# Accepted Manuscript


ATP release mechanisms of endothelial cell-mediated stimulus-dependent hyperalgesia

Elizabeth K. Joseph, Paul G. Green, Jon D. Levine

PII: S1526-5900(14)00696-8

DOI: 10.1016/j.jpain.2014.04.005

Reference: YJPAI 2931

To appear in: *Journal of Pain*

Received Date: 11 February 2014

Revised Date: 25 March 2014

Accepted Date: 8 April 2014

Please cite this article as: Joseph EK, Green PG, Levine JD, ATP release mechanisms of endothelial cell-mediated stimulus-dependent hyperalgesia, *Journal of Pain* (2014), doi: 10.1016/j.jpain.2014.04.005.

# ATP release mechanisms of endothelial cell-mediated stimulus-dependent hyperalgesia

**Elizabeth K. Joseph, Paul G. Green and Jon D. Levine**

Departments of Medicine and Oral Surgery, and Division of Neuroscience
University of California at San Francisco, San Francisco CA 94143-0440

**Running title:** ATP release mechanisms in endothelin hyperalgesia

**Disclosures:** This work was supported by NIH grant AR063312. The authors report no conflicts of interest.

**Address correspondence:** Jon D. Levine MD PhD, Departments of Medicine and Oral & Maxillofacial Surgery, University of California at San Francisco, San Francisco CA 94143-0440. **E-mail address:** Jon.Levine@ucsf.edu

**Abstract and Perspective**

Endothelin-1 acts on endothelial cells to enhance mechanical stimulation-induced release of ATP, which in turn can act on sensory neurons innervating blood vessels to contribute to vascular pain, a phenomenon we have referred to as stimulus-dependent hyperalgesia (SDH). In the present study we evaluated the role of the major classes of ATP release mechanisms to SDH: vesicular exocytosis, plasma membrane associated ATP synthase, ATP-Binding Cassette (ABC) transporters, and ion channels. Inhibitors of vesicular exocytosis (i.e., monensin, brefeldin A and bafilomycin), plasma membrane associated ATPase (i.e., oligomycin and pigment epithelium-derived factor-derived peptide 34-mer) and connexin ion channels (carbenoxolone and flufenamic acid), but not ABC transporters (i.e., dipyridamole, nicardipine or $CFTR_{inh}$-172) attenuated stimulus-dependent hyperalgesia. These studies support a role of ATP in SDH, and suggest novel targets for the treatment of vascular pain syndromes.

**Perspective:** Endothelin-1 acts on endothelial cells to produce mechanical stimulation-induced hyperalgesia. Inhibitors of three different ATP release mechanisms attenuated this stimulus-dependent hyperalgesia. These studies provide support for a role of ATP in stimulus-dependent hyperalgesia, and suggest novel targets for the treatment of vascular pain syndromes.

**Key words:** Endothelin-1; ATP release; vascular pain; endothelium

## Introduction

We have recently discovered a novel mechanism by which endothelial cells, which line the lumen of blood vessels, contribute to peripheral vascular pain mechanisms [29-32]. We demonstrated two separate mechanisms by which endothelin-1 (ET-1) causes hyperalgesia; intradermal administration of ET-1 decreases nociceptive threshold to a similar magnitude when measured for the first time at 15 or 30 min after administration, however, when readings are taken every 5 min, in the interval between 15 and 30 min, there is a progressive decrease in nociceptive threshold, i.e., the mechanical stimulation of the testing itself enhanced ET-1 hyperalgesia [30]. This phenomenon, referred to as stimulus-dependent hyperalgesia (SDH), is elicited by two prominent vasoactive compounds, ET-1 and epinephrine, acting at their cognate receptors on endothelial cells to produce a state in which mechanical stimulation now produces enhanced release of ATP that, in turn, acts at P2X3 receptors on sensory neurons [9]. One key feature of SDH that remains to be elucidated is the mechanism mediating ATP release, in response to the mechanical stimulus in the ET-1 and epinephrine sensitized endothelial cell.

There are several mechanisms that have been described by which ATP is released from cells, including: 1) vesicular exocytosis (ATP is found in high concentration in synaptic vesicles where it is thought to function as a co-transmitter [4]), 2) plasma membrane associated ATP synthase (this enzyme is found in the plasma

membrane as well as mitochondrial membrane [8, 10]), 3) ATP-binding cassette (ABC) transporters [41, 50], and 4) various ion channels [23, 25, 41, 55] (*e.g.*, connexin hemichannels, pannexin channels, maxi-anion channels, volume regulated anion channels and P2X7 receptors). Several of these mechanisms have already been demonstrated to be present in endothelial cells [40, 41] and, importantly from a practical standpoint, there is a relatively well-developed pharmacology available for the study of these mechanisms. Therefore, in the present study we evaluated the role of these ATP release mechanisms in ET-1 induced SDH.

## Materials and Methods

### Animals

Experiments were performed on male Sprague Dawley rats (200–250 g; Charles River). Animals were housed three per cage, under a 12 h light/dark cycle, in a temperature- and humidity-controlled environment. Food and water were available *ad libitum*. All behavioral nociceptive testing was performed between 10:00 A.M. and 4:00 P.M. All experimental protocols were approved by the University of California, San Francisco Committee on Animal Research and conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used and their suffering.

### Nociceptive testing

Rats were acclimatized to the experimental environment and behavioral testing procedures, before performing experiments. To acclimatize rats to the testing environment, they were brought to the experimental area in their home cage and left in their cage for 15–30 min, after which they were placed in a restrainer (cylindrical transparent acrylic tubes that have side openings to allow extension of the hind limbs from the restrainer, for nociceptive testing). Rats were left undisturbed in the restrainer for another 15–30 min before nociceptive testing was started.

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter (Stoelting®), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its paw; the experimenter was not blinded to treatment group. Baseline nociceptive threshold, was defined as the mean of three readings taken at 5-min intervals, determined prior to all experiments. Hyperalgesia was defined as a decrease in mechanical nociceptive threshold, here presented as percent reduction from baseline [% reduction in threshold = (pretreatment threshold - post-treatment threshold)/ (pretreatment threshold)] × 100. Each paw was treated as an independent measure; both paws of the same rat received the same treatment [2, 3, 36]. Each experiment was performed on separate groups of rats. Inhibitors were injected intradermally into the dorsum of both hind paws at the site of nociceptive testing (to determine whether the inhibitors

alone affected nociceptive threshold), 15 min before the administration of ET-1 and paw-withdrawal thresholds compared, before and after drug treatment. The doses used in this study are based on a very large number of studies that have used 1 μg as a screening dose for intradermal drugs [14, 29-33], all inhibitors were administered at concentrations known to inhibit their ATP release mechanisms [1, 13, 16, 17, 48, 62].

***Drugs***

*Vesicular exocytosis mechanisms*

The role of exocytosis (*e.g.*, vesicular exocytosis) mechanisms was studied by administering monensin (Sigma Chemical Co., St. Louis, MO), which interferes with vesicle formation from the Golgi formation [4, 39], bafilomycin A1 (VWR International, Brisbane CA), an inhibitor of vacuolar $H^+$-ATPases that produce proton gradients in endoplasmic reticulum thereby reducing the driving force for uptake of ATP into the vesicle [20] and brefeldin A (Sigma Chemical Co) which blocks the activation of a subset of ADP-ribosylation factors and thereby inhibiting vesicle trafficking [38].

*ATP-binding cassette (ABC) transporters*

ATP-binding cassette (ABC) transporters have been implicated in ATP release, including the multidrug resistance gene product, MDR1 (also known as P-

glycoprotein, ABCB1 [59]), and the cystic fibrosis transmembrane conductance regulator CFTR (ABCC7) [58]. We tested for a contribution of ABC transporters to SDH by administering ABC transporter inhibitors dipyridamole (Sigma Chemical Co.), an inhibitor of MDR1/P-glycoprotein/BCRP [52], and nicardipine (Santa Cruz Biotechnology, Paso Robles, CA), a dihydropyridine in different chemical class from dipyridamole, that is another potent inhibitor of MDR1/P-glycoprotein [52], as well as breast cancer resistance protein [67]. We also tested the highly selective inhibitor of CFTR, $CFTR_{inh}$-172 (Santa Cruz Biotechnology), a thiazolidinone compound that acts directly on the CFTR [57, 58].

*Ion channels*

Finally, the role of ion channels (*e.g.*, connexin hemichannels, pannexin channels, maxi-anion channels, and volume regulated anion channels) has been commonly studied by administering the gap junction inhibitor flufenamic acid (Sigma Chemical Company), an inhibitor of Connexin 43 [22], and carbenoxolone (Sigma Chemical Company), a non-specific gap junction blocker [12].

*Plasma membrane ATP synthase*

In addition to its synthesis by mitochondria, ATP is also synthesized by a plasma membrane ATP synthase. The role of plasma membrane ATP synthase was evaluated by administering the ATP synthesis inhibitor oligomycin (Sigma Chemical Co.) [46] and PEDF (pigment epithelium-derived factor peptide 34-mer,

Phoenix Pharmaceutical, Brisbane, CA) a ligand of cell-surface F1 ATP synthase, that only inhibits *extracellular* ATP synthesis [13, 47], to rule out the mitochondrion as the site of ATP synthase inhibition.

All drugs were administered intradermally (i.d.) in a volume of 5 µl using a 30-gauge hypodermic needle attached to a micro-syringe (Hamilton, Reno, NV) by PE-10 polyethylene tubing. All inhibitors were administered 15 min prior to ET-1 (Fisher Scientific, Houston, TX) and nociceptive thresholds measured (four times), at 15, 20, 25 and 30 min post ET-1. The *per se* effect of all the inhibitors were separately evaluated and none had a significant effect on paw-withdrawal threshold of the naïve rats (data not shown). Monensin[1], oligomycin [62], PEDF [13], carbenoxolone [16], flufenamic acid [17], brefeldin A and bafilomycin [48] were given at concentrations that have been shown to inhibit ATP release or degradation.

**Statistics**

The dependent variable in experiments evaluating cutaneous nociceptive threshold was change in paw withdrawal threshold from the pretreatment baseline threshold. Group data are represented as mean ± SEM. Statistical significance was determined by one- or two-way repeated-measures ANOVA, followed by Dunnet's *post hoc* test. $p < 0.05$ was considered statistically significant.

**Results**

*Vesicular exocytosis*

We administered three inhibitors of vesicular release mechanisms, monensin, brefeldin A and bafilomycin, to evaluate the role of this release mechanism in endothelial cell mediated ET-1-induced SDH. Rats received either vehicle (0.9% sodium chloride), monensin, bafilomycin or brefeldin A, 15 min before ET-1 administration. Nociceptive threshold was evaluated every 5 min beginning 15 min after ET-1, the standard protocol for detecting SDH [30, 31]. In rats pretreated with vehicle, ET-1 hyperalgesia increased with each subsequent test of mechanical threshold, indicating the presence of SDH, as previously described [30]. However, in rats pretreated with either monensin, brefeldin A or with bafilomycin, this enhancement of hyperalgesia by mechanical stimulation was abolished (Figure 1). Monensin also affects ET-1 hyperalgesia, a phenomenon we have previously observed with $\beta_2$-adrenergic and $5HT_{1B/D}$ receptor antagonists [31], presumably due to action on the nociceptor terminal.

*ATP-binding cassette (ABC) transporters*

We administered inhibitors of three ATP-binding cassette (ABC) transporters, dipyridamole, nicardipine and $CFTR_{inh}$-172, to evaluate the role of ABC transporters in endothelial cell mediated SDH. Rats received vehicle (0.9% sodium chloride, or 10% DMSO in 0.9% saline for $CFTR_{inh}$-172), dipyridamole, nicardipine or $CFTR_{inh}$-172 15 min before ET-1. Nociceptive threshold was evaluated every 5 min beginning 15 min after ET-1. Neither dipyridamole,

nicardipine nor $CFTR_{inh}$-172 affected the development of SDH, but $CFTR_{inh}$-172 significantly attenuated ET-1–induced hyperalgesia (Figure 2).

*Ion channels*

We administered two ion channel inhibitors, flufenamic acid (a voltage gated sodium channel blocker) and carbenoxolone (an interneuronal gap junction blocker), to evaluate the role of ion channels in endothelial cell mediated SDH. Rats received vehicle (0.9% sodium chloride), flufenamic acid or carbenoxolone 15 min before ET-1. Nociceptive threshold was evaluated every 5 min beginning 15 min after ET-1. Flufenamic acid and carbenoxolone pretreatment completely prevented the development of SDH, and flufenamic acid attenuated ET-1 hyperalgesia (Figure 3).

*Plasma membrane associated ATP synthase*

Finally, we administered two inhibitors of plasma membrane associated ATP synthase, oligomycin and PEDF, to evaluate the role of ATP synthase in endothelial cell mediated SDH. Rats received either vehicle (0.9% sodium chloride or 10% DMSO in 0.9% saline for oligomycin), oligomycin or PEDF 15 min before ET-1. Nociceptive threshold was evaluated every 5 min beginning 15 min after ET-1. Both oligomycin and PEDF pretreatment prevented both ET-1 hyperalgesia and the development of ET-1–induced SDH (Figure 4).

**Discussion**

The intradermal administration of ET-1 produces mechanical hyperalgesia, which is further enhanced by repeated testing with a threshold nociceptive intensity mechanical stimulus, a phenomenon that we have termed stimulus dependent hyperalgesia (SDH) [30]. While ET-1 hyperalgesia is mediated by its action at ET receptors on the primary afferent nociceptors [29], we hypothesized that SDH is mediated via ET receptors on endothelial cells. Since a role of the endothelial cell in vascular pain has been postulated [6, 44, 51, 56], we used a method employed in the cardiovascular and renal vascular literature to impair endothelial function: administration of octoxynol-9 to the luminal side of blood vessels, *in vitro* [11, 26] and *in vivo* [54]. We observed that this method of impairing endothelial function eliminates ET-1–induced SDH while leaving ET-1 hyperalgesia undiminished [29, 31].

A signal feature of SDH is that it is produced by repeated mechanical stimulation. Since endothelial cells are activated by mechanical stimuli to release ATP [4, 6, 43, 65], and ATP acts on P2X3 receptors on nociceptors to mediate pain, we hypothesized that ET-1 acts on endothelial cells to enhance mechanical stimulation-induced ATP release, giving rise to SDH. In this study, we examined the contribution of ATP release mechanisms to SDH.

We observed that inhibitors of ATP release via vesicular mechanisms (bafilomycin A, monensin, brefeldin A), and ion channel-dependent mechanisms (flufenamic acid, carbenoxolone) significantly attenuate SDH. We also observed

that inhibition of ATP synthase activity with oligomycin A, an inhibitor of the ATP synthase, as well as by PEDF-34-mer, which is specific for ecto F1-ATP synthase on the plasma membrane [47]. However, we did not observe attenuation in ET-1–induced SDH with dipyridamole, a potent inhibitor of the ABC transporters, including P-glycoprotein (ABCB1) [60], multidrug resistance-associated protein (ABCC1) [28], and breast cancer resistance protein (ABCG2) [64], or by nicardipine (a dihydropyridine, different chemical class from dipyridamole) another potent inhibitor of P-glycoprotein [52] and breast cancer resistance protein [67]. $CFTR_{inh}$-172, a potent and highly selective antagonist for the CFTR transport mechanism, also failed to attenuate ET-1 induced SDH. However, $CFTR_{inh}$-172 attenuation of ET-1 hyperalgesia may be related to the role CFTR plays in mediating the release of ATP from dorsal root ganglia (DRG) [34]; presence of $CFTR_{inh}$-172 may decrease the release of ATP from DRG in response to increased extracellular levels of ATP [42].

Since the drugs used in this study have actions other than inhibition of a specific ATP release mechanism, we used more than one class of drug for each release mechanism (i.e. bafilomycin A, monensin and brefeldin A for vesicular release, and flufenamic acid and carbenoxolone for ion channel-dependent release). This approach is used routinely in the endothelial cell literature to establish the role of these mechanisms in ATP release [4, 15, 17, 24, 40, 41, 63]. Of note in this regard, specifically identified following high throughput screening of 50,000 molecules, $CFTR_{inh}$-172 is a potent and highly selective antagonist for the CFTR transport

mechanism [35]. Our observations implicate multiple ATP release mechanisms in response to mechanical stimulation, as seen in other cell types [20, 39, 61].

While release of ATP from endothelial cells in response to mechanical stress is well-established [4, 5, 40, 43, 65], the specific mechanisms mediating release are still under investigation [41]. Our observation that monensin, bafilomycin and brefeldin A inhibit ET-1–induced SDH support the hypothesis that vesicular release mechanism, presumably ATP release based on our previous P2X3 antagonist studies [31], is involved in the expression of SDH. This hypothesis is consistent with histological evidence that ATP is present in vesicles in endothelial cells, and release of ATP from cultured endothelial cells exposed to a shear stress is blocked by monensin [4]. Of note, it is also possible that the compounds used in this study to inhibit ATP release, may have action on cell types in the periphery other than the endothelial cell. For example, gene products of glial cells, such as Schwann cells, have been hypothesized to play a role in nerve injury and sensory nerve function [7, 19], while a role for immune cells (e.g. neutrophils, macrophages, T cells) in acute and chronic inflammatory and neuropathic pain is well-established [21, 45, 53]. Other cell types, such as keratinocytes [18, 37] and mast cells [27, 49] are also implicated in peripheral nociceptive mechanisms. It is likely that actions of mediators, released from these cell types, on nociceptors contribute to regulating nociceptor function in chronic pain.

We also observed that inhibition of ATP synthesis by oligomycin or PEDF-derived peptide 34-mer (which is selective for extracellular ATPase), prevented

both ET-1 induced hyperalgesia as well as SDH. While it might be assumed that inhibition of extracellular ATP synthesis would be independent of mechanical stimulation-induced release of ATP, it has previously been shown that inhibition of cell surface ATP synthase with angiostatin, piceatannol, or anti-ATP synthase antibody (none of which cross the plasma membrane, and so have no effect on mitochondrial ATP synthase activity) markedly decrease mechanical stimulation-induced ATP release [66].

Taken together with our previous observations that ET-1 induced SDH is attenuated by a selective antagonist, for the P2X3 receptor, A-317491, present on the nociceptor and activated by ATP [29], our data provide additional evidence for mechanical stimulation-induced release of ATP from endothelial cells via vesicular and ion channel-dependent mechanisms mediating SDH.

## References


1. Akopova I, Tatur S, Grygorczyk M, Luchowski R, Gryczynski I, Gryczynski Z, Borejdo J, Grygorczyk R: Imaging exocytosis of ATP-containing vesicles with TIRF microscopy in lung epithelial A549 cells. Purinergic Signal 8:59-70, 2012
2. Aley KO, Levine JD: Multiple receptors involved in peripheral alpha 2, mu, and A1 antinociception, tolerance, and withdrawal. J Neurosci 17:735-744, 1997
3. Aley KO, Levine JD: Role of protein kinase A in the maintenance of inflammatory pain. J Neurosci 19:2181-2186, 1999
4. Bodin P, Burnstock G: Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 38:900-908, 2001
5. Burnstock G: Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. J Anat 194:335-342, 1999
6. Burnstock G: Purinergic regulation of vascular tone and remodelling. Auton Autacoid Pharmacol 29:63-72, 2009
7. Campana WM: Schwann cells: activated peripheral glia and their role in neuropathic pain. Brain Behav Immun 21:522-527, 2007
8. Champagne E, Martinez LO, Collet X, Barbaras R: Ecto-F1Fo ATP synthase/F1 ATPase: metabolic and immunological functions. Curr Opin Lipidol 17:279-284, 2006
9. Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN: A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377:428-431, 1995
10. Chi SL, Pizzo SV: Cell surface F1Fo ATP synthase: a new paradigm? Ann Med 38:429-438, 2006
11. Connor HE, Feniuk W: Influence of the endothelium on contractile effects of 5-hydroxytryptamine and selective 5-HT agonists in canine basilar artery. Br J Pharmacol 96:170-178, 1989
12. de Groot JR, Veenstra T, Verkerk AO, Wilders R, Smits JP, Wilms-Schopman FJ, Wiegerinck RF, Bourier J, Belterman CN, Coronel R, Verheijck EE: Conduction slowing by the gap junctional uncoupler carbenoxolone. Cardiovasc Res 60:288-297, 2003
13. Deshpande M, Notari L, Subramanian P, Notario V, Becerra SP: Inhibition of tumor cell surface ATP synthesis by pigment epithelium-derived factor: implications for antitumor activity. Int J Oncol 41:219-227, 2012
14. Dina OA, Joseph EK, Levine JD, Green PG: Mechanisms mediating vibration-induced chronic musculoskeletal pain analyzed in the rat. J Pain 11:369-377, 2010
15. Faigle M, Seessle J, Zug S, El Kasmi KC, Eltzschig HK: ATP release from vascular endothelia occurs across Cx43 hemichannels and is attenuated during hypoxia. PLoS One 3:e2801, 2008
16. Godecke S, Roderigo C, Rose CR, Rauch BH, Godecke A, Schrader J: Thrombin-induced ATP release from human umbilical vein endothelial cells. Am J Physiol Cell Physiol 302:C915-C923, 2012
17. Gomes P, Srinivas SP, Van Driessche W, Vereecke J, Himpens B: ATP release through connexin hemichannels in corneal endothelial cells. Invest Ophthalmol Vis Sci 46:1208-1218, 2005
18. Gopinath P, Wan E, Holdcroft A, Facer P, Davis JB, Smith GD, Bountra C, Anand

P: Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain. BMC Womens Health 5:2, 2005

19. Gosselin RD, Suter MR, Ji RR, Decosterd I: Glial cells and chronic pain. Neuroscientist 16:519-531, 2010
20. Grygorczyk R, Furuya K, Sokabe M: Imaging and characterization of stretch-induced ATP release from alveolar A549 cells. J Physiol 2013
21. Guillot X, Semerano L, Decker P, Falgarone G, Boissier MC: Pain and immunity. Joint Bone Spine 79:228-236, 2012
22. Harks EG, de Roos AD, Peters PH, de Haan LH, Brouwer A, Ypey DL, van Zoelen EJ, Theuvenet AP: Fenamates: a novel class of reversible gap junction blockers. J Pharmacol Exp Ther 298:1033-1041, 2001
23. Hattori F, Ohshima Y, Seki S, Tsukimoto M, Sato M, Takenouchi T, Suzuki A, Takai E, Kitani H, Harada H, Kojima S: Feasibility study of B16 melanoma therapy using oxidized ATP to target purinergic receptor P2X7. Eur J Pharmacol 695:20-26, 2012
24. Hisadome K, Koyama T, Kimura C, Droogmans G, Ito Y, Oike M: Volume-regulated anion channels serve as an auto/paracrine nucleotide release pathway in aortic endothelial cells. J Gen Physiol 119:511-520, 2002
25. Islam MR, Uramoto H, Okada T, Sabirov RZ, Okada Y: Maxi-anion channel and pannexin 1 hemichannel constitute separate pathways for swelling-induced ATP release in murine L929 fibrosarcoma cells. Am J Physiol Cell Physiol 303:C924-C935, 2012
26. Jamal A, Bendeck M, Langille BL: Structural changes and recovery of function after arterial injury. Arterioscler Thromb 12:307-317, 1992
27. Jankowski MP, Koerber HR 2010. In Kruger L, Light AR, eds, *Translational Pain Research: From Mouse to Man*, p. Chapter 2. CRC Press, Boca Raton, FL.
28. Janneh O, Jones E, Chandler B, Owen A, Khoo SH: Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4 T cells and primary human lymphocytes. J Antimicrob Chemother 60:987-993, 2007
29. Joseph EK, Green PG, Bogen O, Alvarez P, Levine JD: Vascular endothelial cells mediate mechanical stimulation-induced enhancement of endothelin hyperalgesia via activation of P2X2/3 receptors on nociceptors. J Neurosci 33:2849-2859, 2013
30. Joseph EK, Gear RW, Levine JD: Mechanical stimulation enhances endothelin-1 hyperalgesia. Neuroscience 178:189-195, 2011
31. Joseph EK, Levine JD: Role of endothelial cells in antihyperalgesia induced by a triptan and beta-blocker. Neuroscience 232:83-89, 2012
32. Joseph EK, Levine JD: Sexual dimorphism in endothelin-1 induced mechanical hyperalgesia in the rat. Exp Neurol 233:505-512, 2012
33. Joseph EK, Reichling DB, Levine JD: Shared mechanisms for opioid tolerance and a transition to chronic pain. J Neurosci 30:4660-4666, 2010
34. Kanno T, Nishizaki T: CFTR mediates noradrenaline-induced ATP efflux from DRG neurons. Mol Pain 7:72, 2011
35. Kelly M, Trudel S, Brouillard F, Bouillaud F, Colas J, Nguyen-Khoa T, Ollero M, Edelman A, Fritsch J: Cystic fibrosis transmembrane regulator inhibitors CFTR(inh)-172 and GlyH-101 target mitochondrial functions, independently of chloride channel inhibition. J Pharmacol Exp Ther 333:60-69, 2010

36. Khasar SG, Miao FJ, Janig W, Levine JD: Vagotomy-induced enhancement of mechanical hyperalgesia in the rat is sympathoadrenal-mediated. J Neurosci 18:3043-309., 1998
37. Khodorova A, Navarro B, Jouaville LS, Murphy JE, Rice FL, Mazurkiewicz JE, Long-Woodward D, Stoffel M, Strichartz GR, Yukhananov R, Davar G: Endothelin-B receptor activation triggers an endogenous analgesic cascade at sites of peripheral injury. Nat Med 9:1055-1061, 2003
38. Klausner RD, Donaldson JG, Lippincott-Schwartz J: Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol 116:1071-1080, 1992
39. Knight GE, Bodin P, De Groat WC, Burnstock G: ATP is released from guinea pig ureter epithelium on distension. Am J Physiol Renal Physiol 282:F281-F288, 2002
40. Koyama T, Kimura C, Hayashi M, Watanabe M, Karashima Y, Oike M: Hypergravity induces ATP release and actin reorganization via tyrosine phosphorylation and RhoA activation in bovine endothelial cells. Pflugers Arch 457:711-719, 2009
41. Lohman AW, Billaud M, Isakson BE: Mechanisms of ATP release and signalling in the blood vessel wall. Cardiovasc Res 95:269-280, 2012
42. Matsuka Y, Ono T, Iwase H, Mitrirattanakul S, Omoto KS, Cho T, Lam YY, Snyder B, Spigelman I: Altered ATP release and metabolism in dorsal root ganglia of neuropathic rats. Mol Pain 4:66, 2008
43. Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, Burnstock G: Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. Proc Biol Sci 241:245-248, 1990
44. Mironidou-Tzouveleki M, Tsartsalis S, Tomos C: Vascular endothelial growth factor (VEGF) in the pathogenesis of diabetic nephropathy of type 1 diabetes mellitus. Curr Drug Targets 12:107-114, 2011
45. Molliver DC, Radeke MJ, Feinstein SC, Snider WD: Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 361:404-416, 1995
46. Nicholls DG, Ferguson S: Bioenergetics, Fourth Edition. 434, 2013
47. Notari L, Arakaki N, Mueller D, Meier S, Amaral J, Becerra SP: Pigment epithelium-derived factor binds to cell-surface F(1)-ATP synthase. FEBS J 277:2192-2205, 2010
48. O'Grady SM, Patil N, Melkamu T, Maniak PJ, Lancto C, Kita H: ATP release and Ca2+ signalling by human bronchial epithelial cells following Alternaria aeroallergen exposure. J Physiol 591:4595-4609, 2013
49. Parada CA, Tambeli CH, Cunha FQ, Ferreira SH: The major role of peripheral release of histamine and 5-hydroxytryptamine in formalin-induced nociception. Neuroscience 102:937-944, 2001
50. Patak P, Hermann DM: ATP-binding cassette transporters at the blood-brain barrier in ischaemic stroke. Curr Pharm Des 17:2787-2792, 2011
51. Pate M, Damarla V, Chi DS, Negi S, Krishnaswamy G: Endothelial cell biology: role in the inflammatory response. Adv Clin Chem 52:109-130, 2010
52. Sakurai A, Onishi Y, Hirano H, Seigneuret M, Obanayama K, Kim G, Liew EL, Sakaeda T, Yoshiura K, Niikawa N, Sakurai M, Ishikawa T: Quantitative structure--activity relationship analysis and molecular dynamics simulation to functionally validate nonsynonymous polymorphisms of human ABC transporter ABCB1 (P-

glycoprotein/MDR1). Biochemistry 46:7678-7693, 2007
53. Sandler SG, Tobin W, Henderson ES: Vincristine-induced neuropathy. A clinical study of fifty leukemic patients. Neurology 19:367-374, 1969
54. Sun ZW, Wang XD, Deng XM, Wallen R, Gefors L, Hallberg E, Andersson R: The influence of circulatory and gut luminal challenges on bidirectional intestinal barrier permeability in rats. Scand J Gastroenterol 32:995-1004, 1997
55. Tan C, Voss U, Svensson S, Erlinge D, Olde B: High glucose and free fatty acids induce beta cell apoptosis via autocrine effects of ADP acting on the P2Y(13) receptor. Purinergic Signal 2012
56. Triggle CR, Samuel SM, Ravishankar S, Marei I, Arunachalam G, Ding H: The endothelium: influencing vascular smooth muscle in many ways. Can J Physiol Pharmacol 90:713-738, 2012
57. Tu J, Le G, Ballard HJ: Involvement of the cystic fibrosis transmembrane conductance regulator in the acidosis-induced efflux of ATP from rat skeletal muscle. J Physiol 588:4563-4578, 2010
58. Tu J, Lu L, Cai W, Ballard HJ: cAMP/protein kinase A activates cystic fibrosis transmembrane conductance regulator for ATP release from rat skeletal muscle during low pH or contractions. PLoS One 7:e50157, 2012
59. Valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC, Higgins CF: Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. Nature 355:830-833, 1992
60. Verstuyft C, Strabach S, El-Morabet H, Kerb R, Brinkmann U, Dubert L, Jaillon P, Funck-Brentano C, Trugnan G, Becquemont L: Dipyridamole enhances digoxin bioavailability via P-glycoprotein inhibition. Clin Pharmacol Ther 73:51-60, 2003
61. Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G: ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. J Clin Invest 115:2412-2422, 2005
62. Wang T, Chen Z, Wang X, Shyy JY, Zhu Y: Cholesterol loading increases the translocation of ATP synthase beta chain into membrane caveolae in vascular endothelial cells. Biochim Biophys Acta 1761:1182-1190, 2006
63. Woodward HN, Anwar A, Riddle S, Taraseviciene-Stewart L, Fragoso M, Stenmark KR, Gerasimovskaya EV: PI3K, Rho, and ROCK play a key role in hypoxia-induced ATP release and ATP-stimulated angiogenic responses in pulmonary artery vasa vasorum endothelial cells. Am J Physiol Lung Cell Mol Physiol 297:L954-L964, 2009
64. Xu H, Kulkarni KH, Singh R, Yang Z, Wang SW, Tam VH, Hu M: Disposition of naringenin via glucuronidation pathway is affected by compensating efflux transporters of hydrophilic glucuronides. Mol Pharm 6:1703-1715, 2009
65. Yamamoto K, Furuya K, Nakamura M, Kobatake E, Sokabe M, Ando J: Visualization of flow-induced ATP release and triggering of Ca2+ waves at caveolae in vascular endothelial cells. J Cell Sci 124:3477-3483, 2011
66. Yamamoto K, Shimizu N, Obi S, Kumagaya S, Taketani Y, Kamiya A, Ando J: Involvement of cell surface ATP synthase in flow-induced ATP release by vascular endothelial cells. Am J Physiol Heart Circ Physiol 293:H1646-H1653, 2007
67. Zhang Y, Gupta A, Wang H, Zhou L, Vethanayagam RR, Unadkat JD, Mao Q: BCRP transports dipyridamole and is inhibited by calcium channel blockers. Pharm Res 22:2023-2034, 2005

**Figure 1. Effect of bafilomycin (vacuolar H-ATPase inhibitor), monensin (inhibitor of vesicle formation) and brefeldin A (inhibitor of vesicle transport) on ET-1 induced mechanical hyperalgesia and stimulus-dependent hyperalgesia (SDH)**

15 min before ET-1, rats received vehicle (5 µl), bafilomycin, monensin or brefeldin A (each 1 µg in 5 µl/paw). Paw withdrawal thresholds were measured 15, 20, 25 & 30 min after ET-1 administration. Bafilomycin, monensin and brefeldin A each significantly inhibited ET-1–induced SDH compared to vehicle treated controls, and monensin also significantly inhibited ET-1 hyperalgesia (*P < 0.001, two-way repeated measures ANOVA, followed by Bonferroni post test, N = 6).

**Figure 2. Effect of dipyridamole, nicardipine and $CFTR_{inh}$-172 (ABC transport inhibitors) on ET-1 induced mechanical hyperalgesia and SDH**

15 min before ET-1, rats received vehicle (5 µl), dipyridamole, nicardipine or $CFTR_{inh}$-172 (all 1 µg in 5 µl/paw). Paw withdrawal thresholds were measured 15, 20, 25 & 30 min after ET-1 administration. Neither dipyridamole, nicardipine nor $CFTR_{inh}$-172 affected ET-1 SDH (P=N.S., two-way repeated measures ANOVA, N = 6), however $CFTR_{inh}$-172 significantly attenuated ET-1 hyperalgesia (2-way ANOVA with Dunnet's post hoc test, *P<0.05). Note that the ET-1 alone data is the same group as in Figure 1.

**Figure 3. Effect of flufenamic acid (voltage gated sodium channel blocker) and carbenoxolone (interneuronal gap junction blocker) on ET-1 induced mechanical hyperalgesia and SDH**

15 min before ET-1, rats received vehicle (5 µl), flufenamic acid or carbenoxolone (both 1 µg in 5 µl/paw). Paw withdrawal thresholds were measured 15, 20, 25 & 30 min after ET-1 administration. Both flufenamic acid and carbenoxolone significantly inhibited SDH; flufenamic acid, but not carbenoxolone, significantly attenuated ET-1 induced hyperalgesia (*P < 0.001, two-way repeated measures ANOVA, followed by Bonferroni post test, N = 6). Note that the ET-1 alone data is the same group as in Figure 1.

**Figure 4. Effect of oligomycin A (ATP synthase inhibitor) or PEDF-34-mer (extracellular ATP synthase inhibitor) on ET-1 induced mechanical hyperalgesia and SDH**

15 min before ET-1, rats received vehicle (5 µl), oligomycin or PEDF-34-mer (both 1 µg in 5 µl/paw). Paw withdrawal thresholds were measured 15, 20, 25 & 30 min after ET-1 administration. Both oligomycin and PEDF-34-mer significantly inhibited SDH as well as ET-1 hyperalgesia compared to vehicle treated controls (*P < 0.05, two-way repeated measures ANOVA, followed by Bonferroni post test, N = 6).