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| Citation       | Barr, Travis P., Phillip J. Albrecht, Quanzhi Hou, Alexander A. Mongin, Gary R. Strichartz, and Frank L. Rice. 2013. Air-stimulated ATP release from keratinocytes occurs through connexin hemicannels. PLoS ONE 8(2): e56744. |
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| Published Version | doi:10.1371/journal.pone.0056744                                                                                                                                                                                                                                   |
| Citable link    | http://nrs.harvard.edu/urn-3:HUL.InstRepos:10622937                                                                                                                                                                                                                  |
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Air-Stimulated ATP Release from Keratinocytes Occurs through Connexin Hemichannels

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Abstract
Cutaneous ATP release plays an important role in both epidermal stratification and chronic pain, but little is known about ATP release mechanisms in keratinocytes that comprise the epidermis. In this study, we analyzed ATP release from cultured human neonatal keratinocytes briefly exposed to air, a process previously demonstrated to trigger ATP release from these cells. We show that exposing keratinocytes to air by removing media for 15 seconds causes a robust, long-lasting ATP release. This air-stimulated ATP release was increased in calcium differentiated cultures which showed a corresponding increase in connexin 43 mRNA, a major component of keratinocyte hemichannels. The known connexin hemichannel inhibitors 1-octanol and carbamoylxyloolone both significantly reduced air-stimulated ATP release, as did two drugs traditionally used as ABC transporter inhibitors (glibenclamide and verapamil). These same 4 inhibitors also prevented an increase in the uptake of a connexin permeable dye induced by air exposure, confirming that connexin hemichannels are open during air-stimulated ATP release. In contrast, activity of the MDR1 ABC transporter was reduced by air exposure and the drugs that inhibited air-stimulated ATP release had differential effects on this transporter. These results indicate that air exposure elicits non-vesicular release of ATP from keratinocytes through connexin hemichannels and that drugs used to target connexin hemichannels and ABC transporters may cross-inhibit. Connexins represent a novel, peripheral target for the treatment of chronic pain and dermatological disease.

Introduction
Unlike most cells in the body, keratinocytes lie at the interface with the external environment where they form the outermost layer of the skin, the epidermis. The epidermis is a dynamic, stratified structure formed by continually proliferating and differentiating keratinocytes that surround the sensory nerve endings of several subtypes of C- and Aδ-fibers [1]. These fibers play an important role in tactile sensation and nociception and express numerous ligand-gated receptors that can regulate their signaling [2,3]. Keratinocytes have been implicated in mechanosensation and thermosensation, as well as peripheral pain mechanisms due to their release of molecules that activate such receptors, including β-endorphin, calcitonin gene-related peptide (CGRP) and ATP [4,5,6].

Cutaneous ATP release is an important signal for epidermal homeostasis as well as the generation of acute and chronic pain. Signaling among keratinocytes through the release of ATP influences their proliferation and differentiation, thereby playing a major role in the creation of the stratified structure of the epidermis and maintaining epidermal homeostasis [7]. During acute tissue injury, such as cuts and abrasions, excessive ATP release from damaged keratinocytes causes pain by activating excitatory purinergic receptors on nociceptive sensory nerve endings [8,9,10]. Lower levels of ATP released by keratinocytes during epidermal homeostasis and in response to mild mechanical and thermal stimulation may participate in normal tactile sensation and also contribute to the spontaneous pain and tactile hypersensitivity that occurs under chronic pain conditions, when nociceptive nerve endings become sensitized [11,12]. Release of ATP from keratinocytes may also be increased during chronic pain [5]. Consistent with a contribution of epidermal ATP release to chronic pain, cutaneous administration of purinergic receptor antagonists reduces nociceptive behavior in a variety of animal models of chronic pain [13,14,15,16].

Despite the importance of ATP in epidermal homeostasis, tactile sensation and nociception, little is known about the mechanisms of keratinocyte ATP release. Mechanical and thermal stimulation, low pH and hypo-osmotic stimulation have all been shown to result in ATP release from keratinocytes, but the relevant mechanisms were not identified [11,12,17,18]. Recently, we showed that activation of keratinocyte voltage-gated sodium channels triggers ATP release and that this mechanism appears to be up-regulated under chronic pain conditions [5]. These results may indirectly suggest vesicular release, although such a mecha-

Citation: Barr TP, Albrecht PJ, Hou Q, Mongin AA, Strichartz GR, et al. (2013) Air-Stimulated ATP Release from Keratinocytes Occurs through Connexin Hemichannels. PLoS ONE 8(2): e56744. doi:10.1371/journal.pone.0056744
Editor: Theodore John Price, University of Arizona, United States of America
Received November 2, 2012; Accepted January 14, 2013; Published February 15, 2013
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Funding: This work was funded in part by the Rosenblatt Fund for Neurofibromytosis Research (to FLR) and United States Public Health Service grant R-01 CA080153 (to GRS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Competing Interests: AAM, currently serves as an academic editor for PLOS ONE. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.
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nism has never been demonstrated in keratinocytes. Several non-vascular ATP release mechanisms have been proposed, but many remain controversial and are complicated by the non-specificity of available inhibitors [19,20].

Air exposure has also been shown to cause ATP release from cultured keratinocytes, though this release mechanism was not previously investigated [21]. Keratinocyte interactions with air may be an important signal to trigger epidermal stratification, as cultured keratinocytes will not form a fully stratified epidermis unless they are brought to the air interface [22,23]. Given the importance of keratinocyte ATP release in epidermal stratification and nociception, combined with the lack of information about keratinocyte ATP release mechanisms, the goal of the present study was to characterize air-stimulated ATP release by analyzing its time course, the extent of intracellular ATP depletion and the mechanism involved.

Materials and Methods

Cell Culture

Neonatal normal human epidermal keratinocytes (NHEK, Lonza, Basel, Switzerland) were cultured in chemically defined keratinocyte growth media (KGM-CD, Lonza) supplemented with 0.5% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and maintained at 37°C and in an atmosphere of 95% air/5% CO2. NHEK were plated in collagen coated 35 mm dishes at a cell density between 3,500 and 10,500 cells/cm² and cultured until they were 70–90% confluent (proliferating cultures), 100% confluent (confluent cultures), or cultured until they reached 100% confluence and then further cultured in KGM-CD containing 2 mM calcium for 5 days (differentiated cultures). Cultures were fed every 2–3 days by completely aspirating the media and replacing it with fresh KGM-CD.

Quantitative RT-PCR

RNA was isolated using the Trizol method from 3 proliferating and 3 differentiated cultures that were plated simultaneously. Quality and quantity of isolated RNA were analyzed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA was converted to cDNA using an iScript kit (Biorad, Hercules, CA) with 2 μg total RNA in 20 μL reactions with an Ependorf Mastercycler (Hamburg, Germany) using the following protocol: 5 minutes at 95°C, 30 minutes at 42°C and 5 minutes at 85°C. Expression of genes of interest was determined by qPCR using iTaq fast SYBR green master mix (Biorad) in 5 minutes at 85°C with an Ependorf Mastercycler (Hamburg, Germany) using the same primer/primer set for targets of interest that were synthesized by Invitrogen based on published sequences, validated primers from Qiagen and 3 differentiated cultures that were plated simultaneously. The following inhibitors were added to the media and replaced with fresh KGM-CD.

Air-stimulated ATP Release Assays

Time course experiments. Dishes were rinsed twice with 2 mL of HEPES buffered “basal media” containing (in mM): 135 NaCl, 3.8 KCl, 1.2 MgSO4, 1.3 CaCl2, 1.2 KH2PO4, 10 HEPES and 10 D-glucose, pH 7.4. Rinses were done quickly to minimize air exposure (1–2 seconds). Cells were then pre-incubated at room temperature for one hour to allow degradation of ATP released during rinses. Immediately before air exposure, a 100 μL baseline sample was taken. Cells were then exposed to air by aspirating all of the media and slowly (over 1–2 seconds) pipetting 2 mL of basal media onto the side of the dish after 15 seconds had passed. For controls, only half of the media was exchanged to prevent air exposure and provide a control for potential ATP release from mechanical stimulation. Samples (100 μL) of extracellular media were taken at each time point and immediately placed into wells of a white 96-well plate on ice containing 50 μL mammalian cell lysis buffer solution (ATPlite kit, Perkin Elmer, Waltham, MA) to stabilize ATP. Data were analyzed as changes from baseline release, because an increase in protein expression in differentiated cells prevented normalization to total protein (data not shown).

Intracellular ATP levels after air exposure. Experiments were conducted as described for the time course experiments. At 7 and 60 minute time points (times near peak release and return to baseline), cells were lysed by replacing media with 500 μL of mammalian cell lysis buffer solution. Samples (100 μL) of the lysates were diluted 10-fold in basal media to fit within the calibration range of the ATP assay. ATP content in all samples was calculated as % control at the 7 minute time point.

Pharmacological analysis of air-stimulated ATP release. Cells were treated the same as in time course experiments, except on the second rinse with basal media, only 1 mL was added. Ten minutes before the one hour pre-incubation period was complete, 1 mL of the appropriate inhibitor was added at double the final desired concentration. The following inhibitors of non-vascular ATP release pathways were purchased from Sigma Aldrich (St. Louis, MO) and used at the final concentrations indicated in parentheses: the connexin hemichannel blocker 1-octanol (2 mM), the dual connexin/pannexin hemichannel blocker carbenoxydone (100 μM), the P2X7 channel blocker Brilliant Blue G (BBG, 1 μM), the anion channel blockers phloretin (100 μM), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB, 100 μM) and gadolinium chloride (50 μM), the CFTR transporter inhibitor glibenclamide (200 μM), the MDR1 transporter inhibitor verapamil (10 μM) and the MRPi transporter inhibitor probenecid (1 mM) [24,25,26,27,28,29,30,31,32]. Phlorizin, NPPB, glibenclamide and probenecid were dissolved in DMSO so that the final concentration of DMSO in the test media was ≤0.1%. All other agents used were soluble in basal media. Air exposure was conducted as described for time course experiments, except basal media was now added with or without (vehicle) the appropriate inhibitor. Samples (100 μL) of extracellular media were collected immediately before (baseline) and 7 minutes after air exposure, near the time of peak ATP release.

Quantification of ATP Levels

ATP concentrations were determined using a luciferin/luciferase assay kit (ATP lite, PerkinElmer). This kit has a long reaction half-life and was validated using standard curves of ATP to ensure measurements were stable throughout the time course of experimentation and plate reading. Luminescence was measured using a Victor3 1420 plate reader (PerkinElmer) with a 535 nm emission filter for 2 seconds. Luminescence was measured 3 times for each well and then averaged for a single data point. For each experiment, values were plotted on the line of best fit for a 6-point
logarithmic standard curve (0.01 μM–3.2 μM ATP) using Origin 6.0 (OriginLab, Northampton, MA).

Cell Viability Assay

Confluent cultures were put through the same procedure as described for the pharmacological analysis experiments. Sixty minutes after air exposure, cells were rinsed twice quickly with basal media, fixed with 4% paraformaldehyde, and labeled with propidium iodide (PI, 5 μL/mL) for 90 minutes. Cells were then rinsed three times with PBS for 5 minutes per rinse. Five images were captured of both the light field and PI fluorescence for each dish. Image capture settings were identical for all images and set so that all nuclei considered positive for PI were saturated (i.e. appeared as easily identifiable solid white dots). Light field and PI images were merged and then PI positive and total cells were manually tagged and counted using Neurolucida (MBF Biosciences, Williston, VT).

Assessment of Connexin Hemichannel Activity

Uptake of a connexin hemichannel permeable dye (lucifer yellow, LY) was measured to assess the extent of hemichannel opening in response to air exposure alone and in the presence of ATP release inhibitors. Confluent cultures were treated the same as described for the pharmacological analysis experiments, with 10 minutes before the end of pre-incubation, 500 μL of the 1 mL of remaining media was removed and replaced with 500 μL of media with the appropriate inhibitor at twice the desired concentration or basal media alone. After 10 minutes, 500 μL was again removed and replaced with [3H]-digoxin-containing media (50 nM final concentration with specific activity of 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) with or without the appropriate inhibitor, both at twice the desired concentration. For the air exposure group, all media was removed for 15 seconds and replaced with [3H]-digoxin-containing basal media. After 5 minutes, cells were lysed with 2% SDS/8 mM EDTA and 500 μL samples were counted in 5 mL of Ecolite+/TM Liquid Scintillation Cocktail (MP Biomedicals, Solon, OH) for 5 minutes on a Beckman Model LS8500 (Beckman, Inc; Palo Alto, CA) scintillation counter.

Assessment of MDR1 Transporter Activity

Activity of the MDR1 ABC transporter was determined by measuring the uptake of a radiolabeled substrate of this transporter, [3H]-digoxin. [3H]-digoxin is a membrane-permeable molecule that is excluded from the cell based on active transport by MDR1. Proliferating cultures were treated similarly to pharmacological analysis experiments. Ten minutes before the end of pre-incubation, 500 μL of the 1 mL of remaining media was removed and replaced with 500 μL of media with the appropriate inhibitor at twice the desired concentration or basal media alone. After 10 minutes, 500 μL was again removed and replaced with [3H]-digoxin-containing media (50 nM final concentration with specific activity of 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) with or without the appropriate inhibitor, both at twice the desired concentration. For the air exposure group, all media was removed for 15 seconds and replaced with 1 mL of [3H]-digoxin-containing basal media. After 5 minutes, cells were lysed with 2% SDS/8 mM EDTA and 500 μL samples were counted in 5 mL of Ecolite+/TM Liquid Scintillation Cocktail (MP Biomedicals, Solon, OH) for 5 minutes on a Beckman Model LS8500 (Beckman, Inc; Palo Alto, CA) scintillation counter.

Statistical Analysis

Data were analyzed using Statistica 6.0 (Statsoft). qPCR and intracellular ATP data were analyzed using independent sample t-tests. General linear model two-way ANOVA were used to analyze time course and pharmacological analysis data, with subsequent one-way ANOVA and Dunnette’s post-hoc tests. PI, LY and [3H]-digoxin data were analyzed using one-way ANOVA with Dunnette’s post-hoc tests. Differences were considered significant if p<0.05.

| Target | Sequence (5’ → 3’) | Source |
|--------|---------------------|--------|
| YWHAZ, forward | GCCACAATGTCTTGGGCACTAT | Dr. Spiro Getsios, Northwestern University |
| YWHAZ, reverse | TGGTTTGCTGACAAGAGAGCTT | Dr. Spiro Getsios, Northwestern University |
| Keratin 14, forward | Proprietary, Validated Primer | Qiagen |
| Keratin 10, forward | AAAACCGCAAGATGCTGGAAGCCT | Dr. Spiro Getsios, Northwestern University |
| Keratin 10 reverse | CAAAAGGCCCCATGGGACGTAAGTT | Dr. Spiro Getsios, Northwestern University |
| Loricrin, forward | TTCATAGTGCTACCCAGAGTTG | Dr. Spiro Getsios, Northwestern University |
| Loricrin, reverse | GGGTTGGAGGTAGTTGACAGAA | Dr. Spiro Getsios, Northwestern University |
| Involutin, forward | GGCCTCAGCAGAATTGAG | Invitrogen |
| Involutin, reverse | AGCGTGCTAGCCCTTTGTG | Invitrogen |
| Connexin 43, forward | Proprietary, Validated Primer | Qiagen |
| CFTR, forward | Proprietary, Validated Primer | Qiagen |
| Pannexin 1, forward | Proprietary, Validated Primer | Qiagen |
| Pannexin 2, forward | Proprietary, Validated Primer | Qiagen |
| P2X7, forward | CTTTCTCAAACAGAAGGCAAGA | Invitrogen |
| P2X7, reverse | CAACTCGGTGAGGACGAA | Invitrogen |
| MDR1, forward | CACCCGACTTACAGATGATG | Invitrogen |
| MDR1, reverse | GTGCCATTTAGCTGAAAAGA | Invitrogen |
| MRP1, forward | GGCCAAAGAGACATC | Invitrogen |
| MRP1, reverse | AACCGGAACACAAACAGG | Invitrogen |

Table 1. Primers used for qPCR analyses.
Results and Discussion

Keratinocytes Express Multiple ATP Release Pathways

The presence of potential non-vesicular ATP release pathways in NHEK cultures was determined by qPCR analyses. Transcripts for all investigated ATP release pathways were detected in both proliferating and calcium differentiated cultures (Figure 1). Calcium treated cultures showed significantly higher levels of keratin 10, involucrin and loricrin ($p<0.0001$), confirming increased keratinocyte differentiation. Levels of P2X7 and connexin 43 were also significantly higher following calcium differentiation ($p<0.001$), in agreement with previous studies [7,33]. MRP1 showed a small, but significant decrease in differentiated keratinocytes ($p<0.01$), while the other ABC transporters MDR1 and CFTR were relatively unchanged ($p=0.3124$ and $0.1957$, respectively). A previous study reported increased levels of MRP1 and MDR1 using a longer time period of calcium differentiation [34]. In contrast to evidence from immunostaining studies, mRNA levels for pannexin 1 and pannexin 2 were significantly higher in proliferating keratinocytes ($p<0.0001$ and 0.01, respectively) [35]. Such differences in the localization and measure of mRNA and protein signals may result from non-specificity of antibodies or translational and post-translational protein regulation. We have previously shown that voltage-gated sodium channels have a wider distribution of mRNA compared to immunostaining in keratinocytes [5].

Characteristics of Air-stimulated ATP Release

Exposing keratinocytes to air for 15 seconds caused a robust, long-lasting ATP release that was significantly higher in differentiated cultures (Figure 2a, ANOVA $p<0.01$). This release was not due to cell lysis, as the same procedure did not increase PI staining (Figure 3a, $p=0.99$). This release was also not merely due to mechanical stimulation, as an exchange of half the media in controls did not result in any detectable ATP release. ATP levels in the media were significantly higher than baseline in both cultures at 2, 5, 10, 15 and 25 minutes (Figure 2a, $p<0.01$), with differentiated keratinocytes showing higher ATP levels at five minutes ($p<0.05$). Given the usually rapid metabolism of extracellular ATP, it seems likely that some release continues throughout this time course, although there may also be a contribution of re-phosphorylated AMP and ADP [36,37].

The amount of intracellular ATP remaining in air-exposed cells suggests that the ATP released during this process is a sizable proportion of intracellular ATP, although potential changes in synthesis and metabolism were not analyzed. Seven minutes after treatment, air-exposed cells had a significant reduction (~25%) in total intracellular ATP (Figure 2b, $p<0.01$). Intracellular ATP levels in air-exposed cultures recovered slightly by 60 minutes and were no longer significantly different than control ($p=0.089$). In standard keratinocyte cell culture procedures, feeding cells requires replacement of media every 2–3 days, which routinely involves brief air exposure. The fact that such air-exposed cells maintain viable cultures demonstrates that these cells recover from this stimulus and the lack of increased PI staining shows cell membranes are not compromised (Figure 3a).

Air Exposure Triggers ATP Release Through Connexin Hemichannels

PI labeling confirmed that drugs used to inhibit ATP release pathways cause little, if any harm to the cells (Figure 3a). Only carbenoxolone caused a significant increase in the percentage of PI labeled cells ($p<0.05$) and this still remained less than 3% of total cells. Differentiated keratinocytes had significantly lower baseline extracellular ATP levels compared to proliferating cells (Figure 3b, ANOVA $p<0.05$). This could be due to a change in un-stimulated ATP release or metabolism caused by the calcium differentiation, or simply a consequence of the higher cell density that occurs in differentiated cultures. No inhibitor significantly reduced un-stimulated ATP release, although verapamil resulted in a significant increase ($p<0.05$).

In both proliferating and differentiated keratinocytes, air-stimulated ATP release was almost completely prevented by the connexin hemichannel inhibitors 1-octanol and carbenoxolone (Figure 3c). Carbenoxolone can block both connexins and pannexins, but no inhibitory effect was seen with probenecid, which can also inhibit pannexins [38]. Glibenclamide and verapamil, drugs traditionally used to inhibit ABC transporters, also significantly inhibited air-stimulated ATP release from NHEK cultures (Figure 3c, $p<0.0001–0.05$). The inhibition by both

![Figure 1. mRNA expression of putative ATP release pathways in NHEK.](image-url)
Connexin hemichannels have recently been suggested in airway epithelial cells [40]. To determine whether both the MDR1 ABC transporter and connexin hemichannels were involved in air-stimulated ATP release, uptake of the connexin permeable dye LY and exclusion of the radiolabeled MDR1 substrate \(^{3}H\)-digoxin were measured following air exposure (Figures 4 and 5, respectively). Air exposure significantly increased the uptake of LY (Figure 4, \(p<0.001\)), consistent with an opening of connexin hemichannels in response to this stimulus. This increase in LY uptake was blocked by the connexin hemichannel blocker 1-octanol, but also by the ABC transporter inhibitors glibenclamide and verapamil \((p=0.7814\)–1.0). The increase in LY uptake following air exposure, but not in the presence of drugs that inhibit air-stimulated ATP release, indicates that connexin hemichannels are open during air-stimulated ATP release.

In contrast, \(^{3}H\)-digoxin exclusion experiments showed that activity of MDR1 is inhibited by air exposure, thus it is unlikely that this transporter is involved in air-stimulated ATP release (Figure 5). Air exposure significantly increased intracellular levels of \(^{3}H\)-digoxin \((p<0.0001)\), indicating a reduced capacity of MDR1 to transport this substrate out of the cell. A similar effect was seen for the known MDR1 inhibitors glibenclamide and verapamil in the absence of air exposure \((p=0.0001)\). Unexpectedly, 1-octanol also significantly increased uptake of \(^{3}H\)-digoxin \((p<0.0001)\), suggesting this drug may also inhibit MDR1. Carbenoxolone was without effect \((p=0.9040)\), demonstrating that not all drugs that block air-stimulated ATP release also inhibit MDR1. It seems possible that the reduction in intracellular ATP, required for MDR1 transport, could be responsible for the inhibition of this transporter following air exposure.

Taken together, our data provide strong evidence that air exposure stimulates ATP release from keratinocytes through connexin hemichannels and that the inhibitory effects of the ABC transporter inhibitors glibenclamide and verapamil on this release may be due to non-specific actions on these hemichannels. It is also possible that the inhibitory effects of glibenclamide and verapamil on connexin hemichannels are through an intermediate target. For example, these drugs are known to inhibit ion channels expressed by keratinocytes, which may in turn have an influence on connexin hemichannels [41,42,43,44].

Previous studies have provided compelling evidence that, at least in some cell types, ATP release can occur through connexin hemichannels [45]. Expression of connxin 43, the major connexin in keratinocytes, is increased in suprabasal keratinocytes in vivo [45,46]. Consistent with these observations, our data showed increased levels of connexin 43 mRNA and a corresponding increase in air-stimulated ATP release in differentiated cultures. While connexin 43 is the most highly expressed connexin in keratinocytes, many other subtypes are present and may also contribute to air-stimulated ATP release. More detailed analyses beyond the scope of this study, possibly utilizing siRNA and connexin inhibiting peptides, would be necessary to determine which specific connexin subtypes are involved.

Connexin hemichannels could also be involved in keratinocyte ATP release in response to other tactile stimuli. For example, connexin hemichannels have been shown to be mechanosensitive and connexin inhibitors reduce calcium waves mediated by ATP release in response to mechanical stimulation of cultured keratinocytes [12,47]. While it was concluded that the reduced calcium wave propagation in the presence of these drugs was due to blockade of gap junctions between neighboring keratinocytes, a blockade of ATP-permeable hemichannels that opened in response to mechanical stimulation was not ruled out as a contributing factor.

A significant increase in air-stimulated ATP release was seen in the presence of probenecid in proliferating cultures, although the reason for this is unclear (Figure 3c, \(p<0.05\)). Probenecid did not cause an increase in un-stimulated ATP release, indicating it does not cause ATP release itself (Figure 3b).

### Potential Role for Air-stimulated ATP Release in Epidermal Homeostasis, Barrier Formation and Dermatological Disease

ATP released from keratinocytes through interactions with air may be an important signal in regulating epidermal homeostasis.
ATP plays a complex role in epidermal homeostasis and barrier formation by signaling through P2Y and P2X receptors which are primarily restricted to basal and suprabasal keratinocytes, respectively [7]. This is further complicated by the different affinities of purinergic receptors for ATP metabolites. P2Y1 and P2Y2 activation increases DNA synthesis and cell number in submerged keratinocyte cultures and also increases epidermal thickness in vivo [7]. In contrast, P2X5 and P2X7 activation reduces keratinocyte number in culture and reduces epidermal thickness in vivo.

Cutaneous injection of ATP causes epidermal thickening, indicating P2Y receptor-stimulated proliferation predominates over the anti-proliferative effects of P2X receptors. Along with their apparent anti-proliferative effects, P2X receptors also inhibit terminal differentiation and barrier formation [48,49].

There is evidence that air-stimulated ATP release regulates epidermal homeostasis in vivo. Disruption of the epidermal barrier by tape stripping or acetone, a process that would increase air exposure of keratinocytes, results in increased cutaneous ATP release and signaling. Tape stripping resulted in increased ATP release from skin sections 15 minutes after barrier disruption, while acetone treatment led to ATP dependent increases in epidermal thickness and DNA synthesis in basal keratinocytes [48,50,51]. Moreover, a dry environment also induces epidermal thickening, while a humid environment has the opposite effect [51]. These changes in epidermal thickness may be due to alterations in keratinocyte ATP release through connexin hemichannels in response to humidity in the external environment. In addition, air-stimulated ATP release may participate in wound healing.
Air-stimulated ATP Release from Keratinocytes

Figure 4. Assessment of connexin hemichannel involvement in air-stimulated ATP release. Uptake of the connexin permeable dye lucifer yellow (LY) was measured to confirm that connexin hemichannels are open in response to air exposure and are blocked by the same drugs that prevent ATP release. Cells were pre-incubated with ATP release inhibitors using the same procedure as for the pharmacological analysis assays. LY (500 µM final concentration) was added in the media after air exposure or a change of half the media (control). Sixty minutes later, cultures were rinsed twice with media containing 1-octanol (2 mM) to remove residual LY, but minimize leakage of absorbed LY. LY levels in the cell lysates were then measured. Air exposure significantly increased LY uptake compared to control and the same drugs that inhibited air-stimulated ATP release prevented this increase in LY uptake. This indicates that connexin hemichannels are open during air-stimulated ATP release. Values represent 8 or 9 wells per group +/- SEM, * p<0.05 vs. control. doi:10.1371/journal.pone.0056744.g004

Figure 5. Assessment of MDR1 transporter involvement in air-stimulated ATP release. Exclusion of a radiolabeled substrate of the MDR1 ABC transporter was measured to determine whether activity of this transporter is increased during air-stimulated ATP release. Note that this transporter acts to exclude substrates, so an increase in [3H]-digoxin uptake reflects an inhibition of transporter activity. Confluent NHEK cultures were pre-incubated with ATP release inhibitors using the same protocol as for the pharmacological analysis assays. [3H]-digoxin (50 nM final concentration) was added in the media after air exposure or a change of half the media (control). After 7 minutes, the time of peak air-stimulated ATP release, [3H]-digoxin levels in cell lysates were measured. Air exposure significantly increased [3H]-digoxin uptake, as did the ABC transporter inhibitors glibenclamide and verapamil. The connexin blocker 1-octanol also significantly inhibited uptake, while the other connexin blocker carbenoxolone was without effect. The inhibition of MDR1 following air exposure, combined with the disparate effects of ATP release inhibitors, indicates that this transporter is not involved in air-stimulated ATP release. Values represent 6 wells per group +/- SEM, * p<0.05 vs. control. doi:10.1371/journal.pone.0056744.g005

Potential Role for Air-stimulated ATP Release in Chronic Pain

In addition to a likely role in normal tactile sensation, there is accumulating evidence supporting the ability of keratinocyte signaling to influence pain sensation [53,54]. Keratinocytes surround the terminals of nociceptive sensory neurons and release molecules that sensitize and activate these neurons (e.g. prostaglandin E2 and ATP), as well as those that inhibit their activity (e.g. β-endorphin) [6,55]. We have previously shown that the peripheral anti-nociceptive effects of ETB and CB2 receptor agonists likely involve the release of β-endorphin from keratinocytes, leading to reduced activity of µ opioid receptor-expressing nociceptive fibers [6,56]. Normal tactile sensation, as well as acute and chronic pain, likely involve a balance between these keratinocyte excitatory and inhibitory neuromodulatory mechanisms.

Many of the signaling molecules that are released by keratinocytes help to regulate their own proliferation and differentiation, but also have the potential for contributing to pain sensation, especially when nociceptor sensitivity is enhanced under pathological conditions such as nerve injury. As described above, the keratinocyte cells that make up the epidermis release ATP to regulate epidermal homeostasis, a process that may be influenced by air-stimulated ATP release through connexin hemichannels. The cutaneous release of ATP, in particular, may make an important contribution to chronic pain [2].

Injection of ATP into the skin causes overt pain, most likely through the activation of P2X3 receptors expressed in small diameter neurites that innervate the superficial epidermis [1,10,57]. During inflammation, P2X3 receptors become sensitized in both animal models and human patients [57,58,59,60]. Anti-sense knockdown of P2X3 in DRG results in reduced responses to ATP, as well as reduced thermal and mechanical hyperalgesia in inflammatory and neuropathic pain models [61,62]. Furthermore, subcutaneous injection of P2X antagonists...
(e.g. the P2X3 specific inhibitor A-317491), are effective at reducing ongoing inflammatory and neuropathic pain, supporting a contribution of endogenous ATP release in the skin to chronic pain [13,14,15,16]. P2Y receptors, especially P2Y1 and P2Y2, are also expressed by primary sensory endings and may participate in chronic pain states. For example, knockout of P2Y2 receptors prevents the development of CFA-induced thermal hyperalgesia, possibly through an interaction with TrpV1 [63].

Low levels of keratinocyte ATP release during epidermal stratiﬁcation and in response to innocuous tactile stimulation may help regulate normal tactile sensation, but this release normally does not reach the level necessary to trigger a painful response [2]. In addition, purinergic antagonists often lack effects in acute mechanical and thermal nociceptive testing where an intense, escapable thermal or mechanical stimulus is applied to the skin (i.e. von Frey and hot plate tests) [14]. Thus, cutaneous ATP release does not appear to contribute to pain in the absence of tissue injury.

Under chronic pain conditions such as inflammation and nerve injury, however, nerve endings become sensitized and this normally innocuous level of epidermal ATP may now be sufﬁcient to cause activation that reaches a nociceptive threshold [13]. Consequently, ATP released from keratinocytes in response to mild thermal and mechanical stimulation and during normal epidermal homeostasis may now contribute to tactile hypersensitivity and spontaneous pain.

Despite the likely importance of cutaneous ATP in chronic pain, the source of this ATP has remained unknown. Keratinocyte ATP release through connexin hemichannels represents a major potential source of this pro-algesic molecule. In fact, systemic administration of the connexin inhibitor carbenoxolone has been shown to reduce inflammatory pain in a CFA model, but this route of administration makes it unclear whether these effects could be due to reduced keratinocyte ATP release or through an exclusively central mechanism [64]. Future studies testing the cutaneous effects of connexin hemichannel blockers in animal models of chronic pain will help clarify a role for keratinocyte hemichannels in these conditions.

Conclusion

In this study, we show that brief air exposure causes a robust ATP release from keratinocytes and provide strong evidence that this release occurs through connexin hemichannels. We propose that this mechanism is constantly at play during epidermal homeostasis and may also contribute to chronic pain through activation of purinergic receptors on sensitized epidermal nerve endings. Importantly, such an on-going release of ATP in the epidermis may contribute to spontaneous pain, which has traditionally been the most difficult aspect of human chronic pain to treat. It is also possible that keratinocyte/ATP release in response to thermal and mechanical stimulation involves connexin hemichannels and contributes to tactile hypersensitivity under chronic pain conditions. Chronic pain and dermatological disease can often occur concomitantly and may involve common underlying pathologies in keratinocyte signaling. Further research into ATP release from keratinocytes will likely yield valuable insights into the sensory and structural functions of the epidermis and potentially lead to the identiﬁcation of new targets for the treatment of chronic pain and dermatological disorders.

Acknowledgments

The authors would like to thank Marilyn Dockum for her excellent technical assistance and Dr. Spiro Getsios of Northwestern University (Chicago, IL) for generously providing some of the qPCR primers used in this study.

Author Contributions

Conceived and designed the experiments: TPB. Performed the experiments: TPB. Analyzed the data: TPB. Contributed reagents/materials/analysis tools: JAM AAM GRS FLR. Wrote the paper: TPB.

PLOS ONE | www.plosone.org 8 February 2013 | Volume 8 | Issue 2 | e56744

References

1. Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrpgrd. Neuron 45: 17–25.
2. Dussor G, Koerber HR, Oaklander AL, Rice FL, Moliver DC (2009) Nucleotide signaling and cutaneous mechanisms of pain transduction. Brain Res Rev 60: 24–35.
3. Kwan KY, Glazer JM, Corey DP, Rice FL, Stucky CL (2009) TRPA1 modulates mechanotransduction in cutaneous sensory neurons. J Neurosci 29: 4808–4819.
4. Hou Q, Barr T, Gee L, Vickers J, Wymer J, et al. (2011) Keratinocyte expression of calciomycin gene-related peptide beta: Implications for neuropathic and inflammatory pain mechanisms. Pain. 159: 90–103.
5. Khorshid A, Navarro B, Jousilive LS, Murphy JE, Rice FL, et al. (2003) Endothelin-B receptor activation triggers an endogenous analgesic cascade at sites of peripheral injury. Nat Med 9: 1053–1061.
6. Greig AV, Linge C, Terenghi G, McGrouther DA, Burnstock G (2003) Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. J Invest Dermatol 120: 1007–1015.
7. Burnstock G (2009) Purinergic receptors and pain. Curr Pharm Des 15: 1717-1735.
8. Cook SP, McCleskey EW (2002) Cell damage excites nociceptors through release of cytosolic ATP. Pain 95: 41–47.
9. Wirker K, Spelthagh B, Eles P (2007) P2X3 receptor involvement in pain states. Mol Neurobiol 36: 165–183.
10. Mandali S, Sokabe T, Shihasaki K, Katayosaka K, Mizuno A, et al. (2009) TRPV3 in keratinocytes transmits temperature information to sensory neurons via ATP. Pflügers Arch 458: 1093–1102.
22. Gangatipar P, Paquette-Fidler S, Li A, Rossi R, Kaur P (2007) Establishment of 3D organotypic cultures using human neonatal epidermal cells. Nat Protoc 2: 178–186.

23. Simpson CL, Kojima S, Getzios S (2010) RNA interference in keratinocytes and an organotypic model of human epidermis. Methods Mol Biol 565: 127–146.

24. Anderson CM, Nedergraad M (2006) Emerging challenges of assigning P2X7 receptor function and immunoreactivity in neurons. Trends Neurosci 29: 257–262.

25. Ballestrini P, Di Iorio P, Ciccarelli R, Nargi E, D’Alimonte I, et al. (2002) Gli cells express multiple ATP binding cassette proteins which are involved in ATP release. Neuroreport 13: 1769–1792.

26. Bruzzone R, Barre MT, Jakob NJ, Monyer H (2003) Pharmacological properties of homomeric and heteromeric panxxin hemorrhagic expressed in Xenopus oocytes. J Neurochem 92: 1033–1043.

27. Fan HT, Morishima S, Kidia H, Okada Y (2001) Phloretin differentially inhibits volume-sensitive and cyclic AMP-activated, but not Ca-activated, Cl(-) channels. Br J Pharmacol 133: 1096–1106.

28. Liu HT, Tashmukhamedov BA, Inoue H, Okada Y, Sabirov RZ (2006) Roles of two types of anion channels in glutamate release from mouse astrocytes under ischemic or osmotic stress. Glia 54: 345–357.

29. Minich T, Riemer J, Schulz J, Wielinga P, Wijhaldes J, et al. (2006) The multidrug resistance protein 1 (Mrp1), but not Mrp5, mediates export of glutathione and glutathione disulfide from brain astrocytes. J Neurochem 97: 373–384.

30. Roman RM, Louni N, Braunstein G, Feranchak AP, Simeoni LA, et al. (2001) Evidence for multidrug resistance-1 P-glycoprotein-dependent regulation of cellular ATP permeability. J Membr Biol 183: 165–173.

31. Sharpea JD, Woldh MJ (1992) Effect of ATP-sensitive K+ channel regulators on cysis fibrosis tissue membrane conductance regulator chloride currents. J Gen Physiol 100: 575–591.

32. Ye ZC, Wyeth MS, Bahan-Tekko S, Ransom BR (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci 23: 3389–3506.

33. Goger JA, Paul DL (1994) Expression of gap junction proteins Cx26, Cx31.1, Cx37, and Cx43 in developing and mature rat epidermis. Dev Dyn 200: 1–13.

34. Kielar D, Kaminski WE, Liebisch G, Pelhier A, Wenzel JJ, et al. (2003) Adenosine triphosphate binding cassette (ABC) transporters are expressed and regulated during terminal keratinocyte differentiation: a potential role for ABCA7 in epidermal lipid reorganization. J Invest Dermatol 121: 465–474.

35. Celetti SJ, Cowan KN, Penuela S, Shao Q, Churko J, et al. (2010) Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation. J Cell Sci 123: 1363–1372.

36. Burrell HE, Wlodarski B, Foster BJ, Buckley KA, Sharpe GR, et al. (2005) ABCA7 in epidermal keratinocytes and effects of agonist and antagonist function and immunoreactivity in neurons. Trends Neurosci 29: 257–262.

37. Denda M, Sato J, Masuda Y, Tsujiya T, Koyama, J, et al. (1998) Exposure to a dry environment enhances epidermal permeability barrier function. J Invest Dermatol 111: 858–863.

38. Bao L, Sachs F, Dahl G (2004) Connexin-43 hemichannels are involved in ATP-stimulated ATP release from keratinocytes. J Cell Physiol 208: 51–60.

39. Schmid-Antomarchi H, de Weille J, Fosset M, Lazdunski M (1987) The antidiabetic sulfonylurea glibenclamide is a potent blocker of the ATP-modulated K+ channel in insulin secreting cells. Biochem Biophys Res Commun 146: 21–25.

40. Kang J, Kang N, Lovatt D, Torres A, Zhao Z, et al. (2008) Connexin 43 hemichannels are permeable to ATP. J Neurosci 28: 4702–4711.

41. Scheckenbach KL, Losa D, Dudez T, Bacchetta M, O’Grady S, et al. (2010) P2X7 receptor function and immunoreactivity in neurons. Trends Neurosci 29: 257–262.

42. Denda M, Inoue K, Fuzisawa S, Senda D (2002) P2X7 purinergic receptor antagonist accelerates skin barrier repair and prevents epidermal hyperplasia induced by skin barrier disruption. J Invest Dermatol 119: 1034–1040.

43. Mullin SA, Davis BM, Koerber HR, Reynolds JH, Albers KM, et al. (2008) Overexpression of human connexin 43 causes an organotypic model of human epidermis. Methods Mol Biol 585: 127–146.

44. Hamilton SG, Warburton J, Bhattacharjee A, Ward J, McMahon SB (2000) ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. Brain 123 (P 6): 1238–1246.

45. Hamilton SG, Wade A, McMahon SB (1999) The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. Br J Pharmacol 126: 526–332.

46. Paukert M, Osteroth R, Geisler HS, Brandle U, Glowatzki E, et al. (2001) Adenosine triphosphate binding cassette proteins which are involved in ATP release. J Cell Physiol 208: 51–60.