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ORIGINAL ARTICLE

Pharmacological dissection of the cellular mechanisms associated to the spontaneous and the mechanically stimulated ATP release by mesentery endothelial cells: roles of thrombin and TRPV

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Abstract

Endothelial cells participate in extracellular ATP release elicited by mechanosensors. To characterize the dynamic interactions between mechanical and chemical factors that modulate ATP secretion by the endothelium, we assessed and compared the mechanisms participating in the spontaneous (basal) and mechanically stimulated secretion using primary cultures of rat mesentery endothelial cells. ATP/metabolites were determined in the cell media prior to (basal) and after cell media displacement or a picospritzer buffer puff used as mechanical stimuli. Mechanical stimulation increased extracellular ATP that peaked within 1 min, and decayed to basal values in 10 min. Interruption of the vesicular transport route consistently blocked the spontaneous ATP secretion. Cells maintained in media lacking external Ca^{2+} elicited a spontaneous rise of extracellular ATP and adenosine, but failed to elicit a further extracellular ATP secretion following mechanical stimulation. 2-APB, a TRPV agonist, increased the spontaneous ATP secretion, but reduced the mechanical stimulation-induced nucleotide release. Pannexin1 or connexin blockers and gadolinium, a Piezo1 blocker, reduced the mechanically induced ATP release without altering spontaneous nucleotide levels. Moreover, thrombin or related agonists increased extracellular ATP secretion elicited by mechanical stimulation, without modifying spontaneous release. In sum, present results allow inferring that the spontaneous, extracellular nucleotide secretion is essentially mediated by ATP containing vesicles, while the mechanically induced secretion occurs essentially by connexin or pannexin1 hemichannel ATP transport, a finding fully supported by results from $Panx1^{-/-}$ rodents. Only the latter component is modulated by thrombin and related receptor agonists, highlighting a novel endothelium-smooth muscle signaling role of this anticoagulant.

Keywords ATP release · Mechanically evoked ATP release · Thrombin receptors · PAR agonist analogs · TRPV · Pannexin/connexin hemichannels · Vesicular release

Abbreviations

ATP	Adenosine 5′-triphosphate
ADP	Adenosine 5′-diphosphate
AMP	Adenosine 5′-monophosphate
ADO	Adenosine
2-APB	2-Aminoethoxydiphenylborane
CMD	Cell medium displacement
DMSO	Dimethyl sulfoxide
ECs	Endothelial cells
Gd	Gadolinium III
H-1152P	(S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine
H C 067047	2-Methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-*N*-[3-(trifluoromethyl)phenyl]-1*H*–pyrrole-3-carboxamide
$Panx1^{-/-}$	Pannexin1 knockout
MβCD	Methyl-β-cyclodextrin
NEM	*N*-ethylmaleimide
PAR	Protease-activated receptor
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
VNUT	vesicular nucleotide transporter
Y-27632	(R)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide

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Introduction

The extracellular life of ATP gained remarkable momentum 25 years ago with the discovery of multiple cell membrane receptors for this novel signaling molecule [1, 2]. ATP activates two sets of membrane receptors: ionic channels and metabotropic termed in general purinergic receptors, each with a partial pharmacological specificity that has allowed receptor classification based on agonists/antagonists profiles. Seven clones have been characterized for the P2X receptors, a family of trimeric ion channels relatively specific for ATP and related structural ligands containing a triphosphate chain [3, 4]. Moreover, eight clones of P2Y receptors coupled to the G-protein encompass the purinergic family [4]. In addition, adenosine (ADO) a byproduct of ATP metabolism activates a separate family of four P1 receptors, also coupled to G proteins [4]. Consonant with the ATP extracellular role, nucleotides have a short half-life due to rapid extracellular enzymatic hydrolysis. Most tissues express a set of ecto-ATPases that include CD 39 (Ecto-ATP diphosphohydrolase) and CD 73 (Ecto-5′-nucleotidase), which hydrolyze ATP sequentially to ADP, AMP, and ADO, respectively, accounting for the short ATP signaling role [5]. The analysis of ATP metabolites is of physiological interest since ADP is a P2Y receptors ligand, while ADO exerts separate signaling through the ADO receptors present in the vascular wall, relevant to this proposal interest.

Previous work from our laboratory showed that nucleotides play a prominent role in endothelial signaling to the vascular smooth muscle. Buvinic et al. demonstrated that P2Y receptor activity is coupled to NO-mediated vascular dilatation, inferring that endothelial cells signal through ATP released in auto/paracrine manner [6]. ATP is released from endothelial cells either as auto or paracrine signal and activates endothelial P2X4 and/or P2Y1 or P2Y2 receptors [6, 7]. The latter two receptors are coupled to phospholipase C, which leads to a rise in intracellularly stored calcium which activates eNOS, an enzyme dependent on calcium-calmodulin activation. Based on this finding, we proposed that ATP/ADP are key endothelial players dynamically involved in NO synthesis which participate in vascular wall homeostasis. Since endothelial cells (ECs) are under the influence of shear stress forces, we inferred that ECs via mechanical sensors localized either in the apical surface facing the vessel lumen or basolateral membranes, sense mechanical forces, a stimulus that impact ECs on a sec-to-sec bases. Therefore, the question of the identity and physiology of the putative endothelial mechanosensors has been a matter of keen interest for vascular physiology. The recent discovery of the Piezo1 and 2 as ECs cationic channel mechanosensors [8, 9], in addition to other claimed membrane mechanosensors, offered the opportunity to investigate the intrinsic signaling mechanism and the contribution of physiological ligands that operate concurrently in the vascular wall in vivo. Assuming mechanosensors are a primary target of shear stress forces, a valid question regards the link between mechanosensor activity and extracellular ATP release mechanism. In addition to mechanosensors, we hypothesized that transient receptor potential (TRP) channels expressed ECs and may also participate as modulators of ATP secretion [10].

Two basic mechanisms have been proposed to account for the extracellular nucleotide release and are partially documented in several cell types: (i) vesicular release, as better characterized in the nervous system [11] and (ii) non-selective channels such as the connexin and/or pannexin1 hemichannels [12]. In ECs, it is not clear whether the ATP secretion occurs via vesicles, such as the Weibel-Palade bodies, identified by anatomists and cell biologists to contain proteins and likely also ATP. These ECs vesicles are released either by a constitutive (basal component) or by a receptor or phorbol ester-operated mechanism(s) [13]. Most meaningfully, quinacrine-labeled ATP containing vesicles were shown in HUVECs which release ATP either spontaneously or by hypoxia-related mechanism [14]. Considering the charged nature of ATP, a nucleotide transporter is necessary to accumulate this molecule inside vesicles. In this regard, a vesicular nucleotide transporter (VNUT) was first described in the brain and later in peripheral tissues as the adrenals or pancreas [15]. In ECs, ATP is also released via connexin or pannexin1 hemichannels, [16, 17], notwithstanding the role of these channels in the spontaneous versus the mechanically induced ATP release from endothelial cells has not been addressed. In view of the role of thrombin in hemostasis, we raised the hypothesis that this ligand may play a significant role in the auto/paracrine role of ATP in endothelium smooth muscle signaling [18–20].

It is our working hypothesis that the mechanisms that govern the spontaneous and the mechanically induced ATP release and its subsequent ectoATPase hydrolysis by ECs are distinct and may be differentiated by stimuli and signals that trigger ATP secretion. At the light of this proposal, this communication describes a series of experiments systematically aimed at identifying putative mechanisms involved in the spontaneous and the mechanically induced ATP/metabolites release from ECs. We also addressed whether thrombin, an endogenous blood component relevant to prominent vascular diseases implicated in the coagulation cascade, modifies ATP secretion and whether TRPs are involved in the spontaneous or the mechanically induced ATP secretory routes. Based on the use of pharmacological agents, we assessed the role of (i) the vesicle secretory pathway in ATP secretion, (ii) connexin and pannexin1 hemichannels using a set of blockers and ECs from Panx1$^{-/-}$ mice implicated on ATP release mechanism(s), (iii) TRP channel ligands and/or mechanosensor blocking agents and their modulator role in endothelial cell ATP overflow, and (iv) thrombin, and related protease-activated receptor (PAR) peptide analogs on basal and mechanically induced

ATP secretion mechanism(s). Based on in-vitro protocols, using primary cultures of ECs, we conclude that the spontaneous ATP release is mainly associated to a vesicular release component, likely modulated by TRPV subtypes. In contrast, the mechanically induced release is essentially due to connexin and pannexin1 hemichannel activity which we infer is regulated by PAR ligands. Overall, this distinction permits analyzing the route of ATP secretion from ECs in physiology and pathophysiological conditions, highlighting the role of ATP secretion in endothelium smooth muscle signaling.

Materials and methods

Animals sources

Adult male Sprague Dawley rats (250–300 g) bred at the Animal Reproduction Facility of the Faculty of Biological Sciences of the P. Catholic University of Chile were used throughout. Rat handling followed animal care and welfare guidelines according to NIH (USA) standards were strictly adhered to. The Universidad de Santiago Ethical Committee for the use of animals in biological research approved the specific protocols designed and supervised our strict adherence to the subscribed guidelines through the local Ethical Committee of the Faculty of Chemistry and Biology. Every possible effort to reduce the number of animals sacrificed was enforced. Endothelial cells (ECs) from a same rat were used in parallel to assess each protocol and its control with and without mechanical stimulation. ECs derived from at least two separate rats per routinely used per experiment. The number of protocol wells examined is indicated as "n;" these values derived from two to three separate ECs batches seeded from separate animal mesenteries.

Panx1$^{-/-}$ mice were bred at Universidad de Santiago Animal Reproduction Facilities; animals were donated by Prof. Acuña-Castillo who collaborated in these studies. The Chilean colony of pannexin1 knockout C57BL/6 mice (Panx1$^{-/-}$) was introduced by Prof. J.C. Saéz and was maintained at the Animal Reproduction Labs at the P. Catholic University of Chile. As controls, C57BL/6 mice (WT) from Jackson Laboratory were used; WT were also donated by Prof Acuña-Castillo. Molecular biology assays confirmed the complete deletion of pannexin1 as originally reported by Bargiotas et al. [21]; additional Shoji et al. [22] studies further supported Panx1$^{-/-}$ obliteration. Only critical experiments were performed with these knockout rodents; four Panx1$^{-/-}$ and six controls were assessed in total avoiding excess animal sacrifices. Ethical guidelines applied to all rodent studies; strict adherence was mandatory during protocol progression.

ECs harvesting for primary culture

The procedure reported by Ashley et al. to isolate ECs was followed with minor modifications as briefly detailed [23]. Rats were anesthetized with a mixture of ketamine (75 mg/kg) plus xylazine (5 mg/kg) i.p. Once anesthetized, a mid-line abdominal incision was practiced; the superior mesenteric artery was localized and cannulated with polyethylene tubing; perfusion with Tyrode solution ensued (mM: 118 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 23.8 $NaHCO_3$, 11.1 D-glucose), added with penicillin (200 U/mL), streptomycin (0.2 mg/mL), and amphotericin-B (0.5 μg/mL) at room temperature (20 °C). Few minutes thereafter, the vascular bed of each mesentery was carefully excised from the intestines; the mesentery was always isolated from a live rat. The whole procedure lasted less than 5 min since rats were deeply sedated. Once the mesentery was removed from the abdomen, rats were sacrificed under deep anesthesia by bleeding and pneumothorax. The pancreas was removed from the isolated mesentery and discarded. The mesentery was incubated at 37 °C for 1 h under mild stirring in 5 mL of Tyrode buffer supplemented with the antibiotics plus 0.1% BSA and 2 mg/mL collagenase I. Thereafter, the cell suspension was centrifuged at 3000 rpm at 4 °C for 10 min to eliminate mesentery fat and gross tissue debris. The cellular pellet was dissolved in M-199 medium plus antibiotics and centrifuged 1500 rpm at 4 °C for 5 min. The new pellet was re-suspended in 36 mL medium 199 plus antibiotics supplemented with 20% fetal bovine serum plus 20 μg/mL endothelial cell growth supplements. ECs are seeded in two 24 multi-well plates, and incubated in a tissue chamber at 37 °C until reaching approx. 80% confluence within 3 days. This procedure greatly favors the enrichment and growth of ECs, as assessed by expression of positive marker proteins (von Willebrand, eNOS) and the absence of smooth muscle markers (α-actin), as well as for the characteristic cell cobblestone pattern.

ECs from the Panx1$^{-/-}$ mice The same procedures and protocols to harvest primary cultures of rat mesenteric ECs were adjusted to mice mesenteric ECs. We need to recognize that the number of ECs harvested per mouse was considerable less and grew slower than rat ECs. For this reason, only 8–10 wells of a 24 multi-well plates were seeded per mouse; after 12–14 days, cell confluence rarely exceeded 60%. Parallel protocols were conducted in four separate occasions; in each case, a Panx1$^{-/-}$ mice plus one or two controls were examined in parallel.

Cell media sampling to assess extracellular ATP/metabolites

Prior to the performance of each protocol, ECs were incubated in Tyrode buffer supplemented with 10 mM HEPES at 37 °C

for 1 h; this practice avoided regular cell media which contains 1 μM ATP. In the 24 multi-well plates, parallel protocols and corresponding controls were conducted. Some wells were maintained without mechanical stimulation and represent spontaneous secretion. Other wells were subjected to mechanical stimulation; 200 μL aliquots of the cell media were routinely collected 1 min after mechanical stimulation by the cell media displacement (CMD) procedure. In parallel, the same volume was collected from non-mechanical stimulated, to assess the level of basal, spontaneous, ATP secretion. These aliquots were placed in ice until chemical derivatization, to synthesize fluorescent ATP/metabolites. Some specific protocols assessed the time course of extracellular ATP/metabolites secretion; in these cases, cell media aliquots were sampled 1, 3, 5, 10, and 15 min following CMD procedure. After protocol completion, the wells were rinsed with Tyrode buffer, supernatants were discarded. The plate was placed at −20 °C until Bradford protein determinations were performed in each individual well. ATP/metabolites were quantified as described in the analytical procedures.

Studies with Panx1$^{-/-}$ mice were conducted following the same experimental conditions that operated for the rat series of protocols. Spontaneous levels of extracellular ATP/metabolites were retrieved in some of the wells; in other wells, upon the CMD procedure, extracellular media samples were collected only 1 min after stimuli.

ECs mechanical stimulation procedure

Two procedures were used as prototype mechanical stimulation protocols to elicit extracellular ATP secretion:

a) *Cell media displacement* (*CMD*) was the most frequently mechanical stimuli applied. Half (200 μL) of the total cell media volume (400 μL) was gently pipetted up and down for three consecutive times during less than 3 s, on the walls of each well using a P1000 micropipette. This procedure did not cause detectable cell death as evidenced by the detection of lactic dehydrogenase assays in the cell media, or trypan blue or Evans blue uptake as recognized cell markers of cell death. Trypan blue and Evans blue elicited the detection of 0.061 and 0.044% of dye uptake, respectively, in control assays while a similar value of 0.044 and 0.040% in ECs subjected to CMD procedure. These values allow discarding CMD-induced cell death. Cell media samples were collected following CMD to assay extracellular ATP/metabolites released. This protocol was adapted from the original Lazarowski et al. report [24]. Other investigators such as Romanello et al. or Hovater et al. also used this method to elicit extracellular ATP/metabolites secretion from several cell types [25, 26]
b) *Picospritzer puffs* delivered as a single 300 μL cell media puff of 500 ms duration, to the central portion of each cell well. To assess the validation of this procedure, multiple control assays with Indian ink instead were performed in wells devoid of cells to establish the best geometry for the stimuli delivery. Bernoulli's equations were used to estimate the magnitude of the applied forces considering the particular design and geometry of the puff delivery cannula. The calculation of the force generated per puff on the surface of the cell culture was equivalent to that assumed to operate in situ by post capillary veins, only when the procedure was operant protocol and was performed as indicated. Aliquots of cell media were sampled 1, 3, 5, 10, and 15 min after the buffer puff to assay the time course of extracellular ATP/metabolites secretion.

Protocols aimed to assess the putative mechanisms involved in the spontaneous or CMD-induced ATP secretion

I. Role of vesicles in the ATP secretory process To ascertain whether the spontaneous and/or the CMD-evoked release extracellular ATP involve the participation of vesicles, we used several drugs that inhibit different steps of the vesicular transport route as well as the role of the vesicular nucleotide transporter (VNUT).

a) *Monensin*, inhibitor of vesicular formation from the Golgi apparatus [27]. A 11 mM solution was prepared in ethanol, which at the moment of the experiment was diluted in Tyrode-HEPES buffer. ECs were incubated with 10 μM monensin for 1 h prior to the CMD stimulus. Parallel vehicle controls were assessed.
b) *Nocodazole*, a microtubule depolymerization agent that disrupted vesicular transport [28]. To this aim, a 33.2 mM stock nocodazole solution was dissolved DMSO and diluted in Tyrode-HEPES buffer (0.1% DMSO) at the moment of the experiment. ECs were incubated with 33 μM nocodazole for 3 h, prior to the CMD stimulus. Parallel vehicle controls were performed.
c) *Brefeldin A*, disrupts vesicular trafficking by inhibiting protein transport from the endoplasmic reticulum to the Golgi apparatus [27]. A stock solution of 21.4 mM brefeldin A was prepared in DMSO, an aliquot was diluted in Tyrode-HEPES buffer at the moment of the experiment. ECs were incubated with 20 μM brefeldin A for 3 h prior to the CMD-induced stimulation. Vehicle controls were performed in parallel.
d) *N-ethylmaleimide* (NEM), inhibitor of vesicular fusion with the plasma membrane [27]. A stock 7.2 mM was prepared in Tyrode-HEPES buffer and diluted at the moment of the experiment in the same buffer. ECs were incubated with 1 or 100 μM, for 15 min prior to the CMD stimulation.

e) *Evans blue*, a putative antagonist of the vesicular nucleotide transporter (VNUT) [15]. ECs were incubated during 30 min with 3–10 nM of Evans blue; parallel controls were conducted. Evans blue stock and dilutions were prepared in Tyrode-HEPES.

II. Role of connexin and/or pannexin1 hemichannels in the ATP secretion To assess whether the spontaneous and/or the CMD-evoked extracellular ATP release involves connexin or pannexin1 hemichannels, three compounds with differential sensitivity for these channels were used.

a) *18 β-Glycyrrhetinic acid*, a putative connexin channel blocker [16], was used at 5 μM, following a 15-min incubation. This agent was dissolved in DMSO and diluted in Tyrode-HEPES buffer to 0.04% DMSO, a concentration that did not interfere with ATP secretion, based in parallel vehicle controls.
b) *Probenecid*, 30 or 100 μM of this channel inhibitor with relative specificity for pannexin1 over connexin channels [17], was assessed following a 60 min of incubation; parallel control protocols were performed to assess spontaneous and CMD-induced ATP/metabolites released extracellularly. In addition, 100 μM probenecid was also investigated in ECs from Panx1$^{-/-}$ mice. Parallel controls examined either the spontaneous or the CMD-evoked nucleotide/metabolite release. A 1 mM probenecid stock solution was dissolved in 1 M NaOH and diluted in Tyrode-HEPES buffer to reach a final 20 μM; pH was adjusted to 7.4.
c) *Carbenoxolone*, a preferential pannexin1 channel blocker at concentrations lower than 100 μM [29]. ECs were incubated for 20 min with either 5 or 20 μM. This chemical was dissolved in water and diluted in Tyrode-HEPES buffer for ECs application.

Extracellular calcium reduction To differentiate the participation of hemichannels in basal and CMD-evoked extracellular ATP secretion, experiments were conducted in ECs maintained in cell media lacking calcium [30–32]. To this aim, ECs were grown in cell media containing normal calcium and later incubated for 10 min in Locke solution prepared with and without calcium. The composition of the Locke solution was (mM) 154 NaCl, 5.4 KCl, 2.3 $CaCl_2$-$2H_2O$, 1.5 $MgCl_2$-$6H_2O$, 5 HEPES, 5 glucose, pH = 7.4. The buffer without calcium had the same composition except for calcium and magnesium chloride; the buffer was adjusted at pH = 7.4. Following the 10 min ECs incubation in this media, CMD protocols and the parallel corresponding controls were performed.

III. Participation of TRPV, mechanosensors, and/or stretch-activated channels

1. To differentiate the alleged role played by TRP channels in the basal versus the CMD-evoked ATP secretion, pharmacological agents were used to activate or block TRPV1, TRPV3, and TRPV4, channels reported to be expressed in ECs [33–36]

a) *2-APB*: This chemical was used to activate TRPVs within a range of 2-APB concentration. A stock solution of 100 mM 2-APB in DMSO was prepared and diluted in Locke solution to attain 30 μM in the cell incubation media. The final DMSO concentration did not exceed 0.03%, which proved innocuous in parallel control assays. Cells were incubated with 2-APB for 10 min; controls examined the effect of the vehicle alone compared to the drug treatment. In addition, basal and CMD-induced ATP/metabolites were examined in the cell incubation media. To assess the simultaneous participation of hemichannels and TRPs in both the spontaneous and mechanically evoked ATP secretion, an additional protocol was conducted incubating ECs in media lacking external calcium for 10 min plus the 30 μM 2-APB addition. Proper parallel control protocols were performed.
b) *Ruthenium red*: This trivalent cation blocks TRPV1, TRPV2, and TRPV3 [37]. To this aim, ECs were incubated with 10–130 μM added to ECs for 10 min, prior to the CMD procedure. A 3.3 mM stock solution of ruthenium red was prepared in distilled water and diluted accordingly in Tyrode-HEPES solution.
c) *HC 067047*: To evaluate the participation of the TRPV4 channel in the basal and or CMD-evoked ATP secretory process, we assessed the effect of a purported TRPV4 antagonist in the basal and CMD-evoked extracellular ATP secretion [34]. ECs were treated with 0.3, 1, or 5 μM HC 067047, for 20 min prior to CMD procedure. A 10 mM stock solution was prepared in DMSO and diluted in Tyrode-HEPES immediately prior to the protocol performance. Parallel vehicle controls were conducted.

2. Based on the notion that mechanosensors are primary mechanical stimuli targets, we next aimed at blocking mechanosensors or stretch-activated channels and assessed extracellular ATP release prior to and following CMD-evoked ATP secretion. To this purpose, trivalent ions are used as channels blockers**:**

a) *Gadolinium III* (*Gd*): This ion is used to block piezo1 and other stretch channels [38–40]. ECs were incubated with 1 or 100 μM Gd for 10 min, prior to CMD mechanical stimulation. Moreover, to further explore possible synergic interactions between hemichannels together with mechanosensors, experiments were performed incubating

1 μM Gd plus 20 μM carbenoxolone for 20 min prior to assess the effect of these combination of chemical in both the basal and CMD-induced extracellular ATP secretion. A stock solution of 1 mM Gd chloride was prepared in distilled water and diluted in Tyrode-HEPES buffer for ECs application. The preparation of the carbenoxolone solution was detailed in a previous section.

b) *Lanthanum*: This trivalent ion blocks in a non-selective manner a variety of channels related to calcium influx. A 8 mM stock solution of lanthanum chloride was prepared in distilled water and diluted to 200 μM using Tyrode-HEPES buffer. ECs were incubated for 10 min prior to basal or CMD-induced extracellular ATP secretion.

IV. Role of thrombin and related PARs agonists Since ECs are continually in contact with blood, we examined whether thrombin and PAR agonist peptides [41] participate in extracellular ATP release mechanism. We assessed the effect of PAR agonists and antagonists in ECs with and without mechanical stimulation.

a) *Thrombin*: A stock 100 U/mL in physiological saline was diluted in Tyrode-HEPES solution. ECs were incubated for 1 min with thrombin (0.1–1 U) prior to CMD stimulation; cell media samples were collected 1, 3, 5, 10, and 15 min after the stimulus to assay ATP/metabolites in control ECs or ECs subjected to CMD stimulation.

b) *PAR_1-PAR_2 agonist peptide SFLLRN* (10–100 μM) were incubated during 1 min prior to CMD; samples were collected 1, 3, 5, 10, and 15 min after the stimulus to assay ATP/metabolites. A 1.3 mM stock solution was prepared in distilled water and diluted accordingly in Tyrode-HEPES buffer.

c) *PAR_1 agonist peptide TFLLRN* (10–100 μM) were incubated during 1 min prior to CMD; samples were collected 1, 3, 5, 10, and 15 min after the stimulus to assay ATP/metabolites. A 1.3 mM stock of this peptide was prepared in distilled water and diluted in Tyrode-HEPES to attain the desired concentration.

d) *AFLARAA*, *a putative PAR_1 antagonist*: ECs were treated for 10 min with 50 μM AFLARAA, next 10 μM SFLLRN was added for 1 min prior to CMD. Samples to assess ATP/metabolite were collected 1 min after the stimulus. A 1.3 mM stock solution was dissolved in distilled water and diluted in Tyrode-HEPES buffer.

e) *Carbenoxolone plus thrombin*: To examine as whether the rise in extracellular ATP elicited by thrombin was hemichannel-mediated, ECs were incubated with both 20 μM carbenoxolone for 20 min plus 0.3 U/mL thrombin added the last min of the incubation, prior to the CMD stimulation. Controls assessed the effect of this treatment on the spontaneous extracellular ATP release.

f) *Rho signaling cascade*: To assess the participation of Rho kinase in the intracellular signaling of thrombin, in both the spontaneous as well as the CMD-evoked extracellular ATP secretion, two chemicals were used to inhibit this enzyme, as detailed by Ikenoya et al. and Lingor et al. [42, 43]: (i) H1152P: ECs were treated with 0.01, 0.1, 1 μM H1152P, for 120 min prior to CMD. A stock 2.5 mM solution was prepared in distilled water and later diluted in Tyrode-HEPES buffer. Control evaluated the effect of this compound in the basal extracellular ATP release. (ii) Y-27632: ECs were treated with 0.01, 0.1, 1, 10 μM Y-27632, during 120 min prior to CMD. A 1.3 mM stock solution was dissolved in distilled water and diluted accordingly in Tyrode-HEPES buffer. Control evaluated the effect of this compound in the basal extracellular ATP release.

Lowering the cell membrane cholesterol content Finally, in an effort to clarify whether the spontaneous or CMD-induced ATP release mechanism are related to the membrane cholesterol content or the targets related to the ATP mobilization are localized in lipid rafts, ECs were incubated with Tyrode-HEPES buffer supplemented with 2.5 mM methyl-β-cyclodextrin (MβCD) for 120 min [44]. After this incubation period, cell media was removed and replaced by Tyrode-HEPES buffer; 20 min later, the CMD protocol was applied. Cholesterol content was assayed as described by Norambuena et al. [45]. Parallel controls examined the basal, spontaneous, ATP release.

Analytical procedures to assay ATP/metabolites

ATP and its metabolites present in the ECs media sampled were derivatized by chemical reaction with chloroacetaldehyde to form the corresponding fluorescent purine etheno adducts. A 200 μL aliquot of the cell media was used to react with sample purines to yield the corresponding 1,N6-etheno fluorescent species, by adding 100 μL buffer phosphate-citrate (pH = 4) plus 10 μL of chloro-acetaldehyde. Samples were heated for 40 min at 80 °C [46, 47]. The chemical reaction was stopped by ice incubation for 5 min. After 24 h at 4 °C, an automated Merck-Hitachi HPLC apparatus equipped with fluorescence detector identified and quantified the ethene purines, at excitation and emission wavelengths of 233 and 415 nM, respectively. A 20 μL aliquot of each sample was injected into a Chromolith HPLC column equilibrated with the mobile phase at a flow of 1.5 mL/min (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 5 mM tetrabutylammonium, pH = 6). A calibration curve was performed daily; linearity was routinely attained between 0.0012 and 10 pmol/20 μL purines.

Drugs and chemicals used

Adenosine 5′ triphosphate as the trisodium salt and its metabolic degradation products as the sodium salts, apyrase, carbenoxolone disodium salt, methyl-β-cyclodextrin, 18-β-glycyrrhetinic acid, probenecid, gadolinium III chloride hexahydrate, lanthanum III chloride heptahydrate, ruthenium red (ammoniated ruthenium oxychloride), brefeldin A, monensin, *N*-ethylmaleimide, nocodazole, and Evans blue were obtained from Sigma Chemicals (St. Louis, Mo, USA); 2-APB, H-1152P, and Y-27632 were obtained from Calbiochem (Merck Millipore); HC 067047 were obtained from Tocris (a bio-techne brand); and thrombin-JMI was obtained from Pfizer (New York, USA). SFLLRN, TFLLRN, and AFLARAA were obtained from ANASPEC. The chemicals used to prepare buffers and the mobile chromatography phases were chromatographic grade and were purchased from Merck Chemicals (Darmstadt, Germany).

Expression of results, statistical analysis, and significance level setting

Results are expressed essentially in three different ways: (i) absolute values of ATP/metabolites found in the well cell media (pmoles/mg protein); (ii) as a percentage of each internal control, which could refer to the spontaneous value or the CMD-evoked value, a value exclusive of each protocol; and (iii) as a ratio of the CMD-evoked secretion/basal values, or treated over non-treated values, which permitted calculating the fold increase elicited by CMD and drug treatments.

For statistical analysis, the STATA 14.0 program, Texas, USA, was routinely used. Normal data distribution was determined by the Shapiro-Wilk method. To compare two sets of data, Student *t* test or Mann-Whitney tests were used when appropriate. To compare more than two sets of data, analysis of variance (ANOVA) or Kruskal-Wallis assays were used. The level of significance was defined as $\alpha = 0.05$.

Results

Extracellular ATP release

a. *CMD*: gentle application of the CMD procedure elicited a transient rapid secretion of extracellular nucleotides; ATP peaked within 1 min decaying to basal values in 10–15 min, consonant with ectoATPase's hydrolysis. The time course of the released extracellular ATP is shown in Fig. 1a. The ATP peak is paralleled by an increase in basal ADP and AMP which over 10–15 min also decayed to basal values (data not shown). Extracellular basal and peak ATP/metabolites following CMD stimulation are summarized in Fig. 1b and Table 1. Adenosine levels did not change following CMD; basal ADO value is 12-fold larger than ATP (Table 1).

Consistent with nucleotide hydrolysis, parallel studies showed that cell media addition of 4 U/mL apyrase reduced both the basal ATP level (from 44 ± 2.9 to 0.6 ± 0.6 pmol/mg protein, $n = 10$, $p < 0.0001$, Mann-Whitney test) as well as the CMD-evoked ATP secretion (308 ± 70 to 17 ± 7 pmol/mg protein, $n = 9$, $p < 0.001$, unpaired Student *t* test). Considering biological variability, each experiment has its own control. Notwithstanding, the spontaneous ATP outflow averaged 72 ± 8 pmol/mg protein ($n = 94$); extracellular ATP released following 1 min after CMD, averaged 337 ± 22 pmol/mg protein ($n = 94$, $p < 0.0001$, Mann-Whitney test), which represents a 5 ± 0.3-fold increase in the extracellular ATP level ($n = 94$), values ranged from 1.17- to 14.3-fold. Out of the 94 separate replicas, only in one experiment the stimulation/basal ratio was 1.17, a result which based on out layer statistical criteria was discarded.

b. *Picospritzer puff*: To confirm whether another mechanical stimuli elicited a similar pattern of ATP release, we searched for a reproducible and researcher unbiased independent stimuli. We examined whether the delivery of a single buffer puff caused a comparable rise in extracellular ATP/metabolites. We consistently observed that this stimulus elicited a significant rise in extracellular ATP that peaked within 1 min and showed a time course similar in magnitude and duration as the CMD-evoked stimulation (Fig 1c, d). The picospritzer puff raised 6.8 ± 1-fold extracellular ATP ($n = 14$). On a comparative base, both the spontaneous and the puff-evoked extracellular ATP released were not significantly different from those achieved following CMD stimulation. In view of this similarity, all subsequent assays routinely used the CMD protocol.

I. Role vesicles in the ATP secretory process All four agents used to interrupt vesicular trafficking consistently resulted in a significant reduction of the spontaneous ATP secretion, while only two of them reduced the CMD-induced ATP secretion. Monensin elicited a significant 50% reduction in both the spontaneous as the CMD-induced ATP release (Fig. 2a, b). Basal extracellular ADP was also reduced 45 ± 13% ($n = 8$) while basal AMP and ADO did not change (data not shown). Moreover, following CMD, extracellular ADP, AMP, and ADO values were not changed by monensin (data not shown). Likewise, nocodazole significantly reduced both the spontaneous as well as the CMD-induced ATP secretion (Fig. 2c, d). Spontaneous extracellular ADP and ADO levels were not changed by nocodazole treatment, but AMP levels were

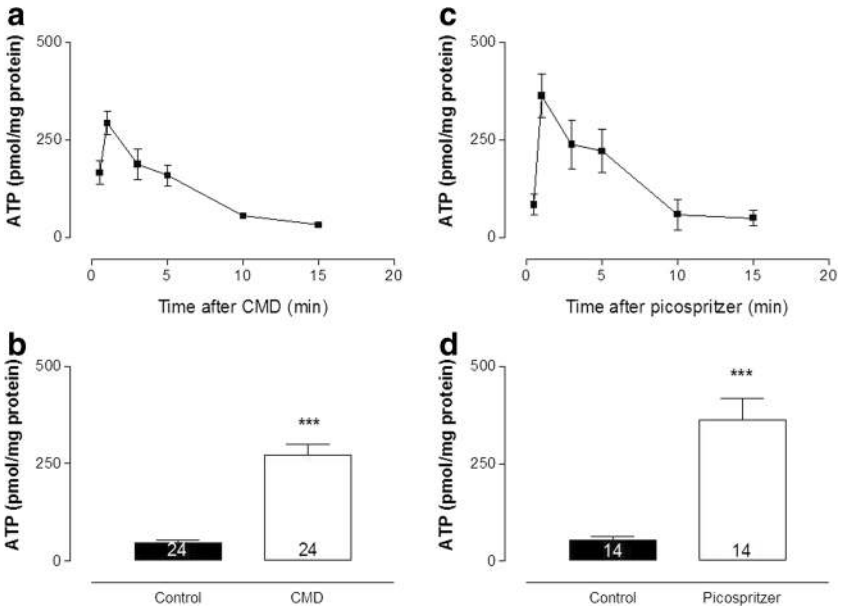

Fig. 1 Spontaneous and mechanically evoked extracellular released ATP in ECs. Comparison of the extracellular ATP released following cell media displacement (CMD) procedure (**a** and **b**) or a picospritzer puff (**c** and **d**). Black columns in **b** and **d** refer to the control, spontaneous (non-mechanical) extracellular ATP, while the white columns in **b** and **d** denote the mechanically evoked nucleotide released; ATP is expressed as pmol/mg protein. Columns represent mean average values, bars, SEM. ***$p < 0.001$, **b**: Mann-Whitney test, **d**: unpaired Student *t* test, numbers inside columns refer to the number of wells examined

reduced (55 ± 13 (n = 9) to 21 ± 9 (n = 12) pmol/mg protein, $p < 0.012$ Mann-Whitney test). Following CMD, extracellular ADP, AMP, and ADO were not changed (data not shown). In a next series of experiments, brefeldin A only inhibited the spontaneously ATP released while not affecting the CMD-evoked secretory component (Fig. 2e, f). Brefeldin A did not modify basal extracellular ADP in spontaneous or following CMD stimulation (data not shown), but increased the spontaneous and CMD-induced AMP secretion (spontaneous increase 1.6 ± 0.3 (n = 8)-fold and after CMD stimulus 1.9 ± 0.3 (n = 8)-fold) and spontaneous ADO level increase was 1.21 ± 0.07-fold (n = 8), and after CMD stimulus, 1.3 ± 0.07 (n = 8). In addition, treatment with 1 or 100 μM NEM reduced by 40 ± 9% (n = 15) and 56 ± 11% (n = 10), respectively, the spontaneous ATP secretion. The CMD-induced ATP secretion was only decreased by 100 μM NEM (31 ± 8%, n = 12). The spontaneous extracellular values of ADP, AMP, and ADO were not modified by 1 nor 100 μM NEM. Following CMD, ADP, and AMP values were not changed. However, the extracellular ADO values after CMD

Table 1 Comparison of the spontaneous and mechanically evoked ATP release and metabolites found in the cell media of cultured ECs maintained in media lacking extracellular calcium, following 30 μM 2-APB addition or both stimuli simultaneously. x ± SEM (pmol/mg protein)

		ATP	ADP	AMP	ADO
Control (18)	S	32 ± 7	235 ± 49	23 ± 6	385 ± 82
	CMD	159 ± 26***	483 ± 96*	64 ± 12**	267 ± 30
	Ratio CMD/S	4.4 ± 0.5	2.1 ± 0.3	3.9 ± 0.9	1.1 ± 0.2
0 Ca^{2+} (18)	S	115 ± 21††	299 ± 42	27 ± 6	795 ± 137††
	CMD	254 ± 66*	453 ± 68*‡	34 ± 6	1016 ± 1191‡‡‡
	Ratio CMD/S	2.3 ± 0.3§§§	1.5 ± 0.2	1.9 ± 0.9	1.2 ± 0.2
2-APB (12)	S	109 ± 20†††	507 ± 136†	67 ± 21†	341 ± 65
	CMD	194 ± 44	372 ± 55	76 ± 20	429 ± 90
	Ratio CMD/S	2.1 ± 0.6ϕϕ	1.3 ± 0.3	2.6 ± 0.9	1.2 ± 0.2
0 Ca^{2+} + 2-APB (12)	S	130 ± 39†	279 ± 46	49 ± 7††	375 ± 62
	CMD	331 ± 57**‡‡	386 ± 59	113 ± 22** ‡,	528 ± 88‡‡
	Ratio CMD/S	2.7 ± 0.5§	1.5 ± 0.2	2.3 ± 0.4	1.5 ± 0.2

Samples for extracellular purine determinations were determined 1 min after the CMD procedure. Numbers in parenthesis indicate the number of wells examined

S spontaneous or non-stimulated, *CMD* cellular medium displacement

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Mann-Whitney test, CMD compared to spontaneous for each condition. †$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$, Mann-Whitney test respect spontaneous control condition. ‡$p < 0.05$;‡‡$p < 0.01$; ‡‡‡$p < 0.001$, Mann-Whitney test respect to CMD control condition. §$p < 0.05$; §§$p < 0.01$; §§§$p < 0.001$, unpaired Student *t* test respect to control ratio. ϕϕ Mann-Whitney test respect to control ratio

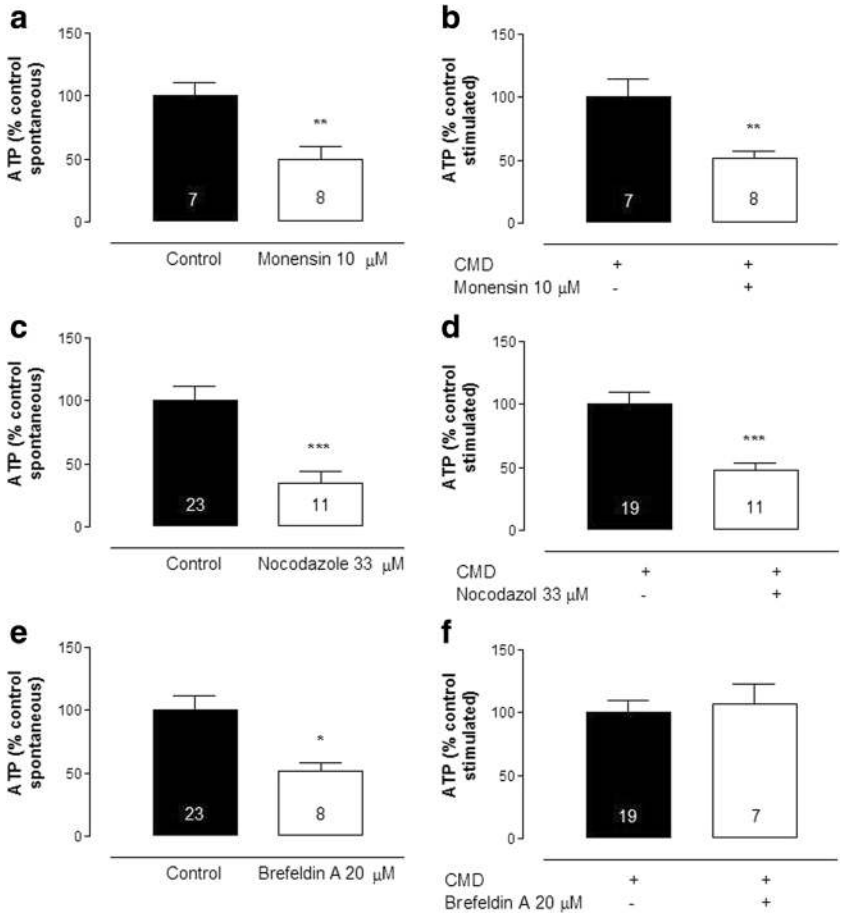

Fig. 2 Drug-induced interruption of the vesicle secretory pathway. Results are expressed as a percentage of the basal vehicle-treated spontaneous secretion (**a**, **c,** and **e**) and as a percentage vehicle treated 1 min after mechanical stimulation CMD (cell medium displacement) procedure (**b**, **d,** and **f**). Columns represent mean average values, bars, SEM. **a**, **c**, **d**, **e,** and **f**: unpaired Student *t* test, **b**: Mann-Whitney test. $*p < 0.05$; $**p < 0.01$; $***p < 0{,}001$. Numbers inside columns indicate the wells examined per drug treatment

increased 5.2 ± 0.8-fold (n = 13) and 9.3 ± 1.2 (n = 13)-fold with 1 and 100 μM NEM, respectively. VNUT participation was examined using Evans blue as a transporter blocker. At the concentrations examined, a significant reduction in the spontaneous ATP release was attained which did not appear concentration related since we observed a 69 ± 7% (n = 6), 63 ± 15% (n = 6), and 64 ± 16% (n = 8) inhibition with 3, 10, and 30 nM, respectively. The CMD-induced ATP secretion was not modified (data not shown). The spontaneous ADP or ADO values were not modified (data not shown), but the spontaneous extracellular AMP value was reduced from 31.7 ± 7 (n = 20) to 10.81 ± 4 (n = 6), 9.34 ± 3.1 (n = 6), and 10.4 ± 3.1 (n = 8) pmol/mg protein by 3, 10, and 30 nM Evans blue, respectively, ($p < 0.05$, Mann-Whitney test). Extracellular ADP, AMP, and ADO values after CMD stimulation were not modified by Evans blue (data not shown).

II. Role of connexins and/or pannexin1 channels in the ATP secretory process All three hemichannel blockers essentially reduced the CMD-induced ATP secretion (Fig. 3a–c) without significantly affecting the spontaneous nucleotide release (data not shown), an indication that these hemichannels are associated to the mechanically induced ATP secretory process. Basal and CMD-induced extracellular ADP, AMP, and ADO secretion was not statistically modified by 18 β-glycyrrhetinic acid (data not shown). The use of a more selective pannexin1 blocker showed that while 30 μM probenecid was inactive, whereas 100 μM reduced 70.5 ± 18% (n = 7) the CMD-induced ATP release (Fig. 3b), and 38 ± 6% (n = 7) extracellular ADP. AMP or ADP levels were not significantly changed (data not shown). Basal extracellular ADP, AMP, and ADO values were not statically modified (data not shown). To confirm previous results, while 5 μM carbenoxolone proved inactive, 20 μM significantly reduced by 49 ± 8% (n = 21) the CMD-induced ATP release (Fig. 3c) and reduced ADP, AMP, and ADO values (25 ± 10, 51 ± 9, and 18 ± 13% (n = 21), respectively). Spontaneous ADP was increased from 83 ± 11 (n = 20) to 135 ± 18 (n = 21) pmol/mg protein ($p < 0.0121$, Mann-Whitney test); ADO was increased from 244 ± 41 (n = 20) to 396 ± 60 pmol/mg protein (n = 21), $p < 0.05$ unpaired Student t test). Extracellular AMP values did not change (data not shown).

To further examine the role of pannexin1 channels in the CMD-induced extracellular ATP overflow, we used a set of $Panx1^{-/-}$ animals and their respective WT controls. Spontaneous extracellular overflow of ATP and related purines was not different when comparing ECs from WT and $Panx1^{-/-}$ mice (Fig. 4a, b, Table 2). Moreover, the relative

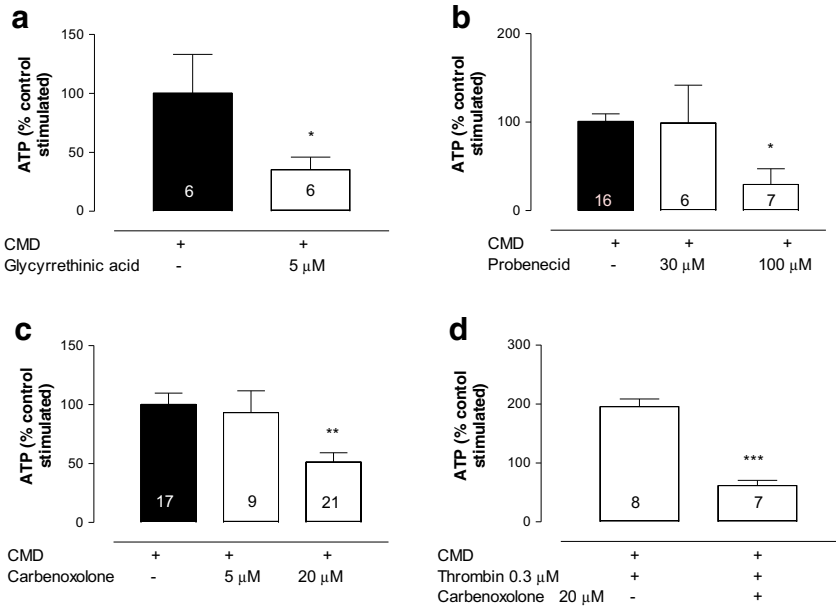

Fig. 3 Connexin and pannexin1 hemichannel blockade reduced the mechanically evoked extracellular ATP released in ECs. Black columns refer to the mechanical stimulation-induced extracellular ATP release in vehicles control; white columns indicate mechanically evoked extracellular ATP released 1 min after mechanical stimulation in the presence of hemichannel inhibitors or thrombin plus carbenexolone. Results are expressed as the % of control ATP released. CMD refers to the cell media displacement procedure. Bars denote the SEM. **a** Release of ATP elicited by CMD in the absence and in the presence of 5 μM 18 β-glycyrrhetinic acid. **b** In the presence of 30 and 100 μM probenecid. **c** In the presence of 5 or 20 μM carbenoxolone. **d** The simultaneous addition of 0.3 U/mL thrombin plus 20 μM carbenoxolone; note that thrombin increased two-fold the control ATP released. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Unpaired Student *t* test for panels **a** and **d**, while the Dunnett's Multiple Comparison Test was used for panels **b** and **c**. Numbers inside columns refer to the number of wells examined per protocol

proportion of purines in the spontaneous overflow differed markedly from those attained in rat ECs. In the case of ECs from these WT mice, AMP values were 3.2 times larger than that those of ATP (Table 2, $p < 0.0071$, Mann-Whitney test); while the ADO values were the smallest and did not differ from those of ATP, an indication that mice ECs must metabolize differently extracellular ATP as compared to rats. In addition, ECs incubation with 100 μM probenecid did not modify the spontaneous ATP overflow neither in WT nor in the Panx1$^{-/-}$ (Fig. 4a, b). CMD induced a 1.67-fold increase in extracellular ATP in WT, whereas in the Panx1$^{-/-}$ ECs, the increase was only 1.14 (Table 2). Moreover, in WT ECs, 100 μM probenecid treatment significantly reduced 41% the CMD-induced ATP overflow (Fig. 4c), a value slightly lower than caused by 100 μM probenecid in rat ECs, an indication that other targets may not be overlooked. In the Panx1$^{-/-}$ ECs, 100 μM probenecid did not modify the CMD-induced ATP overflow (Fig. 4d).

Reduction of extracellular calcium ECs maintained in media lacking extracellular calcium increased the spontaneous extracellular value of ATP, ADP, and ADO (Table 1); extracellular ATP in this set of experiments was 2.7 ± 05-fold higher than parallel controls maintained in media with 2.5 mM external calcium (Fig. 5a). Following CMD stimulation, extracellular ATP was not modified by the lack of calcium (Fig. 5b), while ADP and ADO values were increased (Table 1).

III. Participation of TRPV and mechanosensors and/or stretch-activated channels *TRPV channels* 2-APB was used as a TRPV agonist, ruthenium red as a TRPV blocking agent, and HC 06747 as a selective TRPV4 antagonist to assess the participation of TRPV channels in the spontaneous and CMD-induced ATP release. 2-APB raised spontaneous ATP values (Fig. 5c, Table 1) and ADP values while reducing CMD-induced ATP release (Fig. 5d and Table 1). In an additional protocol, 2-APB incubated in cell media lacking external calcium showed a rise in basal ATP and ADP (Table 1), similar in value to that observed with each treatment alone; no increment in extracellular ADO was observed as shown with the sole 2-APB treatment. Following CMD, extracellular ADP and ADO increased similarly to that observed in medium lacking external calcium (Table 1). Treatment with 30–100 μM ruthenium red did not modify the basal, control, nor the CMD-induced ATP secretion or cell media found metabolites (data not shown); however, 130 μM decreased the spontaneous extracellular ATP, ADP, and AMP values (76 ± 8, 64 ± 11 74 ± 16%, $n = 6$, respectively), while augmented spontaneous extracellular ADO 2.1 ± 0.5-fold ($n = 6$). Following CMD, extracellular values of ATP, ADP, and AMP were not modified by 130 μM ruthenium

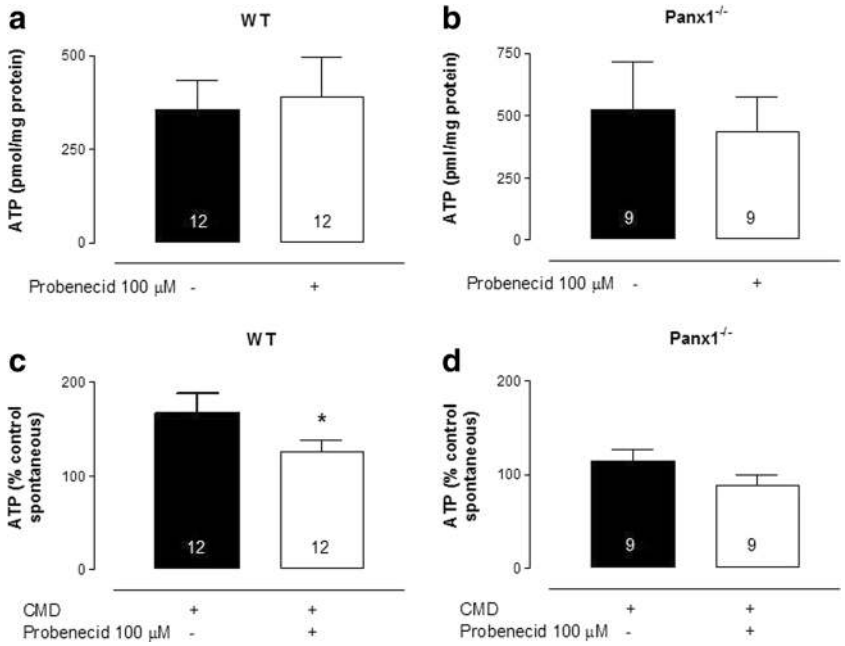

Fig. 4 Endothelial cells from Panx1$^{-/-}$ but not WT mice are resistant to probenecid-induced blockade of ATP overflow. **a** Black column refers to the control, spontaneous ATP secretion while the white column denotes spontaneous extracellular nucleotide observed after ECs incubation in buffer containing 100 μM probenecid. **b** Spontaneous ATP overflow in cells from Panx1$^{-/-}$ mice with and without probenecid treatment. **c** ATP overflow from WT cells 1 min following CMD-induced procedure with and without probenecid treatment. **d** ATP overflow 1 min following CMD in Panx1$^{-/-}$ cells with and without probenecid treatment. **c**, **d** Results are expressed as percentage of the spontaneous released. Bars refer to the SEM; number inside columns represent the replicas performed per group of cells examined. *$p < 0.05$ unpaired Student t test

red, while ADO significantly increased 2.04 ± 0.35-fold ($n = 6$). In a separate set of protocols, we examined whether blockade of a TRPV4 channel modified basal or CMD-induced nucleotide secretion; 0.3 μM HC 067047 decreased spontaneous ATP release from 68.6 ± 6 ($n = 12$) to 43 ± 6 ($n = 9$) pmol/mg protein, $p < 0.01$, unpaired Student t test. Increasing antagonist concentration to 1 μM, further decreased extracellular ATP to 32 ± 8 ($n = 9$) pmol/mg protein, $p < 0.01$, unpaired Student t test. This blocker did not modify the spontaneous extracellular levels of ADP, AMP, or ADO (data not shown).

HC 067047 did not modify CMD-induced secretion of ATP, ADP, AMP, or ADO (data not shown).

Mechanosensors and/or stretch-activated channels

To examine the involvement of Gd-sensitive mechanosensors, in basal and CMD-induced ATP secretion, the effect of 1–100 μM Gd was tested. Spontaneous release of ATP/metabolites was not modified (Table 3), while increasing Gd resulted in a concentration-dependent reduction of CMD-induced extracellular ATP (Fig. 6a). Following the CMD

Table 2 Spontaneous and mechanically evoked ATP/metabolites overflow in the cell media of cultured ECs from wild type (WT) and Panx1$^{-/-}$ mice. x ± SEM (pmol/mg protein)

		ATP	ADP	AMP	ADO
WT mice (12)	S	355 ± 79	825 ± 201	1155 ± 254	283 ± 63
	CMD	591 ± 140	988 ± 278	1350 ± 319	551 ± 168
	Ratio CMD/S	1.67 ± 0.21	1.16 ± 0.12	1.11 ± 0.1	1.61 ± 0.27
Panx1$^{-/-}$ mice (9)	S	523 ± 193	895 ± 220	1681 ± 599	349+ ± 127
	CMD	640 ± 239	770 ± 147	1100 ± 221	161 ± 40
	Ratio CMD/S	1.14 ± 0.12§	0.9 ± 0.19	1.17 ± 0.25	0.86 ± 0.23§

Samples following CMD were collected 1 min after the CMD procedure. Twelve separate wells derived from six WT mice and nine separate wells from 4 Panx1$^{-/-}$ mice

S spontaneous or non-stimulated, *CMD* cellular medium displacement

§$p < 0.05$ (Mann-Whitney test respect to WT mice ratio for ATP and non-paired Student t test respect WT mice ratio for ADO)

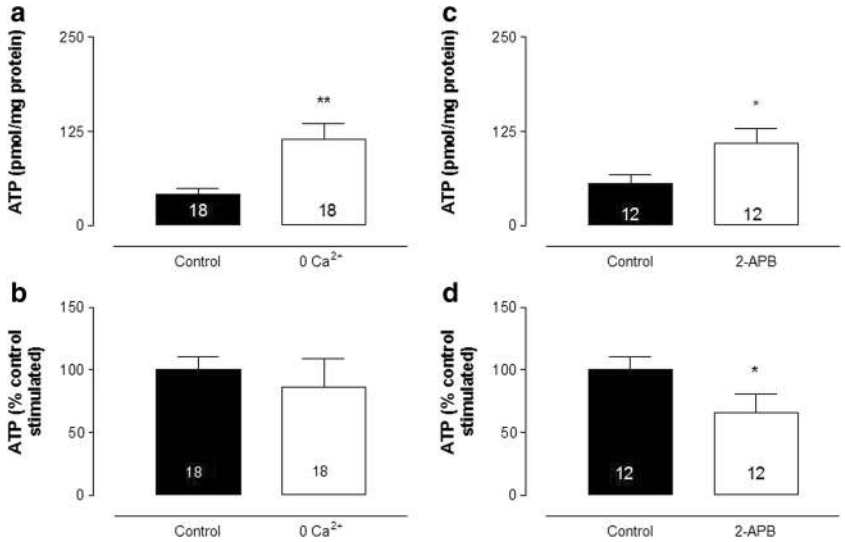

Fig. 5 Spontaneous and mechanically evoked release of extracellular ATP in ECs maintained in buffer lacking external calcium. **a** Black column refers to the control, spontaneous ATP secretion, while the white column denotes spontaneous extracellular nucleotide observed after ECs incubation in buffer without external calcium (0 Ca^{2+}). **b** Black column indicates ATP released by ECs cell media displacement (CMD) in control and in white column ATP released in buffer without external calcium (0 Ca^{2+}), results are expressed as percentage of the control released by CMD. **c** Black column refers to the control, spontaneous ATP secretion, while the white columns denote the spontaneous extracellular nucleotide observed after cell incubation in buffer added with 30 μM 2-APB. **d** Black column denote ATP released by CMD in control and in white column ATP released in buffer with 30 μM 2-APB, expressed as percentage of the control released by this stimulus. **a** $^{**}p < 0.01$, unpaired Student *t* test; **c** and **d**: $^{*}p < 0.05$ Mann-Whitney test. Bars indicate the SEM. Numbers inside columns refer to the number of wells examined per protocol

stimulus, extracellular ADP decreased concentration dependently by 38 ± 8% (n = 18), 39 ± 5% (n = 6), and 76 ± 3% (n = 6) by 1, 10, and 100 μM Gd, respectively. Likewise, the extracellular AMP secretion after CMD was decreased in 55.3 ± 6% (n = 18), 89 ± 4% (n = 6), 83 ± 8% (n = 6) by 1, 10, and 100 μM Gd, respectively. Following CMD stimulus, extracellular ADO levels were not changed. Moreover, in a separate protocol, we assessed the simultaneous blockade of hemichannels plus mechanosensors, by examining the effect elicited by the joint application of

Table 3 Gadolinium III (Gd) plus carbenoxolone do not further reduce the extracellular ATP released by mechanical stimulation of ECs. x ± SEM (pmol/mg protein)

		ATP	ADP	AMP	ADO
Control (12)	S	71 ± 26	77 ± 11	12 ± 3	322 ± 52
	CMD	470 ± 65***	256 ± 31***	69 ± 12***	354 ± 66
	Ratio CMD/S	7.9 ± 1.2	3.5 ± 0.4	6.9 ± 1.3	1 ± 0.1
1 μM Gd (12)	S	85 ± 37	105 ± 15	17 ± 5	471 ± 108
	CMD	341 ± 36***‡	179 ± 33*	34 ± 7**‡‡	380 ± 72
	Ratio CMD/S	4.6 ± 0.8§	1.6 ± 0.2ϕϕϕ	2.2 ± 0.3§§§	0.7 ± 0.1
20 μM carbenoxolone (12)	S	72 ± 21	149 ± 21††	23 ± 8	506 ± 78†
	CMD	244 ± 34***‡‡	190 ± 29	38 ± 5‡	287 ± 51*
	Ratio CMD/S	3.4 ± 0.4§§§	1.2 ± 0.1§§§	2.1 ± 0.3§§§	0.6 ± 0.06 ϕϕ
Gd + carbenoxolone (12)	S	43 ± 17	121 ± 30	18 ± 8	563 ± 116†
	CMD	291 ± 44###£	196 ± 29#	50 ± 5**	299 ± 17#
	Ratio CMD/S	5.5 ± 0.5§	2 ± 0.3§§	3.1 ± 0.6§§	0.8 ± 0.2

Numbers in parenthesis indicate the number of wells examined

S spontaneous or non-stimulated, *CMD* cellular medium displacement

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, Mann-Whitney test CMD respect to spontaneous for each condition. #$p < 0.05$; ###$p < 0.001$, Mann-Whitney test CMD respect to spontaneous for each condition. †$p < 0.05$; ††$p < 0.01$, Mann-Whitney test respect to spontaneous control condition. ‡$p < 0.05$; ‡‡$p < 0.01$, Mann-Whitney test respect to CMD control condition. £$p < 0.05$, Student *t* test respect to CMD control condition. §$p < 0.05$; §§$p < 0.01$; §§§$p < 0.001$, unpaired Student *t* test respect to control ratio. ϕϕ$p < 0.01$; ϕϕϕ$p < 0.001$ Mann-Whitney test respect to control ratio

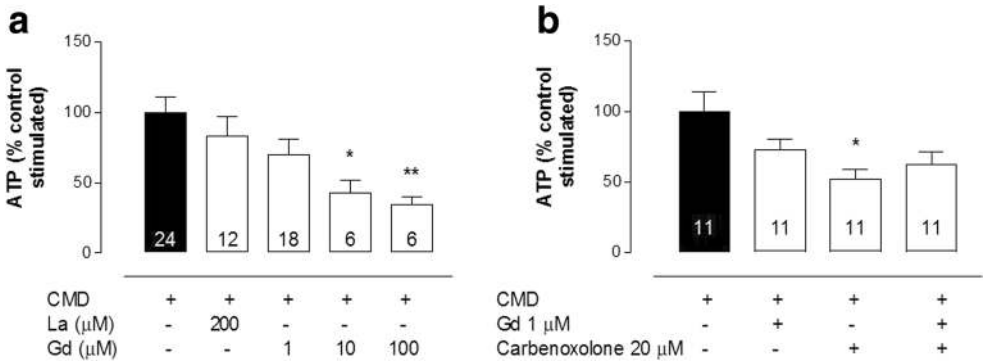

Fig. 6 Gd, a mechanoreceptor inhibitor reduced the extracellular ATP released elicited by mechanical stimulation of ECs. Black columns refer to the average ATP released by CMD (cell media displacement) procedure; results are expressed as the % of ATP released. White columns denote the mechanically evoked ATP released to the extracellular media in: **a** lanthanum (La) or Gd, alleged piezol and two and related mechanosensor channel inhibitors. Dunn's test, Kruskal Wallis ANOVA, $*p < 0.05$; $**p < 0.01$. **b** Gd plus carbenoxolone. Dunn's test, Kruskal Wallis ANOVA. $*p < 0.05$. All comparisons are with regard to the internal control values. Bars denote the SEM. Numbers inside columns refer to the number of wells examined

1 μM Gd plus 20 μM carbenoxolone. Much to our surprise, no additive effects on the spontaneous or the CMD-induced extracellular ATP release was observed (Fig. 6b, Table 3). Moreover, when carbenoxolone was applied either alone or together with Gd, it increased the spontaneous ADO levels (Table 3). Lanthanum was chosen as a control of trivalent cation specificity; 200 μM Lanthanum did not modify neither the spontaneous nor the CMD-induced extracellular ATP/metabolites secretion (Fig. 6a) or its extracellular metabolites (data not shown).

IV. Role of thrombin and related PARs agonists PAR agonists, apparently through PAR_2 activation, facilitate the release of extracellular ATP elicited following CMD stimulation without affecting spontaneous extracellular ATP values. The addition of 0.3 U/mL thrombin (PAR_1-PAR_2 agonist) increased CMD-induced extracellular ATP release (Fig. 7a), without eliciting a significant change in the spontaneous extracellular ATP/metabolites. Moreover, extracellular ADP and ADO also increased following CMD by 192 ± 17 and 239 ± 34% ($n = 8$ for each), respectively. ATP/metabolites rose accordingly and

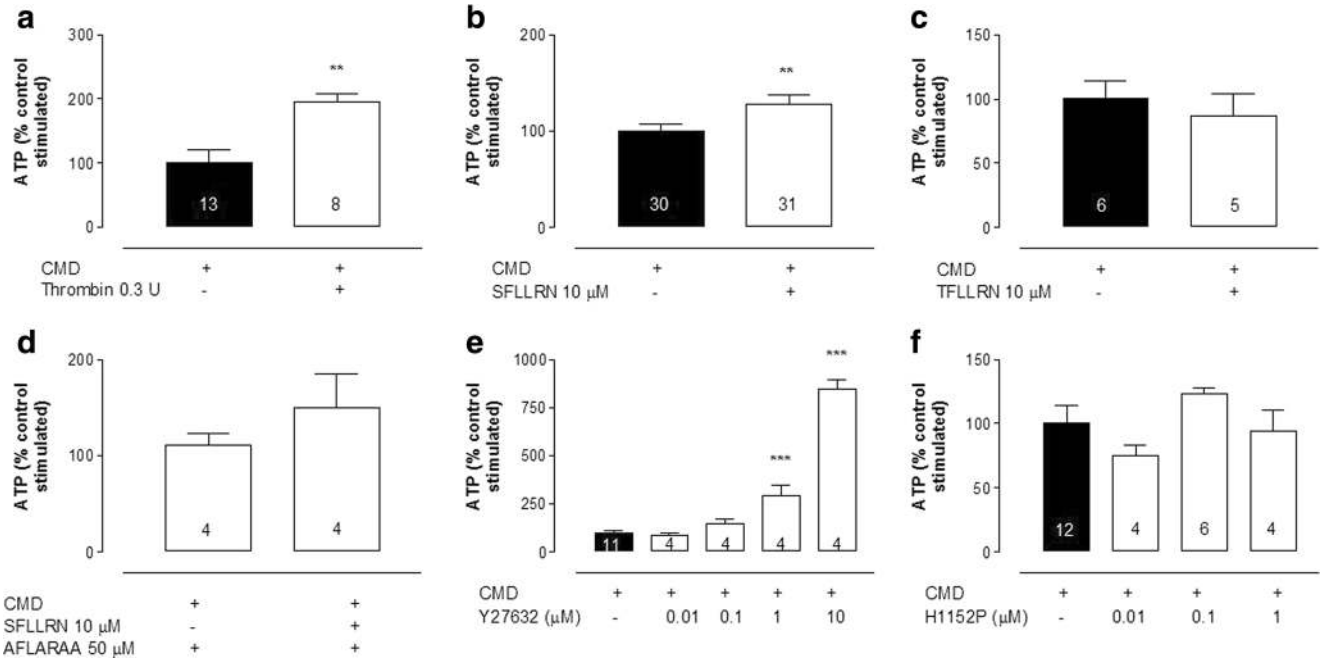

Fig. 7 Mechanically induced extracellular ATP released was increased by thrombin and a related PAR-agonist, and the Rho kinase inhibitor Y27632 in ECs. Black columns refer to the ATP released by CMD (cell media displacement) procedure and expressed as the % of control values. White columns denote the mechanically evoked nucleotide released in the presence of either 0.3 U/mL thrombin (**a**), PAR agonists SFLLRN and TFLLRN, respectively (**b** and **c**), the PAR antagonists AFLARAA (**d**) or two Rho kinase inhibitors, Y27632 (**e**) or H-1152P (**f**). Bars denote the SEM **a**, **b** $**p < 0.01$ unpaired Student *t* test; **e** $***p < 0.001$ Dunnett's Multiple Comparison Test. Number inside columns refer to the number of wells examined

decayed to basal values, within 10–15 min as also observed in non-treated ECs (data not shown). The CMD-induced extracellular ATP rise was mimicked, although to a lesser extent, by the synthetic peptide SFLLRN (Fig. 7b), a claimed PAR_1-PAR_2 agonist. In contrast, TFLLRN, a PAR_1 agonist, did not modify the extracellular ATP release following CMD (Fig. 7c), suggesting PAR_2 activation. Consistently, the PAR_1 antagonist, AFLARAA, did not modify per se the spontaneous nor the CMD-induced ATP secretion (data not shown), nor antagonized the effect of SFLLRN on CMD-induced extracellular ATP secretion (Fig. 7d), strengthening the proposal of PAR_2 involvement. In addition, carbenoxolone reduced the CMD-induced ATP release elicited in the presence of 0.3 U/mL of thrombin (Fig. 3d), a result compatible with the notion that the rise in extracellular ATP elicited by PAR agonism is mediated by pannexin1 channel activity. In an effort to assess whether Rho signaling participates in the thrombin signaling, two compounds were used as enzyme blockers: (i) Y27632, which per se did not modify the spontaneous extracellular ATP/metabolites values in absence or presence of 0.1–10 μM SFLLRN (data not shown). Notwithstanding, CMD stimulation elicited a concentration-dependent rise in extracellular ATP/metabolites, reaching with 10 μM Y27632, an 8.4-fold increase over the control CMD procedure (Fig. 7e and Table 4). To clarify if the Rho-kinase pathway participates in the PAR CMD-induced increase in ATP release, 10 μM SFLLRN was co-applied together with 0.01–10 μM Y27632; 0.01 μM Y27632, a concentration of inhibitor that did not modify per se the ATP released, did not change the CMD-induced ATP secretion as compared to SFLLRN alone (Table 4). (ii) H1152P, a more selective Rho-kinase inhibitor than Y27632, did not modify the spontaneous extracellular ATP nor extracellular nucleotide metabolites (data not shown) nor the CMD-induced ATP release in the absence (Fig. 7f) or presence of 10 μM SFLLRN (data not shown). The study proposed to assess that intracellular Rho kinase signaling is not conclusive based on antagonist specificity and the large extracellular rise in ATP elicited by CMD-stimulation.

Lowering the cell membrane cholesterol MβCD induced a 52.7 ± 12% (n = 5) reduction of the membrane cholesterol content. Treatment caused a 4 ± 0.6-fold increase in basal extracellular ATP from 41 ± 15 (n = 12) to 166 ± 24 (n = 12) pmol/mg protein, $p < 0.0001$ Mann-Whitney test). Likewise, extracellular ADP increased significantly 3 ± 0.4-fold (n = 12), AMP increased 5.6 ± 2-fold (n = 12) while ADO values were not modified (data not shown). Following CMD stimulation, extracellular ATP increased 5-fold, from 251 ± 63 (n = 12) to 1026 ± 267 pmol/mg protein (n = 12, $p < 0.0029$ Mann-Whitney test), ADP increased 2.9 ± 0.3-fold (n = 12), AMP increased 2.5 ± 0.4-fold (n = 12) while ADO values were not modified, (data not shown). These results allow discarding non-specific cell membrane disorder since extracellular ADO was not altered and nucleotides increased in the same proportions.

Discussion

Primary in vitro cultures of ECs consistently reveal a spontaneous, basal, extracellular ATP secretion and react to mechanical stimulation by further increasing extracellular ATP/metabolites. In response to mechanical stimulation, we routinely quantified a characteristic rapid and transient increase in extracellular ATP of short-lived nature. The ATP surge is reduced by ecto-ATPase hydrolysis to ADP, AMP, and ADO as evidence by kinetic analysis. ATP and metabolites are

Table 4 Y 27632, a Rho-kinase inhibitor increased extracellular ATP released and metabolites found in the cultured ECs media. x ± SEM of % of control CMD

		ATP	ADP	AMP	ADO
Control (12)		100 ± 14	100 ± 12	100 ± 13	100 ± 5
Y27632(4)	0.01 μM	91 ± 12	122 ± 12	132 ± 22	67 ± 1‡‡‡
	0.1 μM	149 ± 19‡	142 ± 21	86 ± 24	75 ± 4‡‡
	1 μM	288 ± 60‡‡‡	253 ± 33‡‡‡	253 ± 53‡‡‡	120 ± 6‡
	10 μM	844 ± 48‡‡‡	486 ± 42‡‡‡	318 ± 82‡‡‡	245 ± 22‡‡‡
SFLLRN (12)	10 μM	135 ± 14‡	127 ± 9‡	112 ± 14	98 ± 5
Y27632 + 10 μM SFLLRN (4)	0.01 μM	161 ± 9‡##	177 ± 21‡‡#	163 ± 27‡	78 ± 7‡
	0.1 μM	257 ± 13‡‡‡##	284 ± 22‡‡‡##	276 ± 22‡‡‡###	83 ± 5 ‡
	1 μM	218 ± 11‡‡‡	253 ± 15‡‡‡	201 ± 8‡‡‡	134 ± 14‡‡
	10 μM	710 ± 63‡‡‡	466 ± 42‡‡‡	428 ± 41‡‡‡	221 ± 21‡‡‡,

Numbers in parenthesis indicate the number of wells examined

‡$p < 0.05$; ‡‡$p < 0.01$; ‡‡‡$p < 0.001$, unpaired Student t test respect to CMD control condition. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$, unpaired Student t test respect to CMD in the presence of the respective concentration of Y27632 alone

relevant to endothelium-smooth muscle signaling; ATP/ADP are P2Y receptor ligand which induce NO release, while ADO activates its own smooth muscle set of receptors with characteristic vasodilator effects of clinical relevance. ECs from both rats and mice respond to mechanical stimulation by triggering a signaling cascade that results in increased extracellular ATP overflow which ultimately leads to NO synthesis [48, 49], a chemical intimately related to vascular tone regulation and vascular biology integrity. Although other authors reported that mechanical stimuli cause extracellular ATP release in different cell types [50], we aimed at dissecting pharmacologically the cellular bases inherent to the spontaneous versus the mechanically stimulated ATP release process. Two main conclusions derive from the present findings: (i) the spontaneous, release of extracellular ATP/metabolites relies principally on vesicular secretion, while the CMD-stimulated secretory component is based partly on connexin and/or pannexin1 hemichannel activity as consistently supported by the ECs derived from the Panx1$^{-/-}$ knockout rodents. (ii) In support of the physiological significance of the secretory mechanism, present findings show that TRPV receptors expressed in ECs modulate preferentially the non-mechanical triggered ATP release, while thrombin through PAR2 modulate exclusively the mechanically induced component of ATP secretion. A graphical abstract and the conceptual bases of our proposal is illustrated in Fig. 8; highlighting our conclusions which are extensively discussed in the next paragraphs.

As already indicated, the basal, non-mechanical ATP release, operates spontaneously and depends primarily on vesicular release; interruption at different stages of the vesicular export route concurs to demonstrate over 50% blockade of this extracellular ATP release component not necessarily affecting the mechanically induced secretory process; 1 μM NEM, consistently showed maximal inhibition of the basal ATP secretion component through the inactivation of NEM-Sensitive Factor (NSF) implicated in a wide range of membrane fusion steps in the secretory and endocytic cell pathways [51]. In keeping with the Bodin and Burnstock original research [52], we repeated experiments using NEM and monensin, but examined vesicular traffic blockers including brefeldin A and nocodazole, two well-accepted secretory route blockers. In conjunction with NEM, nocodazole and Brefeldin A also reduced the basal ATP secretion. Care was exercised to follow attentively protocols reporting the use of these agents, to bypass the role of preformed vesicles. Furthermore, we examined the role of the vesicular nucleotide transporter (VNUT) described by Sawada et al. [15]. Evans blue was used as a prototype blocker described to inhibit with nanomolar potency vesicular ATP transport [15]. ECs vesicles proved sensitive to 3–30 nM Evans blue, allowing us to suggest that VNUT expression in ECs and its functional role necessary for vesicular ATP accumulation. Relevant to the present findings, ATP containing vesicles were identified in HUVECs, using quinacrine to fluorescently label the ATP content [14]. Weibel-Palade bodies were also observed in HUVECs, a cargo that contains von Willenbrand factor. In these same cells, nocodazole reduced in vitro von Willenbrand secretion [53] a finding similar to the present results, allowing the proposal that ATP may be stored in Weibel-Palade bodies. Consistent with the proposal that the spontaneous ATP overflow relies predominantly on vesicular secretion, Panx1$^{-/-}$ mice showed that the spontaneous overflow was not altered in ECs from these rodents.

Continuing with the mechanism underlying basal ATP release, we examined the contribution of TRP channels to the basal ATP secretory process. Considering TRP subtypes, we relied on pharmacological characterization. Consistent with Hu et al. [54], 2-APB is a TRPV receptor agonist which in

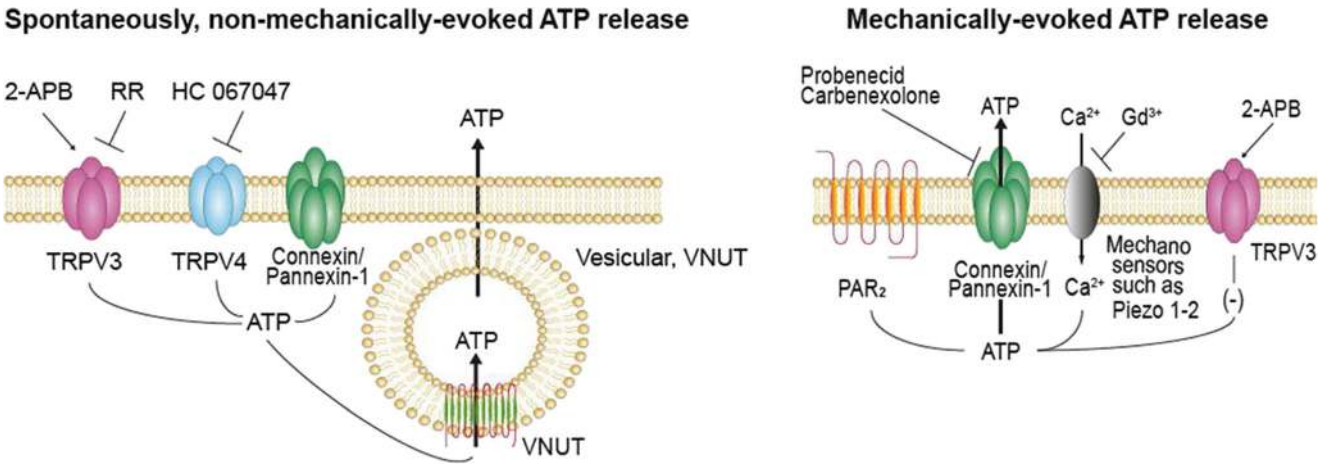

Fig. 8 Working model cartoon showing salient features of the spontaneous (left) and mechanically induced ATP secretory process (right). While the basal, spontaneous released relies on vesicular ATP that express a vesicular nucleotide transporter (VNUT) and is modulated by TRPV channels, the mechanically evoked ATP secretion is essentially based on connexin and pannexin1 hemichannels and is modulated by PAR_2 receptor activity. *RR,* ruthenium red, *TRPV* transient receptor potential subtype V3 and V4. *RR*, HC 067047 and Gd^{3+} are inhibitors. 2-APB is an alleged TRPV3 agonist, while HC 067047 a selective TRPV4 antagonist

our experiments doubled basal extracellular ATP values while slightly reducing CMD-induced ATP secretion. This sole observation allowed us to further distinguish the spontaneous from the CMD-operated ATP release as illustrated in Fig. 8 cartoon. Based on the 2-APB concentration used, we deduce that 2-APB activates TRPV3 channels in ECs [33, 36, 55]. Confirming the notion that ECs express TRPV4, it became challenging to examine whether an antagonist such as HC 067047 modifies ATP secretion. Present results show that TRPV4 antagonism lowered basal ATP release without modifying the CMD-evoked secretory component. Based on the 2-APB plus HC 067047 results, we infer the participation of TRPV3 and TRPV4 in basal ATP secretion, suggesting that an as yet non-determined stimuli triggers a TRPV tone participating in basal ATP secretion. In further support of a putative TRPV3/4 tone, Pires et al. and Seki et al. recently reported that TRPV3/4 allows calcium entrance in cerebral and mesenteric endothelial cells, respectively, leading to vasodilatation mediated by hyperpolarizing factor release [34, 56]. Notwithstanding, we are aware that 2-APB targets other proteins which may help to account for the slight decrease in CMD-evoked ATP release. For example, 2-APB inhibits the entry of capacitive calcium to ECs [57]. Moreover, and in agreement with TRPV participation, ruthenium red [58], another alleged and unspecific TRPV antagonist also reduced exclusively the spontaneous ATP level release, further supporting our proposal illustrated in Fig. 8.

Moreover, we also noticed that lowering cell media calcium resulted in a significant rise in spontaneous extracellular ATP while the CMD-stimulated ATP secretion was not modified. The reduction in extracellular calcium is sensed by ECs which respond eliciting calcium waves mediated by connexin hemichannel activity associated with ATP release [30–32]. It has not escaped our attention that increased calcium waves might also favor vesicular exocytosis, hypothesis that remains to be examined. A repeated observation showed that ECs incubated under reduced extracellular calcium, markedly increased extracellular ADO, as was also observed in spinal astrocytes [58]. It is plausible that hemichannels or ADO transport blockade results in increased extracellular ADO. However, the mechanics of extracellular ADO concentration has not been fully explained in ECs. Our observation that 2-APB added to ECs maintained in low external calcium was not additive, notwithstanding that both stimuli increased basal ATP secretion is not accounted at present. It appears that ECs respond only to TRPV activation, since we failed to observe an extracellular ADO increase, and the ADP value is in all similar to that of 2-APB alone. Altogether, this collection of findings concur to indicate that globally the mechanism that operates non-mechanical ATP release appears restricted to a minor pool of vesicular ATP which accounts for half to a third of the extracellular ATP level and appears to be under TRP control. Although an unequivocal interpretation of these findings is not immediate, we rely on the literature data proposing 2-APB as a TRPV3 agonist [54, 55]. Unfortunately, no commercial TRPV3 channel blocker is available to confirm this proposal.

The ATP secretion elicited by mechanical stimulation operates within a time-scale of seconds. On a comparative basis, mechanical stimuli triggered either by CMD or by a picospritzer puff resulted in a similar rise in extracellular ATP with a common decay course. ECs in vivo are exposed throughout life to shear stress forces; we infer that mechanosensors like the Piezo1 and 2 or sensors such as primary cilium or the lateral proteins cadherins, occludins, or even PECAM proteins could likely be primary targets of shear stress force transduction [59]. In an effort to evaluate the putative role of these sensors, Gd, a non-selective mechanosensor blocker, reduced the mechanically evoked ATP release component but not its spontaneous counterpart, demonstrating some sort of specificity. Since Gd was described to block Piezo1 activity [39], we infer that mechanosensors are linked to the CMD-evoked nucleotide release, although the intricacies of the signaling process remain unknown. In view that CMD-evoked release is blocked in at least 50% by connexin and/or pannexin1 blockers, we conclude that cell mechanosensors are associated to the mechanism of ATP secretion related to hemichannels, as shown by the present results. In support that mechanosensors trigger the intracellular signal leading to hemichannel-mediated ATP secretion, there is no additivity when Gd was co-applied with carbenoxolone as deduced from the fact that the mechanosensor starts the signaling to elicit hemichannel-mediated ATP secretion. In support of our contention, ECs express pannexin1 and connexins 37, 40, and 43 subtypes [16]. Moreover, present data using ECs from Panx1$^{-/-}$ mice confirmed the relevance of pannexin1 hemichannels for the CDM-mediated release mechanism and the lack of probenecid inhibition in the ECs from the pannexin1 KO mice. These results directly support our hypothesis that pannexin1 hemichannels are required only for the extracellular ATP overflow following CMD but not the spontaneous overflow as observed in the control WT mice. Moreover, and consistent with our proposal, ECs from Panx1$^{-/-}$ were insensitive to 100 μM probenecid, validating our notion that the extracellular ATP released elicited by CMD requires pannexin1 hemichannels sensitive to this preferential pannexin1 blocker.

In addition, and tangential to the main focus of this communication, we accumulated evidence in support of the observation that pannexin1 blockade resulted in augmented basal extracellular ADO, but not the CMD-triggered condition. As to whether these preferential pannexin1 blockers also inhibit ADO transporters await examination. Alternatively, it is plausible that these hemichannels and ADO transporters are physically close and functionally coupled, an exciting working hypothesis opened for future studies. This is the first report

that basal ATP release is not sensitive to metal inhibitors or hemichannel blockers as illustrated in the scheme of Fig. 8. Overall, these findings allow proposing that mechanically evoked ATP secretion is mediated essentially by hemichannels rather than vesicles, although we cannot rule out a minor mixed secretory component. In the present results using MβCD to reduce ECs cholesterol, we observed a marked increase, and in the same proportion, of the spontaneous as the mechanically evoked ATP release, while ADO extracellular levels remained unaltered. Although at present this result remains unexplained, we deduce that ECs mechanosensor(s) are not localized in cholesterol-rich lipid rafts membrane.

We next intended to identify physiological ligands that may modulate the mechanically induced ATP secretory component. The present findings indicate that thrombin and the PAR hexapeptide SFLLRN but not TFLLRN, a rather selective PAR_1 agonist, allowed us to tentatively infer that PAR_2 is a likely thrombin target in ECs which may contribute to enhance the mechanically triggered ATP release. This observation may have physiopathological consequences in the clinic following injured blood vessels. By no means have we implicated that mechanical stimulation of ECs conditions thrombin release to the blood stream. Consistent with this interpretation, AFLARAA, a PAR_1 antagonist, was ineffective to block SFLLRN activity, supporting the involvement of PAR_2 in the thrombin effect. Recent work described that thrombin either activates directly a PAR_2 [60] or the dimerized PAR_1-PAR_2 heteromer [19, 41]. To ascertain the role of Rho kinase activity as part of the intracellular PAR signaling, we evaluated the participation of this intracellular cascade through the use of specific Rho blockers. Present results showed that neither H1152P nor Y27632 Rho inhibitors blocked the effect of PAR agonism on CMD stimulation. Moreover, the inhibitors did not modify basal ATP release as was observed with PAR agonists. On the contrary, 0.01–10 μM Y27632 elicited a concentration-dependent rise in extracellular ATP, ADP AMP, and ADO following mechanical stimulation. Overall, this Y27632 effect was similar to MβCD treatment, except that the dextrin did not elicit a rise in AMP or ADO. The substantial 8.4-fold increase in extracellular ATP values masked the possible effect of SFLLRN on PAR agonism. In agreement with our observation, Y27632 was reported to increase ATP release following erythrocyte deformation caused by low oxygen tension. Thuet et al. interpreted this finding indicating that Rho kinase pathway participates in actin cytoskeleton assembly, increasing erythrocyte deformation, and therefore ATP release [61]. In the same way, we deem that mechanical CMD in ECs incubated with Y27632 may alter cytoskeleton inducing the observed ATP release. At present, we cannot discard an indirect effect, since H1152P, a more specific Rho kinase inhibitor [62], did not elicit the huge ATP outflow as Y27632. The mediation of Rho kinase in PAR signaling is not clear. Notwithstanding, the large ATP secretion elicited by CMD in the presence of Y27632 indicates ECs capacity to release ATP, and it becomes an internal standard to support the magnitude of the rise of extracellular ATP allowing an estimation of the intracellular secretory ATP pool available. Altogether, we conclude that thrombin has effects other than its classical role in hemostasis, a finding of relevance to vascular physiology [60, 63]. In view that clotting leads to thrombin formation, and eventually to reduction of local tissue circulation, thrombin favors the EC response to increase extracellular ATP release, leading to increase NO production, a relevant vasodilator.

It has not escaped our attention that the common cell biology practice of protocols that require either changing the cell media or the pipetting of chemicals result in extracellular ATP release; this signal may alter experimental results if not paralleled by proper controls. Summing up, ECs release extracellular ATP via two distinct secretory routes that we dissected using pharmacological tools plus $Pannx1^{-/-}$ knockout mice. While the basal, spontaneous release relies mainly on a vesicular component, the CMD-evoked secretion is principally related to pannexin1 or other hemichannel activity. Finally, the finding of a synergic response between CMD-evoked ATP secretory procedure and PAR2 agonism highlights the physiological and medical relevance of the ATP secretion mechanism to vascular biology.

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Compliance with ethical standards

Conflicts of interest M. Verónica Donoso declares that she has no conflict of interest.
Felipe Hernández declares that he has no conflict of interest.
Tania Villalón declares that she has no conflict of interest.
Claudio Acuña-Castillo declares that he has no conflict of interest.
J. Pablo Huidobro-Toro declares that he has no conflict of interest.

Ethical approval The Universidad de Santiago Ethical Committee for the use of animals in biological research approved the specific protocols designed and supervised our strict adherence to the subscribed guidelines through the local Ethical Committee of the Faculty of Chemistry and Biology.

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