; Valdivia, Chile) were cultured under standard conditions. Cells were cultured in medium consisting of DMEM-F12 low glucose (5 mM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin/streptomycin, non-essential amino acids and HEPES (20 mM). Human collecting duct cells (HCD, passage number 20-30), kindly donated by Dr Pierre Ronco (Hôpital Tenon, Paris, France), were cultured in DMEM-F12 supplemented with FBS 2% (v/v), penicillin/streptomycin, transferrin (5 μg mL⁻¹), sodium selenite (50 nM), glutamine (2 mM), dexamethasone (50 nM), insulin (5 μg mL⁻¹), and HEPES (20 mM). Both cell types were cultured at 37°C in an incubator with 5% (v/v) CO₂ and humidified atmosphere, trypsinized and passaged when they reached 80% confluence.

2.3 | Isolation of EVs

HK2 and HCD EVs for cell treatments were isolated using a differential ultracentrifugation protocol. In brief, HK2 and HCD cells were seeded in 8 T-175 flasks (2x10⁶ cells per flask) and cultured for 24 hours up to 30%-40% confluence as described by Sinha et al. Afterward, cells were rinsed with PBS and 20 mL per flask of EV-free medium were added. FBS-free or low FBS (1% (v/v), EV-depleted) medium was used for HK2 cells or HCD cells, respectively. FBS was depleted of EVs using ultracentrifugation at 100 000 g for 18 hours as previously described. Cells were incubated for 24 hours. Subsequently, the culture medium was collected and centrifuged at 300 g for 15 minutes to remove the remaining cells. The supernatant was centrifuged at 2000 g for 30 minutes and again at 12 000 g for 45 minutes in order to remove dead cells, apoptotic bodies, and cell debris, respectively. The resulting supernatant was filtered using a Ministart 0.22 μm syringe filter (Sartorius, Göttingen, Germany) and ultracentrifuged at 120 000 g for 70 minutes (Sorvall WX + Ultra Series; AH629-36 swinging bucket rotor). The resulting pellet was resuspended in PBS, washed, and ultracentrifuged at 100 000 g for 70 minutes. The final pellet was resuspended in 100 μL of filtered PBS. Unless otherwise stated, EV suspensions for cell treatments were obtained by pooling six different preparations obtained as described above. Suspensions were characterized according to the guidelines of the International Society for Extracellular Vesicles by nanoparticle tracking analysis, transmission electron microscopy, and western blot analysis. Aliquots were stored at −80°C until further analysis or use for cell treatments.

2.4 | Nanoparticle tracking analysis

To quantify vesicle size and concentration of the EV suspensions, Nanoparticle Tracking Analysis (NTA) was performed using a NanoSight NS300 and NanoSight NTA 3.2 software (Malvern Instruments Ltd, Malvern, UK). Samples were optimized for analysis by diluting them in filtered PBS within a range of concentrations to obtain around 20-100 particles per field. Once the appropriate dilution was obtained, samples were introduced in a low-volume flow cell (Malvern Instruments Ltd, Malvern, UK) using an automatic syringe pump. Camera level and detection threshold were optimized to obtain an image with enough contrast to identify individual particles and minimum background noise during recordings (camera level = 13; detection threshold = 3; flow speed = 50 arbitrary units). Particles were tracked by passing a laser beam through the liquid sample, and the scattered light was detected and captured in short videos by a sCMOS camera (3 videos of 30 seconds each). The Brownian motion of particles was determined on a frame-by-frame basis and the distance moved by particles was used to determine EVs size and concentration using the Stokes-Einstein equation.

2.5 | Transmission electron microscopy

Transmission electron microscopy (TEM) was performed to verify the shape and size of EVs isolated using the protocol described above. Briefly, 15 μL of the EV suspension were absorbed onto carbon-coated copper grids (200mesh) for 10 minutes. Samples were negatively stained with 2% (w/v) uranyl acetate solution for 1 minute. Grids were visualized at 87 000x in a Phillips Tecnai transmission electron microscope at 80 kV, and images were acquired using a SIS CCD Camera Megaview G2. To determine EV size, TEM microphotographs were analyzed using ImageJ (NIH, USA).

2.6 | Western blot analysis

CD63 as an EV marker, ENTPD1 and PANX1 protein expression were determined using western blot analysis. For
this purpose, EVs (for CD63) or cell pellets (for ENTPD1 and PANX1) were resuspended in RIPA buffer (Thermo Fisher Scientific Inc, IL, USA) to extract total proteins and protein concentration was determined using the bicinchoninic acid (BCA) method. Protein lysates were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, CA, USA). Membranes were incubated with primary antibodies: mouse monoclonal anti-CD63 (1:200, sc5275, Santa Cruz Biotechnology, TX, USA), rabbit monoclonal anti-CD39 (ie, ENTPD1) (1:1000, ab108248, Abcam, Cambridge, UK), rabbit polyclonal anti-PANX1 (1:1000, ab139715, Abcam, Cambridge, UK), or mouse monoclonal anti-beta actin (1:10 000, A5441, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. Next, they were washed three times with 0.3% (v/v) TBS-Tween and incubated with the correspondent secondary HRP-linked antibody: rabbit anti-mouse (1:10 000, Ab6728, Abcam, MS, USA) or goat anti-rabbit (1:10 000, #7074, Cell Signaling, MA, USA) for 1 hour at room temperature, washed three times with TBS-T. Finally, proteins were detected with enhanced chemiluminescence using a ChemiDoc Imaging System (Bio-Rad, CA, USA).

2.7 | Cell treatments

To investigate the effect of EVs on extracellular ATP and purinergic signaling, HK2 cells were exposed to HK2 EVs (ie, signaling within the proximal tubule), while HCD cells were exposed to HK2 (ie, signaling from the proximal tubule to the collecting duct) and HCD EVs (ie, signaling within the collecting duct). As a control, the effect of HCD EVs on extracellular ATP levels in HK2 cells was investigated. HK2 and HCD cells were seeded in 12-well plates (5 × 10^4 cells/well, for extracellular ATP measurements) or 6-well plates (1.125 × 10^5 cells/well, for intracellular cAMP and expression analysis) and cultured for 24 hours in complete medium under standard conditions. Then, cells were rinsed with PBS, and starving medium (FBS-free or supplemented with 1% (v/v) of EV-depleted FBS, for HK2 and HCD cells, respectively) was added for 24 hours. Next, medium was replaced with fresh starving medium supplemented with HK2 or HCD EVs (7 × 10^7 particles/mL of culture medium) and cells were incubated for 15 minutes (to study short-term effects) and 6 hours (to study long-term effects). After incubation, aliquots of culture medium were collected for intracellular ATP quantification, and cell pellets were obtained for expression analysis. The expression of αENaC, a well-known target of purinergic signaling, was investigated. Dependence of extracellular ATP changes on the EV uptake by the target cells was studied by co-incubation with dynasore (80 μM), used as an uptake inhibitor. The participation of ENTPD1 in mediating the changes in extracellular ATP was assessed using the inhibitor POM1 (25 μM). All ENTPD1 inhibition experiments included a 30-minutes preincubation with the inhibitor. The association between the regulation of extracellular ATP and αENaC expression by EVs was addressed by co-incubation of cells with EV suspensions under depletion of extracellular ATP with apyrase (24 U mL⁻¹). Furthermore, the relevance of variable extracellular ATP was also determined by incubation with the non-hydrolyzable ATP analogue ATP-γ-S (5 and 50 μM). The participation of P2Y receptors in αENaC regulation by EVs was assessed using suramin (50 μM).

2.8 | Confirmation of EV uptake by target cells

HK2 and HCD-derived EV pellets, isolated as previously described, were resuspended in filtered PBS and stained for 5 minutes with PKH67 green fluorescent cell linker kit (Sigma-Aldrich, MO, USA) and diluent C. The reaction was stopped using PBS-BSA 5% (w/v) for 1 minute. The mix was ultracentrifuged at 100 000 g for 70 minutes, and the pellet washed with PBS and ultracentrifuged twice at 100 000 g for 70 minutes. The final pellet was resuspended in 100 μL of PBS. Stained EVs were incubated on target cells (HK2 and HCD) grown on coverslips for 12 hours. After incubation, cells were washed three times with 0.3% (v/v) paraformaldehyde for 10 minutes at room temperature. As a negative control, cells were exposed to free dye incubated without EVs (PKH67 and 5% (w/v) BSA). Cell nuclei were stained with DAPI for 1 minute, rinsed with PBS, mounted in a glass slide using mounting medium and visualized in an epifluorescence microscope.

2.9 | Quantification of extracellular ATP

To assess the effect of EVs on extracellular ATP levels, cells were treated as described in Section 2.7. Extracellular ATP was measured using the ATP Lite kit (Perkin-Elmer, Waltham, MA, USA). After treatment, aliquots of culture medium were diluted in the lysis buffer provided with the kit and used for ATP determinations by chemiluminescence following the manufacturer’s instructions.

To confirm the participation of EVs in the regulation of extracellular ATP levels, EV suspensions from HK2 and HCD cells generated as described in Section 2.3 were depleted from EVs using the Exosome human CD63 Isolation/detection reagent (Thermo Fisher Scientific Inc, IL, USA). Next, the EV-depleted suspension was added to HCD cells in a volume equivalent to the volume of original suspension leading to a concentration of 7×10^7 EVs/mL of culture medium (ie, 0.02 μL suspension/mL of culture medium). Extracellular ATP levels were measured after 6 hours of...
incubation using the ATP Lite kit (Perkin-Elmer, Waltham, MA, USA). EV-depletion was confirmed by western blot for CD63 (see Section 2.6).

2.10 | Gene expression analysis

Total RNA was isolated from target cells treated with EVs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and real-time quantitative PCR were performed as previously described. RPS11 was used as a reference gene. Primer sequences were identified online to an LTQ-Orbitrap-Fusion mass spectrometer (Thermo Fisher Scientific Inc, IL, USA) with a developing gradient from 7% to 30% (v/v) buffer B (80% acetonitrile, 0.1% (v/v) formic acid in water ).

2.11 | Determination of intrinsic ATPase activity of EV suspensions

Intrinsic ATPase activity of EV suspensions was measured in a reaction medium consisting of PBS supplemented with ATP (0.1 μM) and the necessary volume of EV suspensions (isolated as described in 2.3) in order to achieve final concentrations of 5 × 10^4, 1 × 10^5 and 2 × 10^5 EVs per μL of the reaction medium. Samples of reaction medium were collected at 6 hours of incubation, and ATP quantification was performed as described in 2.9.

2.12 | Proteomics analysis of EV suspensions

Extracts of EVs from HK2 and HCD cells were precipitated with acetone to concentrate the samples. Equal protein amounts of all samples were processed simultaneously using filter-aided sample preparation (FASP). In brief, the samples were mixed with 200 μL 8 M urea (in 0.1 M HEPES pH 8.50) and incubated for 20 minutes in the dark with 100 μL of Tris/HCl (0.1 M, pH 8.50) supplemented with iodoacetamide (50 mM) and urea (8 M). Following, the filters were centrifuged and washed three times with 8 M urea (in 100 mM HEPES pH 8.50), followed by three washes with 50 mM ammonium bicarbonate. Overnight digestion was performed in a wet chamber at 37°C with a 1/100 trypsin:protein ratio. After overnight digestion, samples were acidified with trifluoroacetic acid (TFA) and desalted using Stage tips.

The digested peptides were injected into an Easy-nLC1000 (Thermo Fisher Scientific Inc, IL, USA) connected online to an LTQ-Orbitrap-Fusion mass spectrometer (Thermo Fisher Scientific Inc, IL, USA) with a developing gradient from 7% to 30% (v/v) buffer B (80% acetonitrile, 0.1% (v/v) formic acid in water ) for 214 min prior to washes at 60% then 95% (v/v) buffer B, for a total data collection time of 240 minutes. Raw files were searched against the curated UniProt human proteome database (released June 2017) with MaxQuant versions 1.6.0.1 and its integrated search engine Andromeda with default settings. In addition, match between runs, label-free quantification (LFQ) and iBAQ were enabled. Prior to downstream analysis, proteins flagged as reverse or contaminant hits were filtered out. Proteins were analyzed using the LFQ values analyzed by the 2^-ΔΔCT method. miRNA expression was plotted as fold-change respect to the control (no EV addition).
obtained from MaxQuant using the Perseus software (version 1.6.5.0).47

### 2.13 Effect of EVs on intracellular cAMP

Intracellular cAMP was studied in cells exposed to EVs for 6 hours as described in Section 2.7. After treatment, cell lysates were obtained, and intracellular cAMP was measured using the Cyclic AMP Select ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions.

### 2.14 Effect of aldosterone on EV release and extracellular ATP

HCD cells were cultured as previously described (Section 2.2) in complete medium for 24 hours followed by 24 hours of incubation in starving medium (ie, with 1% (v/v) of EV-free and charcoal-stripped FBS).48 Subsequently, cells were stimulated with aldosterone (100 nM) for 24 hours.49,50 Afterward, the culture medium was collected and EVs were isolated. The number of EVs under the different experimental conditions was assessed by nanoparticle tracking analysis, as described in Section 2.4. The effect of EVs from control and aldosterone-conditioned cells on extracellular ATP in HCD cells was studied by exposing the target cells to the same volume of EV suspension (0.02 μL EVs per μL culture medium). Extracellular ATP was determined as described (Section 2.9.).

### 2.15 Effect of uEVs from patients with primary aldosteronism on extracellular ATP and αENaC expression in HCD cells

To study whether uEVs from patients with increased aldosterone production and mineralocorticoid activation exert a differential effect on extracellular ATP and αENaC expression, uEVs were isolated from urine samples of patients diagnosed with primary aldosteronism (PA). The diagnosis of PA was performed according to the endocrine society guidelines51 with serum aldosterone to plasma renin activity ratio (ARR) > 25 and suppressed plasma renin activity (<1 ng mL⁻¹ h⁻¹), after which primary aldosteronism patients (n = 12) and healthy controls (n = 14) were selected. Subjects with underlying comorbidities, or using glucocorticoids, contraceptives or drugs that interfere with the renin-angiotensin aldosterone system (eg, spironolactone) were excluded. Baseline characteristics of both groups of subjects are detailed in Supporting Information 1. Informed consent was obtained from all participants according to the guidelines of the Declaration of Helsinki and Ethics Committee of the Faculty of Medicine, Pontificia Universidad Católica de Chile (CEC-MedUC #180309002).

Second-morning urine samples (15 mL), collected between 8:00 and 10:00 AM and mixed with 1 mL of 1X Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany), were used to isolate uEVs through a differential ultracentrifugation protocol previously described.52 In brief, 12 mL of urine were centrifuged at 17 000 g, 4°C for 15 minutes. The resulting supernatant (SN1) was stored at room temperature, and the pellet was resuspended in 50 μL of DTT (3.2 M) and 200 μL of isolation solution (10 mM triethanolamine and 250 mM sucrose). Afterward, the mix was vortexed and centrifuged at 17 000 g, 4°C for 15 minutes. The resulting supernatant was mixed with SN1 and centrifuged at 200 000 g, 20°C for 90 minutes. The final supernatant was discarded, and the pellet was resuspended in 100 μL of PBS and stored at −80°C until further analysis.

The uEV suspensions from healthy controls and PA patients were generated by pooling similar volumes from the different uEVs preparations, and protein concentration from each suspension was determined using the BCA method. Afterwards, HCD cells were cultured as described in 2.7, and exposed to 10 μg mL⁻¹ of uEV suspensions from healthy controls or PA patients for 6 hours. After the treatment, extracellular ATP levels were determined as in 2.9 and SCNN1A expression as in 2.10.

### 2.16 Statistical analysis

All data are expressed as mean ± SEM. Statistical differences between groups were assessed with the Student's t-test or one-way ANOVA for two or more than two experimental groups, respectively. Analyses were performed with GraphPad Prism 7.0e (GraphPad Software, San Diego, CA, USA).

### 3 RESULTS

#### 3.1 Characterization of EV fractions

Size distribution analysis showed a peak at 84 and 98 nm, with a mean size of 86.7 ± 1.9 and 90.0 ± 0.6 nm for HK2 and HCD EVs, respectively (Figure 1A,B). All particles bigger than 0.22 μM were filtered out during the isolation process. EV concentration in prepared EV suspensions was $3.8 \times 10^8 \pm 2.69 \times 10^7$ particles mL⁻¹ for HK2 EVs and $3.41 \times 10^8 \pm 5.84 \times 10^7$ particles mL⁻¹ for HCD EVs. Western blot analysis confirmed the presence of the EV marker CD63 in both HK2 and HCD EVs (Figure 1C). Transmission electron micrographs for both HK2 and HCD EVs showed the
characteristic cup-shaped morphology and a size range between 50 and 150 nm (Figure 1D,E). Fluorescence microscopy of target cells incubated with PKH67-labeled EVs demonstrated uptake of HK2 EVs by HK2 cells (Figure 1F), HK2 EVs by HCD cells (Figure 1G), and HCD EVs by HCD cells (Figure 1H) under our experimental conditions. No fluorescent signal was observed in cells exposed to a similarly processed mix of PKH67 and 5% (w/v) BSA without EVs (Supporting Information 2).

3.2 HK2 and HCD EVs modulate extracellular ATP levels in HCD cells

The addition of HK2 EVs to HK2 cells (Figure 2A) did not significantly modify extracellular ATP levels after 15 minutes of incubation. Similarly, the addition of neither HK2 (Figure 2B) nor HCD (Figure 2C) EVs to HCD cells for 15 minutes altered extracellular levels of ATP levels. Exposure of HK2 cells to HK2 EVs also did not
HK2 and HCD EVs regulate extracellular ATP levels in HCD cells. Extracellular ATP was studied in HK2 cells exposed to HK2 EVs for 15 min (A) and 6 hours (D), in HCD cells exposed to HK2 EVs for 15 min (B) and 6 hours (E) and in HCD cells exposed to HCD EVs for 15 min (C) and 6 hours (F). The final concentration of EVs in the culture medium was 7x10⁷ EVs/mL culture medium. Results (mean ± SEM) are expressed as fold-change of the extracellular ATP levels respect to cells not exposed to EVs (−EVs). *Different from −EVs, P < .05, n = 4 (independent experiments). Dependence of the EV effects on the uptake by the target cells was determined using dynasore (80 μM) as an inhibitor of the EV uptake, or DMSO (control cells) in HK2 (G) and HCD cells (H). Results (mean ± SEM) are expressed as fold-change of the extracellular ATP levels respect to control cells not exposed to EVs (−EVs). a: different from control −EVs, b: different from control + EVs, c: different from dynasore −EVs. P < .05, n = 4 (independent experiments)
significantly affect extracellular ATP levels at 6 hours of incubation (Figure 2D). However, at the latter time point, HCD cells exhibited a significant increase in extracellular ATP levels after exposure to similar HK2 EV suspensions (Figure 2E). Intra-EV ATP content for HK2 EVs was below the limit of detection, thus indicating that the previous effects cannot be attributed to the release of ATP from the EV lumen into the extracellular space of the target cells (data not shown). Exposure of HCD cells to HCD EVs led to a significant decrease in extracellular ATP levels (Figure 2F). These findings indicate a different response of collecting duct cells depending on the segment of origin of the vesicles. Treatment of HCD cells with suspensions from HK2 and HCD EVs subject to EV depletion did not modify extracellular ATP levels (Supporting Information 4A,B).

Incubation of HCD cells with HK2 EVs in the presence of dynasore prevented the effects of the EVs on extracellular ATP respect to the situation without dynasore (Figure 2G), suggesting an uptake-dependent mechanism. On the contrary, co-incubation with dynasore did not prevent the decrease in extracellular ATP in HCD cells exposed to HCD EVs, indicating an uptake-independent effect (Figure 2H).

3.3 | Molecular mechanism of extracellular ATP upregulation by HK2 EVs

In order to gain further insight into the possible causes and consequences of extracellular ATP upregulation by HK2 EVs in HCD cells, the mRNA expression of PANX1 (ie, ATP secreting channel), ectonucleotidases and purinergic receptors was studied. Addition of HK2 EVs to HCD cells led to a significant downregulation of ENTPD1 expression (Figure 3A). No significant changes were observed in the mRNA levels PANX1 or purinergic receptors P2RY1, P2RY2, and P2RY11 (Figure 3A). ENTPD1 protein expression was significantly downregulated in HCD cells treated with HK2 EVs (Figure 3B). In order to assess if the downregulation of ENTPD1 by HK2 EVs could explain the higher extracellular ATP levels, POM1 was used to inhibit ENTPD1 activity during incubation with EVs. Addition of POM1 prevented the increase in extracellular ATP by HK2 EVs (Figure 3C), suggesting that downregulation of ENTPD1 may indeed be responsible for increased extracellular ATP levels in HCD cells. Nine miRNAs with the potential to regulate ENTPD1 in our experimental models were selected based on in silico analysis and literature evidence (Supporting Information 3). Among them, miR-205-3p and miR-505-3p (Figure 3D) were significantly upregulated in HCD cells exposed to HK2 EVs, probably explaining the decreased expression of their target mRNA ENTPD1. All other candidate miRNAs studied were not significantly affected by exposure to EVs (Figure 3D).

3.4 | Molecular mechanism of extracellular ATP downregulation by HCD EVs

The addition of HCD EVs to HCD cells resulted in the downregulation of PANX1 (Figure 4A) mRNA expression, while no changes were observed in ENTPD1 or purinergic receptors P2RY1, P2RY2, and P2RY11 (Figure 4A). PANX1 protein expression in HCD cells was not affected by HCD EVs (Supporting Information 4D). ENTPD2 expression in HCD cells was below the limit of detection (data not shown).

Since an uptake-independent mechanism was demonstrated (Figure 2H), we determined the intrinsic ATPase activity of the EVs. Incubation of EVs in a noncellular system supplemented with ATP showed ATPase activity for HCD vesicles after 6 hours of incubation (Figure 4B), while no changes were observed for HK2 vesicles (Supporting Information 4E). These observations may explain the uptake-independent decrease of extracellular ATP, once HCD EVs are added to HCD cells. Moreover, HCD EVs decreased extracellular ATP levels in HK2 cells (Supporting Information 4F). Although this experimental condition may not resemble the direction of pro-urine and, therefore, EV flow along the nephron, the results support an effect of HCD EVs mainly determined by ATPase activity due to the cargo of the vesicles and not by the nature of the target cell.

The protein cargo of EVs isolated from HK2 and HCD cells was analyzed using mass spectrometry (raw data available upon request). The results showed that 34 proteins were exclusively expressed in HK2 EVs, 137 in HCD EVs, and 26 proteins were common for both types of vesicles (Figure 4C). From all the proteins identified, only the protein 14-3-3 has been previously reported to exhibit ATPase activity. A heat map was generated using label-free quantification (LFQ) intensities to illustrate the higher abundance of 14-3-3 subunits in HCD EVs compared to HK2 vesicles. 14-3-3 α, e, γ, θ and ζδ were identified in HCD EVs, while only 14-3-3 ζδ was detected in HK2 EVs (Figure 4D).

3.5 | Effect of EVs on αENaC expression in HCD cells

ENaC is a well-known sodium channel mainly located in renal epithelia and associated to purinergic regulation. We observed a significant downregulation of SCNN1A (ie, gene codifying subunit α of ENaC) after exposure of HCD cells to HK2 EVs (Figure 5A). On the contrary, exposure of HCD cells to HCD EVs resulted in a significant SCNN1A upregulation (Figure 5B). Incubation of HCD cells with apyrase (ie, total removal of extracellular ATP) significantly increased the SCNN1A expression. Furthermore, apyrase prevented the effects of HK2 and HCD EVs on SCNN1A expression in HCD cells (Figure 5C). Treatment with γ-S-ATP resulted
in a significant downregulation of SCNN1A (Figure 5D). Prevention of the effects of EVs by the P2YR inhibitor suramin indicates involvement of purinergic signaling in SCNN1A regulation by the vesicles (Figure 5E). These observations strongly suggest an inverse association between extracellular ATP levels and SCNN1A expression in our experimental model, whereby this relation can be modulated by the addition of EVs. To gain further insight into the molecular mechanisms leading to SCNN1A regulation upon exposure of HCD cells to EVs and the consequent alteration

(A) HK2 EVs → HCD cells

(B) HK2 EVs → HCD cells

(C) HK2 EVs → HCD cells

(D) HK2 EVs → HCD cells
in extracellular ATP levels, intracellular cAMP levels were measured. In fact, HK2 EVs increased intracellular cAMP levels in HCD cells (Figure 6A). On the contrary, HCD EVs decreased intracellular cAMP levels (Figure 6B), again highlighting the opposite regulatory effect of proximal tubule and collecting duct EVs.
3.6 | Aldosterone modulates the release and activity of EVs on HCD cells

We demonstrated an inverse association between extracellular ATP levels and αENaC mRNA expression upon the exposure to EVs (Figure 5). Aldosterone is known to modulate extracellular ATP levels and αENaC functionality. To investigate whether aldosterone can contribute to the regulation of purinergic signaling by EVs, as observed in this study, the effect of aldosterone on EV release and on the ability of EVs to modify extracellular ATP was determined in HK2 and HCD cells. No significant changes were observed in the EV release by aldosterone-treated HK2 cells with respect to control cells (Figure 7A). However, aldosterone significantly increased EV release in HCD cells compared to the control conditions (Figure 7B). Furthermore, EV suspensions obtained from aldosterone-treated HCD cells led significantly to lower extracellular ATP levels in HCD cells compared to EVs isolated from control HCD cells (Figure 7D). On the contrary, no significant effects on extracellular ATP levels were observed when EVs from control and aldosterone-conditioned HK2 cells were added to HCD cells (Figure 7C). Moreover, incubation of HCD cells with uEVs from PA patients also decreased extracellular ATP (Figure 7E) and upregulated
αENaC (Figure 7F) with respect to cells incubated with uEVs from healthy donors. These findings highlight the influence of aldosterone on the regulation of purinergic signaling and αENaC by EVs.

4 | DISCUSSION

Purinergic signaling constitutes a major regulatory mechanism involved in renal function. Moreover, EVs from extrarenal tissues have shown to act as purinergic signaling regulators by modulating extracellular ATP levels. Our current study constitutes the first report describing the modulation of renal purinergic signaling by EVs. Moreover, our data, showing regulation of purinergic signaling in collecting duct cells by proximal tubule EVs, indicates the potential of EVs to mediate intersegment communication along the nephron.

Extracellular ATP in HCD cells increased with long-term exposure to HK2 EVs (Figure 2E). Furthermore, the prevention of this effect by dynasore (Figure 2G) suggests a mechanism dependent on the endocytosis of the EVs by HCD cells, as dynasore inhibits dynamin 2, a key component of clathrin-mediated endocytosis and caveolin-dependent endocytosis. Concomitant downregulation of ENTPD1 could explain decreased hydrolysis and, therefore, higher extracellular ATP levels. Effects on extracellular ATP were only observed after 6 hours of incubation, without changes after a short-term incubation. In this regard, the regulation of ENTPD1 at the mRNA and protein level may account for the longer incubation time required for the effects to take place. An increase in extracellular ATP upon ENTPD1 downregulation has been described in extra-renal models, where extracellular ATP levels modulated by CD39 regulate P2Y2R-mediated purinergic signaling. Additionally, cancer-derived exosomes were described to regulate CD73, another ectonucleotidase, when added to dendritic cells. Our results constitute the first study describing the modulation of ENTPD1 by EVs in the kidney.

MiRNAs are known negative regulators of mRNA expression. Additionally, miRNAs transfer via EVs is a common mechanism of cell-cell communication, also in the kidney. In the current work, we observed an upregulation of miR-205-3p and miR-505-3p in HCD cells exposed to HK2 EVs (Figure 3C). Our data are in line with previous studies reporting the presence of both miRNAs in HK2 cells. Furthermore, miR-205 was also described in human UEVs. Since both miRNAs target ENTPD1 mRNA, their upregulation may explain ENTPD1 downregulation, whereby the transfer of the miRNAs from HK2 to HCD cells via EVs appears as a likely mechanism. This, however, would need to be ultimately confirmed by using inhibitors against these particular miRNAs. Considering the role of extracellular ATP in renal physiology and in the development of pathologies such as renal injury, polycystic kidney disease and diabetic nephropathy, this could potentially yield novel therapeutic targets to be explored.

Contrarily to the effects upon addition of HK2 EVs, incubation of HCD cells with HCD EVs decreased extracellular ATP levels (Figure 2F), being this effect not prevented by the co-incubation with dynasore (Figure 2H). Although dynamin 2-independent uptake mechanisms have been described and cannot be fully ruled out, processes dependent on clathrin and caveolin, which are inhibited by dynasore, constitute the most frequent internalization mechanisms for EVs. Therefore, our results support an uptake-independent process in HCD EVs-mediated effects. In principle, a decrease in PANX1 expression by EVs (Figure 4A) may have also contributed to explaining the decrease in extracellular ATP upon addition of HCD EVs to HCD cells. However, the downregulation was not reproduced at the protein level (supporting information 4D), indicating a minor role of ATP secretion in the differences observed after incubation with EVs. Furthermore, positive feedback regulation of PANX1 by extracellular ATP has been described. In that sense, the downregulation of PANX1 mRNA after the addition of EVs could be a consequence of reduced extracellular ATP levels rather than a
cause. Also, our data demonstrates an ATP-hydrolyzing activity by HCD EVs (Figure 4B), unlike HK2 EVs, which is in line with previous evidence for cancer-derived, \(^{22}\) and saliva exosomes. \(^{55}\) Proteomics analysis of HK2 and HCD EVs clearly exhibited an enrichment in different subunits of the protein 14-3-3 in HCD vesicles. Noteworthy, subunits \(\gamma\), \(\varepsilon\), and \(\zeta\) have demonstrated ATPase activity. \(^{53}\) Altogether, these findings support an uptake-independent mechanism, where HCD EVs hydrolyze extracellular ATP in HCD cells due to intrinsic ATPase activity present in their cargo.

Inhibition of \(\alpha\)ENaC by extracellular ATP is a well-known process under physiological \(^{24}\) and pathophysiological conditions.
conditions. Previously reported evidence points to a short-term regulation at the channel activity level upon the increase in extracellular ATP levels and activation of purinergic receptors. Our findings, reveal an inverse association between extracellular ATP and αENaC mRNA expression after the addition of HK2 and HCD EVs (Figure 5), suggesting that this purinergic regulation also takes place at the gene expression level. The latter is in line with previous reports that have shown in collecting duct cells that ENaC activity decreases with increased extracellular ATP. Downregulation of αENaC expression by γ-S-ATP and upregulation by apyrase (ie, total removal of extracellular ATP) further supports this association. Finally, prevention of the effects of the EVs by apyrase and suramin strongly supports a dependence on the modulation of extracellular ATP levels and activation of P2Y receptors, respectively. To gain a deeper insight into the underlying molecular mechanisms, we evaluated intracellular cAMP levels, as a second messenger frequently accompanying P2Y2 and P2Y11 activation by ATP. Our results showed an increase in intracellular cAMP levels by HK2 EVs and a decrease by HCD EVs, in parallel with an increase and a decrease in extracellular ATP levels, respectively. Interestingly, although previous reports demonstrated upregulation of αENaC expression by cAMP in rat submandibular gland epithelial cells and in rat kidney, downregulation of the channel expression by cAMP was, on the contrary, observed in rat lung and in endothelial cells. Discrepancies may be attributed to different signaling pathways and molecules being activated in the various cases, of which cAMP could be only one of them, under the different stimuli applied (eg, hormones, direct exposure to cAMP analogues, etc). In the present work, we demonstrated a direct association between extracellular ATP and cAMP, and an inverse association between their levels and αENaC expression. Furthermore, the participation of P2Y receptors in the regulation of αENaC by EVs was shown, among which downstream signaling via cAMP is well-known. Although further studies should be performed to confirm and elucidate the mechanism inversely associating cAMP levels and αENaC mRNA levels, our findings suggest the participation of this second messenger in the downregulation of the channel expression by ATP and therefore, by EVs.

Previous evidence has demonstrated that the effects of aldosterone on ENaC activity and the consequent sodium re- absorption can depend on the release of ATP. On the other hand, aldosterone has also been shown to increase the release of EVs from endothelial cells, while PA patients have also shown to have more circulating EVs than essential hypertensives. The latter, along with our results (Figure 7), suggests that in renal cells, aldosterone effects on ATP and ENaC might be regulated, at least in part, by the release of EVs to the extracellular space.

Furthermore, sodium intake has been shown to activate ATP release by connexin 30 (Cx30) that in turn decreases ENaC activity in the distal part of the nephron. This also highlights an inverse association between extracellular ATP and ENaC, as displayed by our data, where ENaC expression was reduced in HCD cells upon the addition of HK2 EVs. Overall, it seems plausible that proximal tubule EVs can contribute to this paracrine purinergic signaling in order to regulate the activity and expression of ENaC. Moreover, Cx30-/- K.O mice (ie, lower extracellular ATP) show a hyper-active ENaC, which agrees with our observations where aldosterone stimulates the release of EVs with ATP hydrolytic activity in HCD cells, ultimately downregulating ENaC expression. These results suggest a novel mechanism by which 14-3-3 protein regulates ENaC, in addition to its known effects on the ubiquitin E3 ligase Nedd-4-2, which leads to an increase in ENaC abundance and activity. In agreement with this, uEVs from PA patients, which have higher serum aldosterone levels, activated ENaC and increased sodium re-absorption, decreased extracellular ATP and upregulated the expression of αENaC on HCD cells. In relation with this, a previous study reported the inhibition of EV release by the well-known ENaC inhibitor amiloride. Altogether, this points toward the fact that EVs contribute to different mechanisms that regulate extracellular levels of ATP and ENaC activation, encouraging further studies at this level.

The enclosed nature of EVs, defined by a lipidic bilayer, allows the shuttle of information (determined by the EV cargo) between cells, protected from external factors that may result in an attenuation or significant modification of the message. Previous reports already described the transfer of information between proximal tubule (donor cells) to distal tubule and collecting duct cells (target cells) via EVs. This mechanism allows the donor cell to sense different conditions in its intra- and extracellular environment and transmit an according message to other cells. In the case of purinergic signaling along the nephron, when signaling nucleotides are released to the pro-urine, they can be hydrolyzed by several ectoATPases expressed along the tubules, resulting in a modification of the signaling information before it reaches the purinergic receptors in downstream segments. In this sense, remote regulation via EVs could be an efficient communication mechanism for the transfer of information from proximal to collecting duct cells.

Although our findings provide relevant evidence on the role of EV-mediated signaling between renal epithelial cells, our study bears some limitations, partially inherent to the state-of-the-art in EV research, which should be taken into consideration. For instance, due to the lack of reports on the intratubular concentration of EVs, it is difficult to estimate whether the concentration of vesicles used in our studies mimic the situation in vivo. Noteworthy, we defined the concentrations of EVs based parameters previously used in other studies such as concentration of particles and concentration of proteins. In addition, our data point to a clear...
transcriptional regulation of αENaC by EVs. Further studies should be performed to confirm the impact of these changes at the protein and activity level.

In summary, we demonstrated the regulation of purinergic signaling and αENaC mRNA expression by EVs between proximal tubule and collecting duct cells in vitro as well as within the collecting duct, being the latter sensitive to aldosterone. These findings confirm the regulation of renal purinergic signaling by EVs, as previously demonstrated in other tissues. Furthermore, our work points to a new role for EVs in intercellular communication in the kidney that may not only affect sodium balance, but also other physiological and pathophysiological processes affected by purinergic signaling.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
E.R. Barros Lamus, V. Carotti, C.A. Carvajal, R.J.M. Bindels, J.G.J. Hoenderop, and J.P. Rigalli designed the research; E.R. Barros Lamus, V. Carotti, C. de Vries, F. Witsel, and J.P. Rigalli performed the research; O. Arntz, F. van de Loo, and C.A. Carvajal contributed new reagents or analytic tools; E.R. Barros Lamus, V. Carotti, C. de Vries, F. Witsel, J.G.J. Hoenderop, and J.P. Rigalli analyzed the data; E.R. Barros Lamus, V. Carotti, J.G.J. Hoenderop, and J.P. Rigalli wrote the paper.

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are either presented in the manuscript, available in the Supporting Information of this article or upon request to the corresponding author.

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