ORIGINAL ARTICLE

In Vivo Imaging Demonstrates ATP Release from Murine Keratinocytes and Its Involvement in Cutaneous Inflammation after Tape Stripping

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Adenosine 5′-triphosphate (ATP) release from keratinocytes has been observed in various stress models *in vitro*, but studies demonstrating epidermal ATP release *in vivo* are limited. To visualize extracellular ATP (eATP) *in vivo*, we developed enhanced green-emitting luciferase immobilized on agarose beads (Eluc-agarose). Subcutaneous injection of Eluc-agarose together with ATP into the dorsal skin of BALB/c mice following intraperitoneal luciferin injection produced detectable and measurable bioluminescence using an *in vivo* imaging system. Using Eluc-agarose, we demonstrated *in vivo* that bright bioluminescence was observed from 1 to 20 minutes after repeated tape stripping of murine skin. This bioluminescence was suppressed by the local administration of apyrase. Eluc-agarose bioluminescence was observed only in tape-stripped skin with transepidermal water loss (TEWL) between 100 and 140 $g\,m^2\,h^{-1}$, indicating a loss of bioluminescence with excessive tape stripping (TEWL > 140 $g\,m^{-2}\,h^{-1}$). Histologically, tape-stripped skin with detectable eATP had a viable epidermis and a subepidermal neutrophil infiltrate, and administration of apyrase reduced the inflammatory infiltrate. Neither a viable epidermis nor an upper dermal neutrophil infiltrate was observed after excessive tape stripping. These results suggest that tape stripping prompts ATP release from viable keratinocytes, which facilitates inflammatory cell migration. Eluc-agarose may be useful in the *in vivo* detection of eATP in murine models of skin diseases.

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INTRODUCTION

Tape stripping of the stratum corneum (SC), a long-standing technique used in dermatology research to induce epidermal damage, disrupts the cutaneous epithelial barrier and induces various biological responses in the skin. For instance, it significantly affects the terminal differentiation of keratinocytes (de Koning *et al.*, 2012) and increases epidermal DNA synthesis (reviewed by Man *et al.* (1999)) as part of the homeostatic repair response in the epidermis. Tape stripping also induces the production of a specific array of inflammatory cytokines from keratinocytes, such as tumor necrosis factor-α, IL-8, IL-10, TSLP, IL-33, transforming growth factor-β, IL-1α, vascular endothelial growth factor, IL-1β; endothelial adhesion molecules such as E-selectin and vascular cell adhesion molecule-1 (Wood *et al.*, 1992; Nickoloff and Naidu, 1994; Wood *et al.*, 1996; Elias *et al.*, 2008; Dickel *et al.*, 2010; Oyoshi *et al.*, 2010); chemokines (Onoue *et al.*, 2009); and anti-bacterial peptides (Aberg *et al.*, 2008; Glaser *et al.*, 2009). Furthermore, tape stripping induces Langerhans cell maturation (Takigawa *et al.*, 2001) and migration from the epidermis to the draining lymph nodes (Holzmann *et al.*, 2004). Recently, it has been reported that tape stripping recruits plasmacytoid dendritic cells in addition to neutrophils to the skin (Guiducci *et al.*, 2010).

In addition to inducing mild epidermal injury and inflammation, tape stripping has been used to precipitate disease in mouse models of chronic eczematous dermatitis (Matsunaga *et al.*, 2007), psoriasis (Sano *et al.*, 2005), atopic dermatitis (Jin *et al.*, 2009), and lupus erythematodes (Guiducci *et al.*, 2010). Moreover, it is well known that tape stripping is effective in inducing Koebner phenomenon in psoriatic patients (reviewed by Weiss *et al.*, 2002).

Despite the well-documented use of tape stripping to disrupt the epidermal barrier, the mechanism underlying the biological responses caused by tape stripping remains unclear. It is possible that "endogenous danger signals" or "damage-associated molecular patterns" (DAMPs)—host molecules involved in potentiating the immune response against danger (reviewed by Willart and Lambrecht (2009))—may be responsible for the responses seen after tape stripping.

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Abbreviations: ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-(γ-thio)triphosphate; DAMP, damage-associated molecular pattern; eATP, extracellular ATP; mRNA, messenger RNA; PMN, polymorphonuclear cell; SC, stratum corneum; TEWL, transepidermal water loss

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The following molecules, among others, have been identified as DAMPs: adenosine 5′-triphosphate (ATP), heat shock proteins, hyaluronan, monosodium urate, galectins, thioredoxin, adenosine, high-mobility group box protein 1, IL-1α, and IL-33 (reviewed by Hirsinger *et al.*, 2012). In this study, we focused on the role of extracellular ATP (eATP) in the cutaneous response after tape stripping.

ATP is a ubiquitous carrier of chemical energy and a building block of genetic material in all living organisms (reviewed by Brake and Julius (1996)), and transient increases in eATP occur during cell-to-cell communication in the nervous, vascular, and immune systems (reviewed by Burnstock, 2007). In healthy tissues, cellular release of ATP is tightly regulated and their concentrations are kept low by eATP/ADPases (Kaczmarek *et al.*, 1996; Picher *et al.*, 2003). In damaged tissues, ATP is released from injured cells to initiate inflammation and to amplify and sustain cell-mediated immunity through P2 receptor–mediated purinergic signaling (Bours *et al.*, 2006). In particular, ATP is an essential danger signal as it leads to IL-1β and IL-18 secretion by activating inflammasomes (reviewed by Vitiello *et al.*, 2012).

Keratinocyte release of ATP has been observed *in vitro* in various settings, such as exposure to UV light (Takai *et al.*, 2011), γ-ray (Tsukimoto *et al.*, 2010), mechanical stimulation (Koizumi *et al.*, 2004), and in hypo-osmotic shock (Azorin *et al.*, 2011). To the best of our knowledge, eATP in the skin has been demonstrated *in vivo* only in murine allergic contact dermatitis (Weber *et al.*, 2010) and in graft-versus-host-disease (Wilhelm *et al.*, 2010) using HEK293-pmeLUC cells expressing plasma membrane–targeted luciferase (Pellegatti *et al.*, 2005).

In the present study, we developed cell-free, enhanced green-emitting luciferase immobilized on agarose beads (Eluc-agarose) to enable visualization of eATP *in vivo*, thus avoiding artifacts from using live cells with plasma membrane–targeted luciferase. Using Eluc-agarose, we demonstrated that mechanical skin barrier disruption releases eATP from viable epidermal keratinocytes, which facilitated the migration of inflammatory cells into the dermis.

RESULTS

Generation of luciferase beads and evaluation of bioluminescence

To visualize eATP *in vivo*, we developed Eluc-agarose using Ni-NTA Superflow (Figure 1a). *In vitro* luminescence experiments of Eluc-agarose mixed with luciferin and various concentrations of ATP showed that in the first 30 minutes after addition of ATP, the bioluminescence of the mixture was dependent on the ATP dose (Figure 1b). Next, when we injected Eluc-agarose mixed with 0.1, 1, or 10 mM of ATP following intraperitoneal luciferin injection, only Eluc-agarose mixed with 10 mM ATP produced bright bioluminescence while mixtures with 0.1 or 1 mM ATP produced weak and inconsistent bioluminescence (Figure 1c and d).

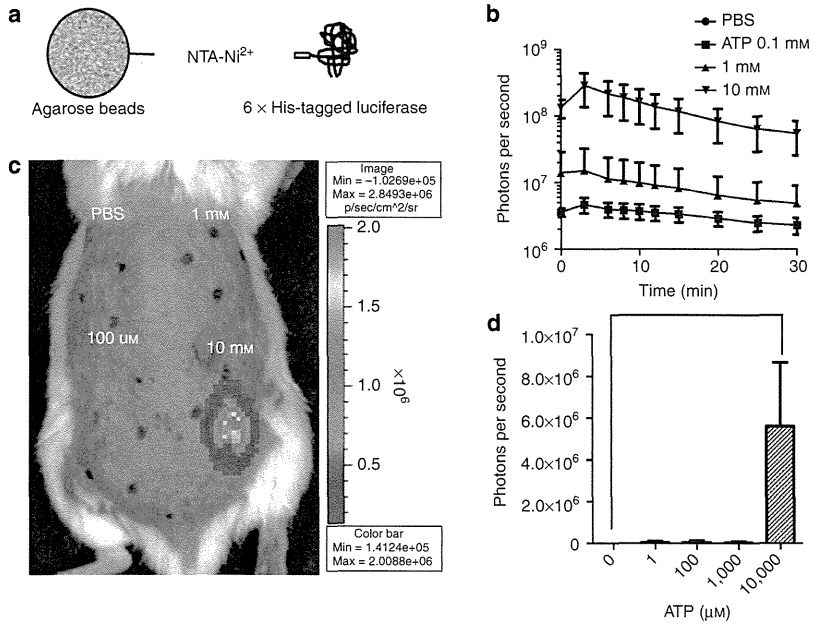

Figure 1. *In vivo* imaging of subcutaneously injected adenosine 5′-triphosphate (ATP) using Eluc-agarose. (**a**) Pictorial representation of green-emitting luciferase immobilized on agarose beads (Eluc-agarose). (**b**) Fifty microliters of Eluc-agarose and 50 μl of d-luciferin were mixed with 100 μl of different concentrations (0.1, 1, and 10 mM) of ATP, and the luminescent signal was measured using the *In Vivo* Imaging System (IVIS). (**c**) Eluc-agarose mixed with 0.1, 1, or 10 mM of ATP was subcutaneously injected into dorsal murine skin following intraperitoneal luciferin injection, and luminescence was visualized using IVIS. The color scale on the right-hand side shows the range of signal intensity in photons per second at the surface of the animals. A representative picture from five independent experiments is shown. (**d**) Graph of bioluminescence intensity versus concentration of exogenous ATP. Data (mean ± SD) from five mice are shown. Statistical significance was calculated using one-way analysis of variance test followed by a Dunnett's post test compared with the control group. PBS, phosphate-buffered saline.

In vivo imaging of eATP in murine skin after tape stripping
As Eluc-agarose can be used to visualize at least 10 mM of eATP *in vivo*, we next examined whether barrier disruption by tape stripping causes ATP release in murine skin. When transepidermal water loss (TEWL) was measured at various times after repeated tape stripping of the dorsal skin of mice, it was found that TEWL correlated with the number of times tape stripping was performed, but the TEWL value could not be accurately predicted by the number of tape stripping repeats (Figure 2a). Therefore, we used the TEWL value as an indicator of the degree of epidermal barrier disruption after tape stripping. When TEWL measurement was performed along with eATP visualization at 2- to 5-minute intervals after tape stripping up to 30 minutes, bright Eluc-agarose bioluminescence was observed only when TEWL was in the range of 100–140 $g\,m^{-2}\,h^{-1}$, with no detectable bioluminescence when TEWL was either $<100\,g\,m^{-2}\,h^{-1}$ or $>140\,g\,m^{-2}\,h^{-1}$ (Figure 2b and c). The maximum bioluminescence detected after optimum tape stripping was significantly different from that of intact skin (Figure 2b). Thus, optimum tape stripping/barrier disruption is required to detect the release of eATP using *in vivo* imaging with Eluc-agarose. Furthermore, bioluminescence was detectable 1 minute after optimum tape stripping and started to diminish 15 minutes later (Figure 2d and e). To exclude the possibility that a loss of activity of Eluc-agarose or luciferin caused the diminished bioluminescence, we injected subcutaneous Eluc-agarose and intraperitoneal luciferin 30 minutes after tape stripping, but the bioluminescence was not restored (data not shown). However, an injection of exogenous ATP resulted in bioluminescence lasting at least 3 hours after a single subcutaneous injection of Eluc-agarose if the luciferin injection was repeated (data not shown).

Histological correlation with eATP bioluminescence
Next, we examined the histopathological changes 24 hours after tape stripping to examine the biological effects of eATP in the tape-stripped skin. No significant histological difference was observed between intact and tape-stripped skin, with a TEWL $<100\,g\,m^{-2}\,h^{-1}$, and there was no detectable bioluminescence (Figure 3a and b). Tape-stripped skin with a TEWL of 100–140 $g\,m^{-2}\,h^{-1}$ and bright bioluminescence showed considerable accumulation of inflammatory cells in the SC, a viable epidermis and a dense subepidermal inflammatory infiltrate (Figure 3c and d). In contrast, tape-stripped skin with a TEWL $>140\,g\,m^{-2}\,h^{-1}$ and no detectable bioluminescence showed no inflammatory cells in the SC, a necrotic or denuded epidermis, and a sparse inflammatory infiltrate in the superficial dermis and moderate inflammation in the deep dermis (Figure 3e). Most of the infiltrating cells in the SC and superficial dermis were polymorphonuclear cells (PMNs), as demonstrated by hematoxylin–eosin and immunohistochemical staining with anti-Ly6G antibody (Figure 3f).

Inhibitory effect of subcutaneously injected apyrase
As tape stripping may result in the release of DAMPs other than ATP, we investigated whether a reduction in ATP attenuates the luminescent signal and accompanying histological changes by injecting apyrase (adenosine diphosphatase) 30 minutes before tape stripping. Subcutaneous injection of apyrase before optimum tape stripping suppressed the bioluminescence (Figure 4a and b) and significantly reduced the corneal and subepidermal inflammatory infiltrate compared with control skin subcutaneously injected with phosphate-buffered saline (Figure 4c, d, and e).

Chemokine messenger RNA expression in the skin after optimum tape stripping and its suppression by subcutaneous injection of apyrase
As PMNs are recruited to the skin after optimal tape stripping and we previously reported that ATP induced CXCL1, -2, and -3 messenger RNA (mRNA) by human epidermal keratinocytes *in vitro* (Ohara *et al.*, 2010), we examined the expression CXCL1, -2, and -3 mRNA together with another neutrophil chemokine, CXCL5, in tape-stripped murine skin with or without prior subcutaneous injection of apyrase (Figure 5). The mRNA expression of CXCL1, -2, and -3, but not CXCL5, was elevated in tape-stripped skin compared with intact skin, while this phenomenon was not observed after excessive tape stripping. Subcutaneous administration of apyrase diminished the high mRNA levels of CXCL1, -2, and -3 seen after optimum tape stripping. These findings suggest that keratinocyte release of eATP after tape stripping stimulates the production of chemokines in an autocrine manner that leads to PMN recruitment to the skin.

Neutrophil recruitment and chemokine mRNA expression after subcutaneous injection of exogenous ATP and ATP analog
Next, we examined the effect of subcutaneous injection of ATP and adenosine 5′-(γ-thio)triphosphate (ATPγS), a hydrolysis-resistant ATP analog, on neutrophil recruitment and chemokine mRNA expression. Cutaneous injection of 10 mM ATP or ATPγS significantly augmented CXCL1, -2, and -3 chemokine mRNA expression 6 hours after injection, and cutaneous neutrophil recruitment was evident histologically 24 hours after injection (Supplementary Figures S1 and S2 online). However, it was noted that the magnitude of the increase in mRNA expression and the degree of neutrophil infiltration were smaller than those seen after tape stripping, possible due to insufficient diffusion of subcutaneously injected ATP into the epidermis or rapid degradation.

DISCUSSION
In this study, we demonstrated that Eluc-agarose could be used to visualize eATP in murine skin *in vivo*. Previously, *in vivo* imaging of eATP was conducted by using HEK293-pmeLUC cells expressing plasma membrane–targeted luciferase (Pellegatti *et al.*, 2005). Despite its success in demonstrating eATP in murine allergic contact dermatitis (Weber *et al.*, 2010) and graft-versus-host-disease (Wilhelm *et al.*, 2010), this method has drawbacks, including the necessity of cell culture, and the unknown effects of the cell graft on its host environment. In contrast, Eluc-agarose is a cell-free system, which avoids the unknown effects of injected cell-based luciferase.

Recent evidence suggests that cells subjected to stress, injury, or those undergoing necrosis secrete various DAMPs;

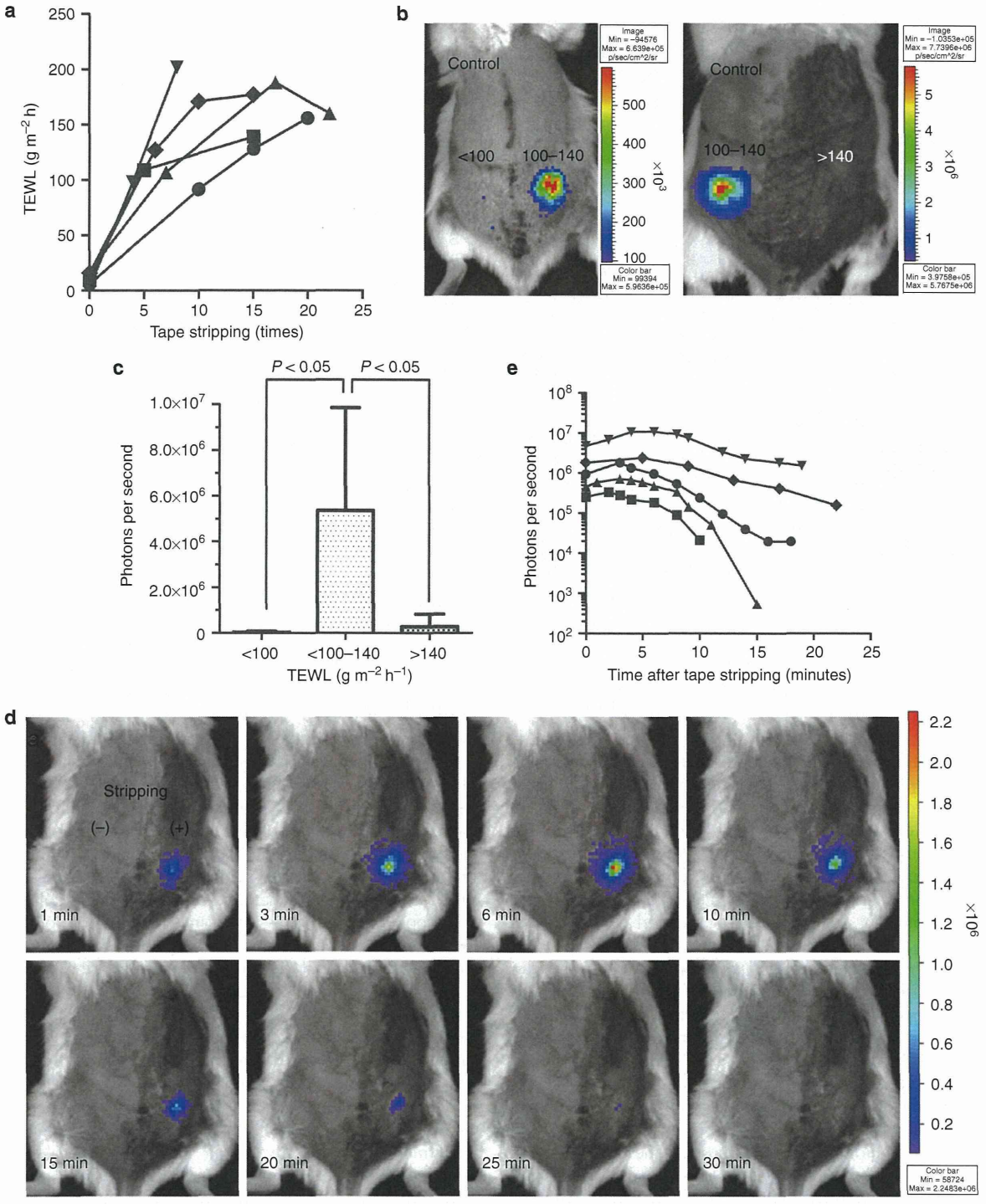

Figure 2. *In vivo* imaging of extracellular adenosine 5′-triphosphate (eATP) after tape stripping using Eluc-agarose. (**a**) Transepidermal water loss (TEWL) positively correlates with the number of times of tape stripping. Each line represents data from one animal, and one representative experiment is shown. (**b**) Bright Eluc-agarose bioluminescence was observed at the site of tape-stripped skin with a TEWL between 100 and 140 $g\,m^{-2}\,h^{-1}$, while no bioluminescence was detected if TEWL was either $<100\,g\,m^{-2}\,h^{-1}$ or $>140\,g\,m^{-2}\,h^{-1}$. The image is representative of data from five mice showing similar results. (**c**) Graph of bioluminescence intensity versus TEWL after tape stripping. Data (mean ± SD) from five mice are shown. Statistical significance was assessed using Student's *t*-test. (**d**) Time course of eATP bioluminescence after tape stripping. Images are from the same mouse and are representative of data from five mice. (**e**) Time course of the bioluminescence intensity from five mice after tape stripping.

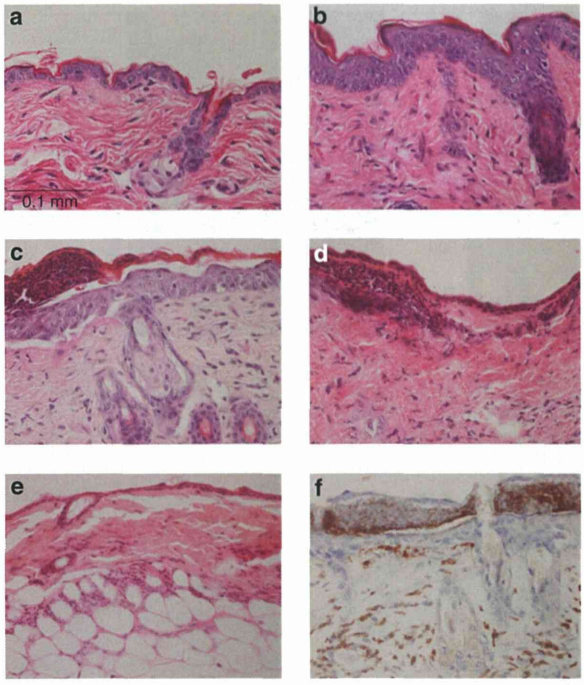

Figure 3. Histological features of tape-stripped skin with different transepidermal water loss (TEWL) values. Histology of (**a**) intact skin, (**b**) tape-stripped skin with TEWL of $<100\,g\,m^{-2}\,h^{-1}$, (**c**, **d**) 100–$140\,g\,m^{-2}\,h^{-1}$, and (**e**) $>140\,g\,m^{-2}\,h^{-1}$ was examined by hematoxylin–eosin staining. Bars = 100 μm. (**f**) Immunohistochemical staining of tape-stripped skin with TEWL between 100 and $140\,g\,m^{-2}\,h^{-1}$ using anti-Ly6G antibody.

thus, we focused on ATP as a representative DAMP molecule in this study. Although ATP release from the organ culture of tape-stripped skin has been reported (Denda *et al.*, 2002), to the best of our knowledge, eATP *in vivo* after tape stripping was previously unreported. Using *in vivo* imaging, it was found that eATP is present in murine skin 1–20 minutes after tape stripping. As the mixture of Eluc-agarose and 10 mM ATP solution—but not 1 mM ATP—produced consistent and significant bioluminescence (i.e., $>5.0\times10^6$ photons per second), the maximum concentration of eATP in the skin after tape stripping was estimated to be ~10 mM as $>5.0\times10^6$ photons per second were detected. Unfortunately, Eluc-agarose mixed with 1 mM ATP did not produce consistent and bright bioluminescence *in vivo*, although the same mixture emitted 1.0×10^7 photons per second *in vitro*. This difference might be due to the rapid diffusion of ATP mixed with Eluc-agarose, hydrolysis of ATP by ecto-ATPase, or low penetration of bioluminescence through the skin. We are currently modifying the procedures to improve the sensitivity of the detection method. If we consider either rapid diffusion of ATP or hydrolysis of ATP as possible reasons, the maximum concentration of eATP after tape stripping might be <10 mM.

It was found that an optimum number of times of tape stripping was necessary to induce detectable eATP in our system, namely, eATP was undetectable if tape stripping resulted in a TEWL $<100\,g\,m^{-2}\,h^{-1}$ or $>140\,g\,m^{-2}\,h^{-1}$. Not surprisingly, eATP was undetectable in tape-stripped skin with a TEWL of $<100\,g\,m^{-2}\,h^{-1}$, suggesting that mild stress or injury was unable to cause detectable release of eATP from keratinocytes. Unexpectedly, excessive tape stripping did not cause detectable release of eATP in the skin. Considering that the epidermis of tape-stripped skin with a TEWL $>140\,g\,m^{-2}\,h^{-1}$ was almost completely denuded, it suggests that eATP could not be produced in the absence of keratinocytes.

Tape-stripped skin with a TEWL between 100 and $40\,g\,m^{-2}\,h^{-1}$ and bright bioluminescence on imaging showed a greater accumulation of inflammatory cells in the SC and dermis than tape-stripped skin with a TEWL $>140\,g\,m^{-2}\,h^{-1}$ and no bioluminescence on imaging. In addition, the latter had a sparse inflammatory cell infiltrate in the superficial dermis. These results suggest that eATP induced by tape stripping has a role in recruiting inflammatory cells to the SC and dermis. Furthermore, subcutaneous injection of apyrase significantly reduced the inflammatory cell infiltrate in the SC and dermis of tape-stripped skin with a TEWL between 100 and $140\,g\,m^{-2}\,h^{-1}$, which confirms the role of eATP in the cutaneous inflammation of tape-stripped skin. To further examine the role of purinergic receptors, we injected a wide spectrum P2 receptor antagonists, suramin, and pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid (Burnstock, 2007) to the skin 10 minutes before tape stripping. However, we could not detect any significant reduction of the inflammatory cell infiltrate (data not shown). The exact reason is unclear, although we speculated that, as our results showed that ~10 mM ATP is present for >10 minutes after tape stripping, rapid diffusion of suramin or pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid might result in levels that were insufficient in suppressing the effects of ATP.

Multiple pathways other than cell lysis participate in ATP release (reviewed by Lohman *et al.* (2012)), including vesicular exocytosis, ATP-binding cassette transporters, connexin hemichannels, and pannexin channels. Recently, it has been demonstrated that ATP is released from dying cells through pannexin channels during apoptosis (Chekeni *et al.*, 2010). It is conceivable that tape stripping induces cell death in the upper epidermis, resulting in ATP release through the above-mentioned mechanism, but the mechanism of keratinocyte cell death after tape stripping and the pathways of ATP release remain to be elucidated.

It is known that ATP and ADP are rapidly cleared while passing through the vascular bed despite their slow metabolism in blood. This is due to the presence of ecto-ATPases, ecto-apyrase, and 5′-nucleotidase activity on the luminal surface of endothelial cells (reviewed by Schwiebert and Kishore, 2001). Therefore, it is difficult to envisage that eATP or its metabolites directly recruit PMNs into the skin. We hypothesized that eATP may act indirectly by stimulating the production of chemokines; therefore, we examined the mRNA expression of CXCL1, -2, -3, and -5, which are representative neutrophil chemokines (Hamilton *et al.*, 2012), in the tape-stripped skin. The results clearly demonstrated that optimum tape stripping significantly augmented CXCL1, -2, and -3, but not CXCL5, mRNA in the skin. However, the relative increase in chemokine mRNA was not observed after excessive tape

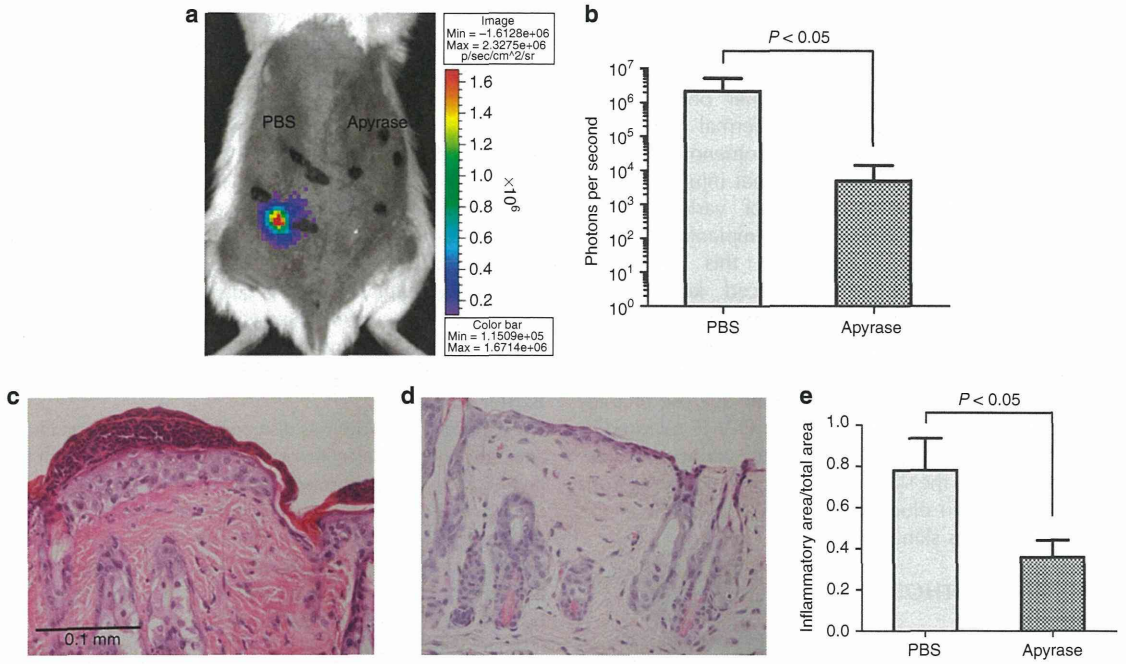

Figure 4. Subcutaneous injection of apyrase diminished extracellular adenosine 5′-triphosphate (eATP) bioluminescence and histological changes associated with tape stripping. (**a**) Apyrase or phosphate-buffered saline (PBS) was injected into the skin 30 minutes before optimum tape stripping, and eATP was visualized using Eluc-agarose. The image is representative of data from nine mice. (**b**) Graph of bioluminescence intensity with or without subcutaneous injection of apyrase before tape stripping. Statistical significance was assessed using Student's *t*-test. Histology of tape-stripped skin (**d**) with or (**c**) without subcutaneous injection of apyrase before tape stripping. Bars = 100 μm. (**e**) The length of the inflamed section was divided by the length of the entire skin biopsy to obtain a ratio. Both sides of the biopsy (10%) were excluded from the calculation. Data (mean ± SD) from nine mice are shown. Statistical significance was assessed using Student's *t*-test.

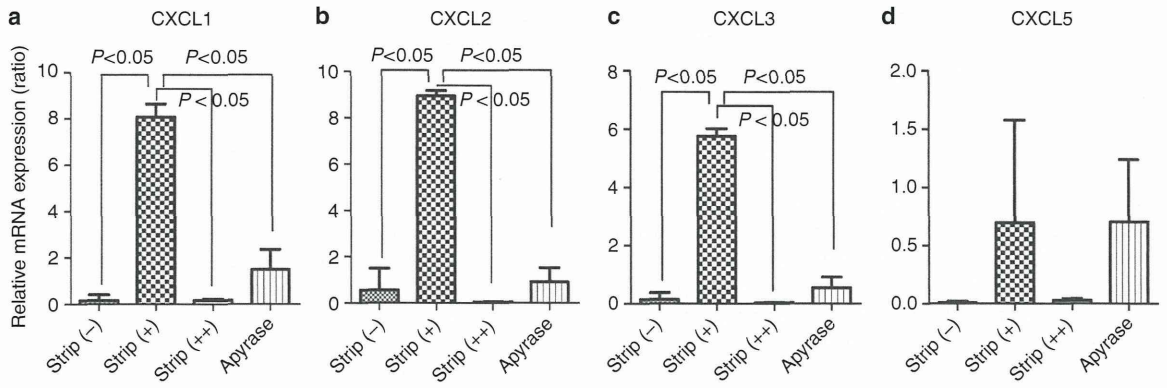

Figure 5. Relative increase in neutrophil chemokine messenger RNA (mRNA) expression in tape-stripped skin is dependent on extracellular adenosine 5′-triphosphate (eATP). We compared CXCL1, -2, -3, and -5 mRNA expression between intact skin, skin after optimum or excessive tape stripping, and skin pretreated with subcutaneous apyrase followed by optimum tape stripping. (**a**) CXCL1, (**b**) CXCL2, (**c**) CXCL3, (**d**) CXCL5. Statistical significance was assessed using Student's *t*-test.

stripping, possibly due to the lack of ATP as a result of excessive loss of keratinocytes.

Tape stripping is a well-known mechanical stimulus that can efficiently induce the Koebner phenomenon in psoriatic patients. Other stimuli such as trauma, bacillus Calmette–Guérin vaccination, drug reactions and dermatoses may also cause the Koebner phenomenon (reviewed by Weiss *et al.*, 2002). It has reported that a patient reacting to one experimental stimulus can react to other Koebner phenomenon–inducing stimuli (Pedace *et al.*, 1969).

Thus, multiple stimuli that induce the Koebner phenomenon may have common signaling molecules, with DAMP being a likely candidate. Several studies suggest the crucial role of the epidermis in the induction of the Koebner phenomenon. Farber *et al.* (1965) demonstrated that a dermal knife blade injury could not induce the Koebner phenomenon except at the entry point of the knife where epidermal injury had also occurred. Similarly, dermal injections of various potent inflammatory stimulators or catgut suture implantation failed to provoke any psoriatic reaction. In this study, we demonstrated that tape stripping induced keratinocyte-derived eATP in the skin, which participated in the recruitment of PMNs. These observations suggest that eATP is one of the triggering factors for psoriasis.

In conclusion, our study showed that Eluc-agarose enables visualization of eATP *in vivo*. As eATP is a DAMP released in various inflammatory skin diseases and after various physical and chemical insults to the skin, we envisage that Eluc-agarose will be a useful tool in elucidating the role of eATP in the pathogenesis of various skin disorders.

MATERIALS AND METHODS

Eluc-agarose

Eluc-agarose was made of a beetle luciferase and Ni-NTA Superflow resin (Qiagen, Valencia, CA). In brief, an expression vector was designed to produce N-terminal 6× histidine-tagged Eluc protein (TOYOBO, Osaka, Japan) without the methionine of the original start codon using pCold II expression vector (Takara Bio, Otsu, Japan) and transfected into BL21 Star (DE3) competent cell, followed by recombinant protein production according to the manufacturer's instructions of pCold. Purification was performed using Ni-NTA agarose (Qiagen). A total of 4 ml of Ni-NTA Superflow slurry was washed with washing buffer (50 mM sodium phosphate (pH 7.0), 300 mM sodium chloride, and 10 mM imidazole). A total of 17 mg of the purified 6× His-tagged Eluc was mixed with the resin and washed with washing buffer (50 mM sodium phosphate (pH 7.0), 300 mM sodium chloride, and 20 mM imidazole) to obtain Eluc-agarose (0.24 mg of Eluc ml^{-1}).

Mice

All mice were handled according to the Regulations for Animal Experiments and Related Activities at Tohoku University. All animal experiments were approved by the ethics committee of Tohoku University. Female BALB/c mice aged 8–12 weeks were purchased from the Charles River Laboratories Japan (Yokohama, Japan). The dorsal hair of mice was shaved 1 day before experimentation.

Evaluation of Eluc-agarose for *in vivo* imaging

To examine whether Eluc-agarose can produce luminescence in the presence of luciferin and ATP, we mixed 50 μl of Eluc-agarose, 50 μl of D-luciferin (Summit Pharmaceuticals International, Tokyo, Japan), and 100 μl of different concentrations (0.1, 1, and 10 mM) of ATP (Amersham Biosciences, Buckinghamshire, UK), and measured the luminescent signal using the *In Vivo* Imaging System (Xenogen, Alameda, CA). Next, to assess whether Eluc-agarose can be used to detect eATP in mice, we tested whether subcutaneous Eluc-agarose produces significant luminescence in response to exogenous ATP. In brief, we injected 150 mg kg^{-1} body weight (15 mg ml^{-1} luciferin) of the substrate d-luciferin into the peritoneal cavity of mice, followed by 100 μl injections of Eluc-agarose with various concentrations of ATP into the dorsal skin of mice 10 minutes later, and *in vivo* imaging was performed immediately afterwards.

In vitro and *in vivo* imaging

Luminescence was visualized using the *In Vivo* Imaging System. The luminescent signal is expressed in photons per second and displayed as an intensity map using Living Image software (Xenogen). The image display is adjusted to provide optimal contrast and resolution in the image without affecting quantitation. For the detection of luminescence, mice were anesthetized with isoflurane/oxygen using the integral anesthetic manifold.

Barrier disruption

The permeability barrier was disrupted by tape stripping. Immediately after the peritoneal injection of d-luciferin, tape stripping of the dorsal murine skin was performed several times using cellophane tape (Nichiban, Tokyo, Japan) and barrier disruption was assessed by measuring TEWL immediately after tape stripping. TEWL was measured using Model H4300 (Nikkiso-YSI, Tokyo, Japan). To examine luminescence after barrier disruption by tape stripping, 150 mg kg^{-1} body weight (15 mg ml^{-1} luciferin) of the substrate d-luciferin was injected into the peritoneal cavity of mice, and 10 minutes later, 100 μl of Eluc-agarose was subcutaneously injected into the dorsal skin and eATP was visualized by *in vivo* imaging. In some experiments, 5 units of adenosine diphosphatase (Apyrase, Sigma-Aldrich, St Louis, MO) in 50 μl phosphate-buffered saline was injected 30 minutes before tape stripping.

Cutaneous injection of ATP and ATP analog

To ascertain that ATP release caused by tape stripping is crucial for continual PMN recruitment, we injected 100 μl of 10 mM ATP or ATPγS (Sigma-Aldrich), a non-hydrolyzed ATP analog resistant to protein phosphatases, to mice skin without tape stripping. The dorsal skin of mice was excised at 6 and 24 hours after injection for RNA isolation and for histology, respectively.

Histology and immunohistochemistry

The dorsal skin of mice were excised at various time periods after tape stripping, fixed in 10% formaldehyde in phosphate-buffered saline, and embedded in paraffin. Formalin-fixed, paraffin-embedded tissue samples were sectioned at a thickness of 4 μm, deparaffinized, and subjected to either hematoxylin and eosin staining or immunohistochemistry. To detect the infiltration of neutrophils, deparaffinized sections quenched with 3% H_2O_2 in methanol were stained with a 1/100 dilution of the anti-Ly6G antibody (Abcam, Cambridge, UK) using ATP-binding cassette staining system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instruction. Diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) was used as a peroxidase substrate and slides were counter stained with Mayer's hematoxylin solution (Fisher Scientific, Fair Lawn, NJ).

In experiments that examined the effect of subcutaneous injection of apyrase, the length of the entire skin biopsy, as well as the length of the inflamed section of skin with either corneal or subepidermal PMN infiltration, was measured using ImageJ software (http://rsbweb.nih.gov/ij/). The length of the inflamed section was divided by the length

of the entire skin biopsy to obtain a ratio. Both sides (10%) of the biopsy were excluded from the calculation.

RNA isolation

Dorsal murine skin was collected 6 hours after tape stripping, frozen with liquid nitrogen, and then crushed with Cryo-Press (MICROTEC, Chiba, Japan). Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Total RNA concentration was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, NC).

Quantitative real-time PCR

Complementary DNAs were synthesized using TaKaRa RNA PCR Kit (AMV) (Takara Bio) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Mx3000p QPCR System (Stratagene, Agilent Technologies Division, Santa Clara, CA). Forward and reverse primers and TaqMan probes were selected using the TaqMan Gene Expression Assay Search (Applied Biosystems, Foster City, CA) and are listed in Supplementary Table S1 online. Quantitative PCR reaction mixtures (total volume 20 μl) contained 10 ng of template complementary DNA, 400 nM of forward and reverse primers, 60 nM TaqMan probe, and 30 nM ROX and Brilliant III Fast QPCR Master Mix (Stratagene). The thermal cycling conditions were: 3 minutes for polymerase activation and complementary DNA denaturation at 95 °C, and 45 cycles of 95 °C for 5 seconds and 60 °C for 20 seconds. Constitutively expressed β-actin served as a normalization control using the ΔΔCt method (Livak and Schmittgen, 2001).

Statistics

Representative data from at least three independent experiments are shown for each set of experiments. Values are presented as mean ± SE. Statistical significance was analyzed using Student's *t*-test or one-way analysis of variance test and *P*-values $\leqslant 0.05$ were considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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ORIGINAL ARTICLE

Nonmetal Haptens Induce ATP Release from Keratinocytes through Opening of Pannexin Hemichannels by Reactive Oxygen Species

Kaoru Onami[1], Yutaka Kimura[1], Yumiko Ito[1], Takeshi Yamauchi[1], Kenshi Yamasaki[1] and Setsuya Aiba[1]

Although extracellular adenosine 5′-triphosphate (eATP) has a crucial role in the sensitization phase of contact hypersensitivity (CHS), the mechanism by which hapten causes keratinocyte cell death and ATP release is unknown. We examined the time course of cell death, reactive oxygen species (ROS) production, and ATP release in HaCaT cells and in normal human keratinocytes after exposure to nonmetal haptens, $NiCl_2$, or irritants. Both haptens and irritants caused cell death of keratinocytes but with different time courses. *N*-acetylcysteine (NAC) significantly reduced only nonmetal hapten-induced cell death as assessed by propidium iodide exclusion. We examined the effects of antioxidants and pannexin (Panx) inhibitors on cell death, ROS production, and ATP release by chemical-treated HaCaT cells. Nonmetal hapten-induced cell death, but not $NiCl_2$- or irritant-related cell death, was dependent on reactivity to thiol residues in the cells. NAC reduced cell death and ATP release, whereas antioxidants and Panx inhibitors did not inhibit cell death but significantly attenuated ATP release. Panx1 small interfering RNA (siRNA) also suppressed ATP release from hapten-exposed HaCaT cells. Intraperitoneal injection of a Panx1 inhibitor attenuated murine CHS. These findings suggest that nonmetal hapten reactivity to thiol residues causes membrane disruption of keratinocytes and ROS production that leads to ATP release through opening of Panx hemichannels.

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INTRODUCTION

Sensitization to contact allergens requires activation of the innate immune system that leads to dendritic cell (DC) activation. However, the mechanisms by which contact allergens activate innate immune signaling pathways are incompletely understood. It is possible that "endogenous danger signals" or "damage-associated molecular patterns" (reviewed in Willart and Lambrecht, 2009) are responsible for activation of the innate immune system in allergic sensitization. The following molecules have been identified as damage-associated molecular patterns: adenosine 5′-triphosphate (ATP), heat shock proteins, hyaluronic acid, monosodium urate, galectins, thioredoxin, adenosine, high-mobility group box protein 1, IL-1α, and IL-33 (reviewed in Hirsinger *et al.*, 2012).

Recent studies using gene targeting in mice demonstrated that Toll-like receptor 2 (TLR2)/TLR4 double-deficient mice (Martin *et al.*, 2008) or purinergic receptor $P2X_7$-deficient mice (Weber *et al.*, 2010) are resistant to allergic contact hypersensitivity (CHS), indicating an essential role of TLR2/TLR4 as well as of purinergic receptor $P2X_7$ in the mouse CHS model. Breakdown products of hyaluronic acid in the range of 1.2 to 500 kDa that are generated during inflammation or tissue damage have been demonstrated to stimulate TLR2 and/or TLR4 in immune cells such as macrophages or DCs (Termeer *et al.*, 2002; Scheibner *et al.*, 2006). Indeed, Esser *et al.* (2012) reported that haptens induce reactive oxygen species (ROS) production by keratinocytes *in vitro* as well as *in vivo*, thereby increasing hyaluronidase activity in the skin that results in the production of low-molecular-weight hyaluronic acid fragments. Combined, these observations suggest that hapten-exposed keratinocytes generate pro-inflammatory low-molecular-weight hyaluronic acid fragments that induce CHS via stimulation of TLR2/TLR4.

Similarly, extracellular ATP (eATP) released by stressed or damaged cells can also activate innate immune responses. The transmembrane ATP receptor $P2X_7$ has been implicated in the post-translational processing of pro-IL-1β and pro-IL-18 via activation of the NLRP3 inflammasome (reviewed by Vitiello *et al.*, 2012). Although Weber *et al.* (2010)

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Abbreviations: ATP, adenosine 5′-triphosphate; CBX, carbenoxolone; CHS, contact hypersensitivity; DC, dendritic cell; DNCB, dinitrochlorobenzene; DPCP, diphenylcyclopropenone; eATP, extracellular ATP; LA, lactic acid; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; 4-NBB, 4-nitrobenzylbromide; NHEK, normal human epidermal keratinocyte; Panx, pannexin; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA; TLR, Toll-like receptor

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demonstrated eATP release in skin painted with hapten, they did not address the mechanism by which hapten-treated keratinocytes release ATP.

In this study, we compared the effects of haptens with irritants on keratinocyte cell death, ROS generation, and ATP release. Although the generation of ROS by hapten-exposed keratinocytes has been demonstrated in several studies (Mehrotra *et al.*, 2005; Esser *et al.*, 2012; Kim *et al.*, 2012), the effect of the generated ROS on cell death and ATP release from hapten-exposed keratinocytes has not been examined. First, we demonstrated that haptens and irritants caused the cell death of the human keratinocyte cell line HaCaT and normal human epidermal keratinocytes (NHEKs), and induced ATP release with different time courses. *N*-acetylcysteine (NAC) significantly reduced cell death of HaCaT cells exposed to haptens but did not affect the death of cells exposed to irritants. Three representative nonmetal haptens, dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), and diphenylcyclopropenone (DPCP), induced the generation of ROS in HaCaT cells that was significantly attenuated by pretreatment with NAC and several antioxidants. Despite the inhibitory effects of NAC and antioxidants on ROS generation, antioxidants suppressed lactate dehydrogenase (LDH) activity and ATP release but did not affect cell death that was assessed using propidium iodide (PI) exclusion. In addition, studies using pannexin (Panx) inhibitors revealed that ATP release from hapten-treated HaCaT cells was through Panx hemichannels. Furthermore, intraperitoneal injection of a pannexin inhibitor, carbenoxolone (CBX), significantly attenuated CHS induced by DNCB. Combined, these results provide an insight into the mechanism by which haptens cause keratinocyte death and ATP release in CHS.

RESULTS

Both haptens and irritants induce HaCaT and NHEK cell death and ATP release but with different time courses

Evaluation of cell death by PI exclusion using flow cytometry and LDH release assay showed that two nonmetal haptens DNCB and 4-NBB, one metal hapten $NiCl_2$, and two irritants SDS and lactic acid (LA) induced HaCaT cell death in a dose-dependent manner (Figure 1a). The minimum concentrations of reagents that were toxic to all cells were 100 μM for DNCB and 4-NBB, 6 mM for $NiCl_2$, 250 μM for SDS, and 34 mM for LA. Haptens and irritants induced cell death with different time courses: maximum cell death was evident 1 hour after irritant exposure but maximum cell death was only achieved after more than 6 hours of hapten exposure as assessed by PI staining. LDH activity in culture supernatants was increased 6 hours after hapten exposure, whereas maximum release of LDH was seen 1 hour after SDS exposure. The LDH activity of LA-treated HaCaT cells could not be measured, possibly because of disturbance of LDH enzyme activity due to the acidity of culture supernatants containing LA.

The time course of ATP release was also different between haptens and irritants. ATP release was evident 6 hours after hapten exposure, whereas maximum ATP release was seen 1 hour after exposure to irritants (Figure 1a). To test whether higher concentrations of hapten induce an earlier release of ATP, HaCaT cells were incubated with increased concentrations of DNCB (from 100 μM to 3.2 mM); however, ATP release earlier than 6 hours after DNCB exposure was not observed (data not shown). To clarify whether the delayed ATP release is limited to HaCaT cells, ATP release in culture supernatants of NHEKs exposed to either haptens or irritants was assessed. Similar to HaCaT cells, the time course of ATP release from NHEKs was also different between haptens and irritants (Figure 1b).

NAC attenuates HaCaT cell death caused by DNCB, 4-NBB, and DPCP but does not affect cell death caused by $NiCl_2$, SDS, or LA

We previously reported that haptens induce a redox imbalance in DCs that stimulates the phosphorylation of p38 mitogen-activated protein kinase and DC activation, and that pretreatment of DCs with NAC corrects the redox imbalance and abrogates the phosphorylation of p38 mitogen-activated protein kinase as well as DC activation (Mizuashi *et al.*, 2005). Therefore, we examined whether NAC could attenuate cell death caused by haptens and irritants. NAC significantly suppressed HaCaT cell death caused by nonmetal haptens DNCB, 4-NBB, and DPCP, as assessed by PI-positive cells, but did not affect cell death caused by a metal hapten $NiCl_2$, or irritants SDS or LA (Figure 2). Similarly, ATP release and LDH activity of HaCaT cells 12 hours after DNCB, 4-NBB, or DPCP exposure were significantly attenuated by NAC, but NAC had little effect on ATP release and LDH activity induced by $NiCl_2$, LA, or SDS (Figure 2).

HaCaT cells exposed to haptens and irritants produce ROS and mitochondrial superoxide anion depending on their thiol reactivity

Next, we examined whether exposure of HaCaT cells to haptens or irritants results in production of ROS using the CM-H_2DCFDA probe (Figure 3a). All nonmetal haptens but not the metal hapten $NiCl_2$ stimulated intracellular ROS production in a dose-dependent manner from 30 minutes to 6 hours after stimulation. ROS production in HaCaT cells exposed to DNCB or DPCP was greater than that in cells exposed to 4-NBB. SDS exposure resulted in the production of ROS in HaCaT cells from 30 minutes to 24 hours after exposure, but the amount of ROS production in the first 6 hours after exposure was much smaller than that caused by hapten exposure. The concentration of SDS that induced cell death of the majority of HaCaT cells did not result in a significant amount of ROS generation within 1 hour after exposure, suggesting that ROS did not contribute to SDS-related HaCaT cell death. LA did not induce ROS in keratinocytes. The positive control H_2O_2 triggered immediate production of ROS in HaCaT cells.

As pretreatment with NAC significantly attenuated HaCaT cell death as well as ATP release, we examined the effects of NAC on ROS production by hapten-exposed HaCaT cells. Pretreatment of HaCaT cells with NAC significantly attenuated ROS production by hapten-exposed HaCaT cells 6 hours after exposure, whereas it did not significantly affect ROS production by SDS- or H_2O_2-treated HaCaT cells (Figure 3b).

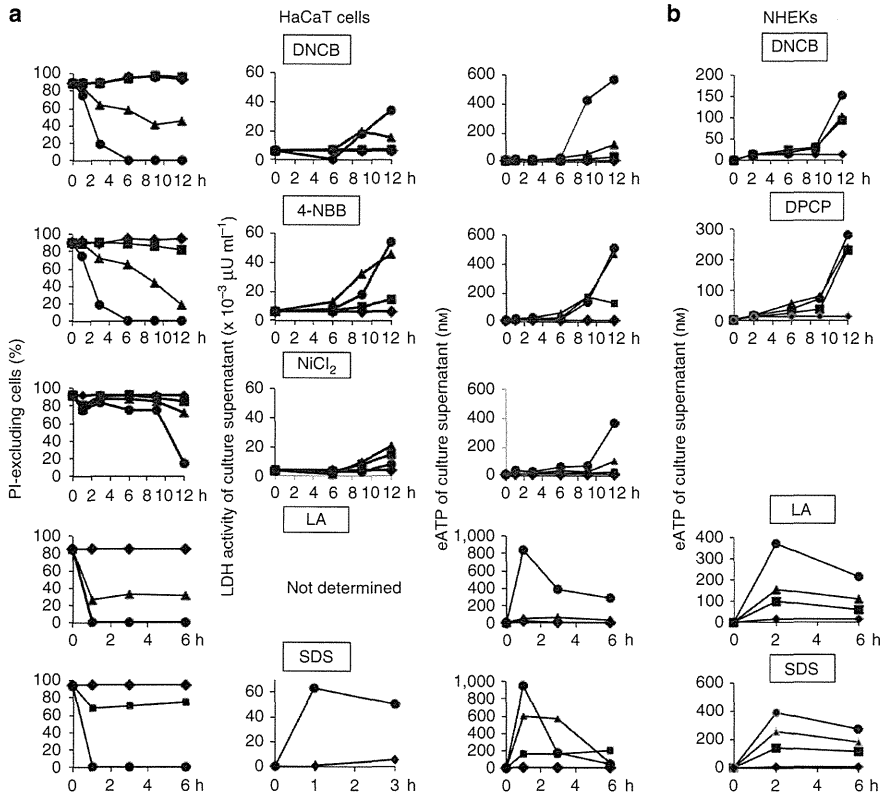

Figure 1. Haptens and irritants cause cell death of HaCaT cells and induce adenosine 5′-triphosphate (ATP) release with different time courses. (**a**) HaCaT cells or (**b**) normal human epidermal keratinocytes (NHEKs) cultured in 24-well plates were treated with graded concentrations of haptens dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), diphenylcyclopropenone (DPCP), or $NiCl_2$, or irritants lactic acid (LA) or SDS, for various time periods. After incubation, propidium iodide (PI) exclusion, lactate dehydrogenase (LDH) activity, and ATP release were examined to assess cell viability. The mean LDH activity and extracellular ATP (eATP) release of triplicate cultures were calculated for each chemical. The symbols ●, ▲, ■, and ♦ correspond to the highest, medium, and lowest concentrations and vehicle control of each chemical, respectively. Chemicals and their concentrations were as follows: DNCB, 4-NBB, and DPCP—100, 50, and 25 μM; $NiCl_2$—6.0, 3.0, and 1.5 mM; SDS—250, 125, and 62.5 μM; and LA—34 and 17 mM. Representative data from three independent experiments are shown.

Next, to clarify the source of ROS in HaCaT cells treated with haptens, we examined whether haptens induce mitochondrial superoxide anion generation using MitoSOX, a mitochondria-targeted ROS-specific fluorescent probe. DNCB, DPCP, and 4-NBB, although weakly, induced mitochondrial superoxide anion production 2 hours after cell exposure (Figure 3c), suggesting that mitochondria contribute to ROS production in HaCaT cells exposed to nonmetal haptens. Treatment with H_2O_2 also induced mitochondrial superoxide anion production. Pretreatment with NAC significantly attenuated mitochondrial superoxide anion production in HaCaT cells exposed to DNCB, 4-NBB, or DPCP, although its inhibitory effect on superoxide anion production by HaCaT cells exposed to 4-NBB was minimum (Figure 3c).

TEMPOL and apocynin do not rescue hapten-treated HaCaT cells from cell death but suppress ROS production and reduce ATP release

Although ROS production after hapten stimulation in the mitochondria of dendritic cells (Migdal *et al.*, 2010) and in the cytosol of keratinocytes (Mehrotra *et al.*, 2005) has been documented, the source of ROS production that causes keratinocyte cell death and ATP release has not yet been determined. Therefore, we examined whether the following reagents affect ROS production in hapten-exposed HaCaT cells: TEMPOL, a whole-cell antioxidant (Wilcox and Pearlman, 2008); MnTBAP, a superoxide dismutase mimetic, catalase mimetic, and peroxynitrite scavenger (Konorev *et al.*, 2002; Batinic-Haberle *et al.*, 2009); allopurinol, a xanthine oxidase inhibitor (Borges *et al.*, 2002); and apocynin, an NADPH oxidase inhibitor (Bedard and Krause, 2007). Results showed that TEMPOL, MnTBAP, and apocynin suppressed ROS production by HaCaT cells exposed to DNCB, 4-NBB, and DPCP, but their inhibitory effect on ROS production by 4-NBB-treated HaCaT cells was not statistically significant (Figure 4a). In contrast, allopurinol failed to suppress ROS production in HaCaT cells exposed to haptens.

We also examined whether MnTBAP or TEMPOL suppresses mitochondrial superoxide anion production by

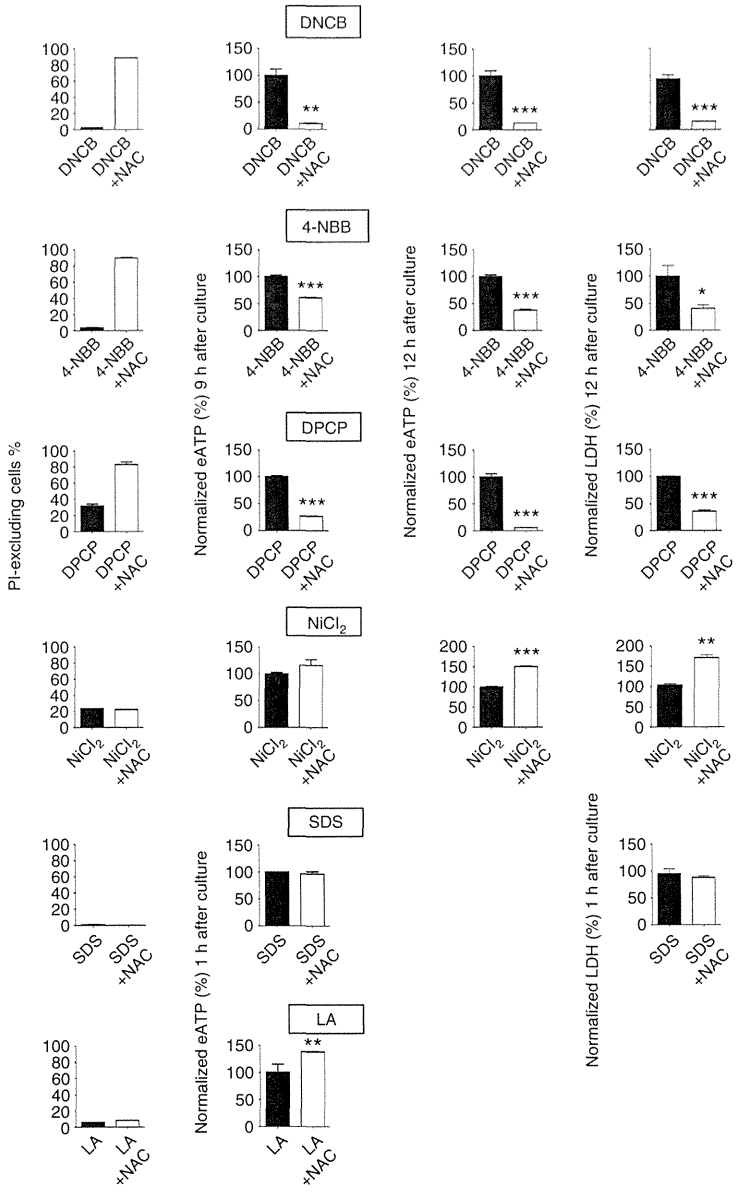

Figure 2. *N*-acetylcysteine (NAC) significantly attenuates cell death and adenosine 5′-triphosphate (ATP) and lactate dehydrogenase (LDH) release by HaCaT cells exposed to dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), or diphenylcyclopropenone (DPCP) but does not affect the death or ATP release of cells exposed to $NiCl_2$, SDS, or lactic acid (LA). HaCaT cells cultured in 24-well plates were either pretreated or not with 2.5 mM of NAC for 30 minutes, followed by exposure to 100 μM of DNCB, 4-NBB, or DPCP, 6 mM of $NiCl_2$, 250 μM of SDS, or 34 μM of LA for various time periods. The effects of NAC on cell death, extracellular ATP (eATP) levels, and LDH activity were assessed 6 hours after culture by propidium iodide (PI) exclusion assay, 9 and 12 hours after culture, and 12 hours after culture, respectively. The mean eATP or LDH activity of triplicate cultures was calculated for each chemical, and results were normalized to the data of hapten-exposed HaCaT cells without NAC. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $**P<0.01$, $***P<0.001$. Representative data from three independent experiments are shown.

hapten-exposed HaCaT cells. In contrast to its effects on ROS production in HaCaT cells, MnTBAP did not suppress mitochondrial superoxide anion production (Figure 4b). TEMPOL suppressed the MitoSOX fluorescence intensity of HaCaT cells exposed to 4-NBB, whereas it did not affect the fluorescence of cells exposed to DNCB or DPCP (Figure 4b).

Next, we examined whether ROS is involved in cell death or ATP release in HaCaT cells exposed to haptens. Assessment

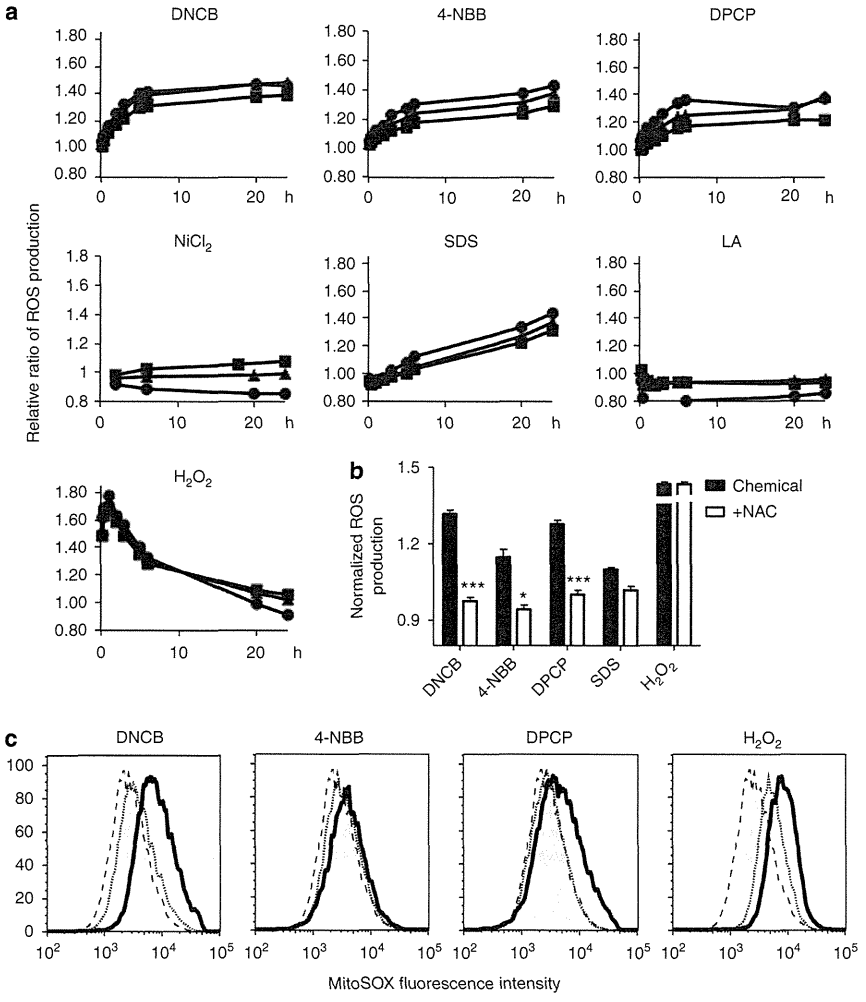

Figure 3. HaCaT cells treated with haptens generate reactive oxygen species (ROS) and mitochondrial superoxide anion in a dose-dependent manner that are significantly reduced by *N*-acetylcysteine (NAC). (**a**) HaCaT cells were treated with graded concentrations of haptens, irritants, or H_2O_2 as a positive control for various time periods. After culture, intracellular ROS were measured using a CM-H_2DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical and expressed as percentage change. The symbols ■, ▲, and ● correspond to the lowest, medium, and highest concentrations, respectively. Chemicals used and their concentrations were as follows: dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), and diphenylcyclopropenone (DPCP)—25, 50, and 100 μM; $NiCl_2$—1.5, 3, and 6 mM; SDS—62.5, 125, and 250 μM; lactic acid (LA)—17 and 34 mM; and H_2O_2—44, 88, and 176 μM. (**b**) HaCaT cells either pretreated or not with NAC were exposed to haptens, SDS, or H_2O_2 for 6 hours. After culture, intracellular ROS were measured with a CM-H_2DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $***P<0.001$. Representative data from three independent experiments are shown. (**c**) MitoSOX-preloaded HaCaT cells were either treated or not treated with NAC, followed by exposure to haptens or H_2O_2. The MitoSOX fluorescence was measured using a flow cytometer. The solid, dotted, and ruptured lines and the shaded area of the histograms represent HaCaT cells treated with hapten alone, hapten + NAC, vehicle control + NAC, and vehicle control alone, respectively.

of cell death by the PI exclusion assay showed that TEMPOL, MnTBAP, apocynin, and allopurinol could not rescue HaCaT cells from cell death after hapten treatment (Supplementary Figure S1 online). TEMPOL, MnTBAP, and apocynin reduced LDH activity and ATP release from DNCB- and 4-NBB-exposed HaCaT cells (Figure 4c and d), and TEMPOL attenuated LDH activity and ATP release from DPCP-exposed HaCaT cells (Supplementary Figure S2 online).

Inhibition of Panx-1 channels significantly suppresses ATP release from hapten-treated HaCaT cells

Multiple pathways other than cell lysis are involved in ATP release (Lohman *et al.*, 2012). It has been demonstrated that ATP release into the extracellular space by dying cells during apoptosis depends on the Panx channel (Chekeni *et al.*, 2010). The release of ATP through Panx hemichannels has also been reported in the setting of ischemia-induced oxidative stress

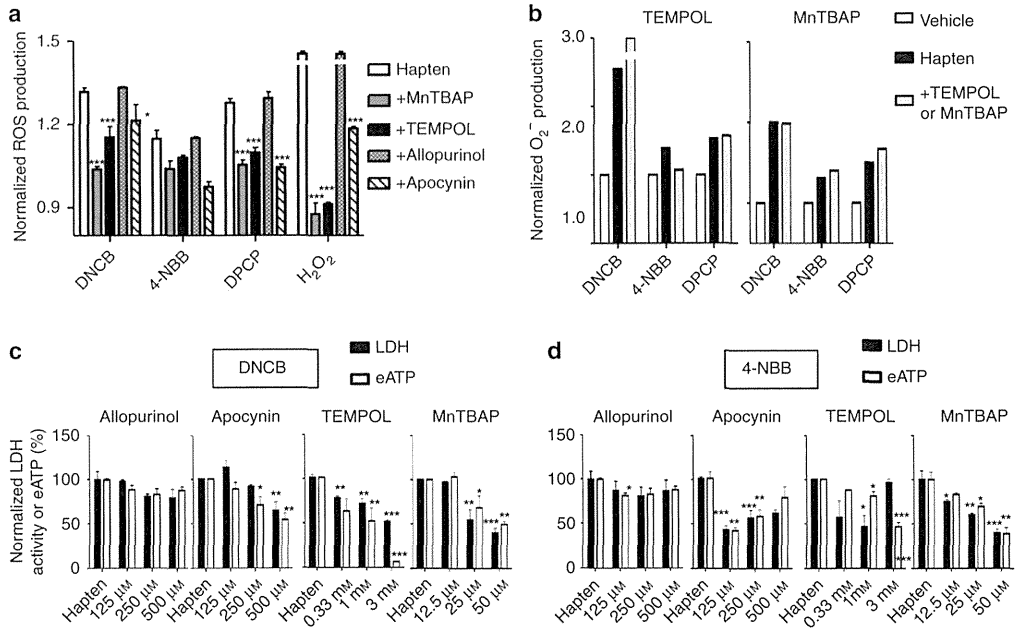

Figure 4. Antioxidants significantly suppress reactive oxygen species (ROS) production by hapten-treated HaCaT cells, and reduce lactate dehydrogenase (LDH) activity and adenosine 5′-triphosphate (ATP) release without decreasing the number of propidium iodide (PI)-positive cells. (**a**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens or H_2O_2. At 6 hours after culture, intracellular ROS were measured using a CM-H_2DCFDA probe. (**b**) MitoSOX-preloaded HaCaT cells were pretreated with TEMPOL or MnTBAP for 30 minutes, followed by exposure to haptens for 2 hours. The MitoSOX fluorescence was measured using a flow cytometer. Mean fluorescence intensity was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. (**c**, **d**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens. At 12 hours after culture, the LDH activity and ATP release in the culture supernatants were measured by colorimetric and luciferase assays, respectively. The mean LDH activity and ATP release of triplicate cultures were calculated for each chemical, and results were normalized to the data of hapten-treated HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $**P<0.01$, $***P<0.001$.

culminating in the cell death of oligodendrocytes (Domercq *et al.*, 2010). We therefore examined whether CBX, a nonspecific Panx inhibitor (Suadicani *et al.*, 2006; Ma *et al.*, 2009), can suppress ATP release from hapten-treated HaCaT cells (Figure 5a). Interestingly, CBX significantly decreased ATP release from hapten-treated but not irritant-treated HaCaT cells in a dose-dependent manner. Furthermore, CBX suppressed LDH release from DNCB-treated but not 4-NBB- or DPCP-treated HaCaT cells (data not shown). Evaluation of cell death by the PI exclusion assay showed that CBX was unable to rescue hapten-exposed HaCaT cells from cell death.

To exclude the possibility that CBX reduced levels of intracellular ATP, thereby decreasing ATP release from hapten-treated cells, we examined the concentration of intracellular ATP in DNCB-exposed HaCaT cells. Results showed that CBX at concentrations of 7.8 to 31 μM increased intracellular ATP levels and decreased ATP release, whereas CBX at 62 μM slightly decreased the intracellular ATP level and significantly decreased ATP release (Figure 5b). These findings exclude the possibility that CBX reduces ATP release by depleting intracellular ATP.

To further examine the role of Panx hemichannels in ATP release from hapten-treated HaCaT cells, we examined the effects of another Panx-1 inhibitor, probenecid (Silverman *et al.*, 2008), and a Panx-1 mimetic blocking peptide (Pelegrin and Surprenant, 2006) on ATP release from hapten-treated HaCaT cells. Probenecid significantly suppressed ATP release from DNCB- or 4-NBB-treated HaCaT cells, and Panx1-blocking peptide also significantly inhibited ATP release from DNCB-treated HaCaT cells (Figure 5c and d). We also examined the effect of small interfering RNA (siRNA) against Panx1. Attenuation of Panx1 mRNA expression in HaCaT cells by Panx1 siRNA significantly suppressed ATP release from cells exposed to either DNCB or DPCP (Figure 5e and f).

Inhibition of Panx1 by CBX significantly reduces CHS induced by DNCB

Finally, to explore the role of Panx1 in the induction of CHS, we administered CBX by intraperitoneal injection and induced CHS using DNCB. After challenge with 0.5% DNCB, the ear swelling of mice pretreated with CBX was significantly reduced compared with those of saline-injected control mice, suggesting that CBX attenuated the CHS response (Figure 6). In contrast, CBX treatment did not affect the ear swelling induced by 0.5% DNCB without sensitization.

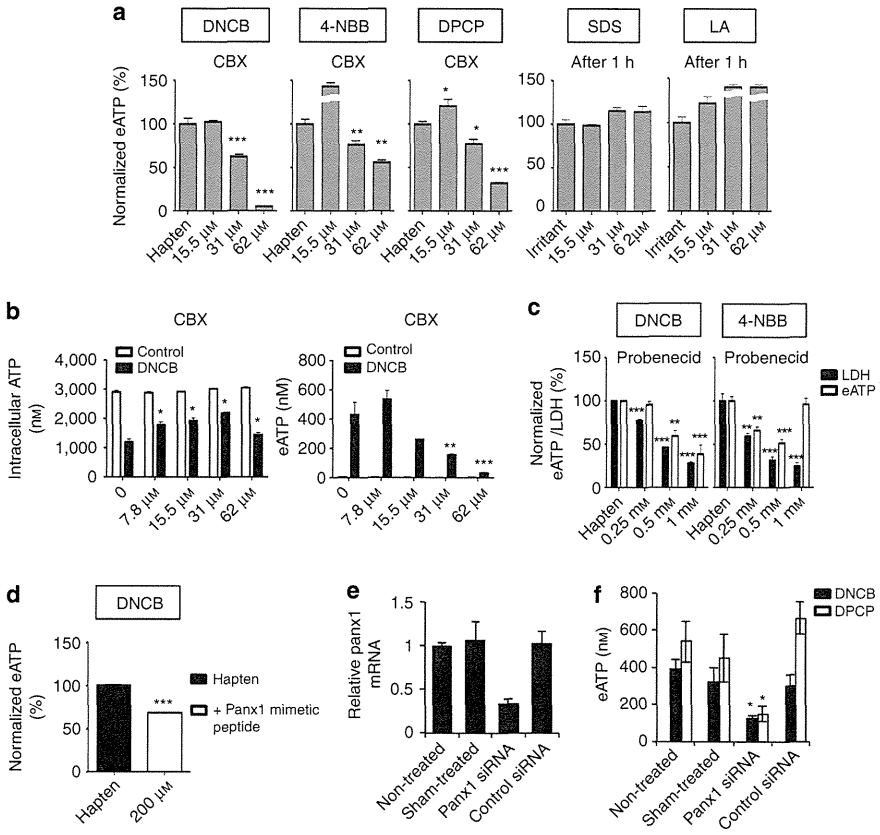

Figure 5. Carbenoxolone (CBX), probenecid, and a pannexin-1 (Panx1) mimetic blocking peptide suppress adenosine 5′-triphosphate (ATP) release from hapten-treated HaCaT cells. (**a**) HaCaT cells were either pretreated or not pretreated with graded concentrations of CBX, followed by exposure to haptens for 12 hours. After culture, ATP in the culture supernatants was measured by luciferase assay. (**b**) The intracellular ATP of HaCaT cells and ATP in the culture supernatants from the same culture were measured by luciferase assay. HaCaT cells were pretreated with (**c**) probenecid or a (**d**) Panx1 mimetic blocking peptide for 30 minutes, followed by exposure to haptens for 12 hours. After culture, ATP or lactate dehydrogenase (LDH) activity in the culture supernatants was measured. The mean extracellular ATP (eATP) and LDH activity of triplicate cultures was calculated for each chemical and results were normalized to the data of hapten-exposed HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $**P<0.01$, $***P<0.001$. HaCaT cells were either treated or not with 10 nM of Panx1 siRNA or control siRNA in transfection reagent. (**e**) After 51 hours of culture, Panx1 mRNA expression in each treatment group was measured by quantitative real-time PCR. (**f**) Cells were subsequently treated with dinitrochlorobenzene (DNCB) or diphenylcyclopropenone (DPCP) for 12 hours and the recovered supernatants were used to measure eATP. Significant differences between treatment groups: $*P<0.05$.

DISCUSSION

In this study, we demonstrated that the haptens DNCB, 4-NBB, DPCP, and $NiCl_2$, and the irritants killed keratinocytes and induced ATP release from keratinocytes with different time courses. This suggests that the mechanism of hapten-induced keratinocyte cell death leading to ATP release is different from that of irritants. Furthermore, keratinocyte cell death caused by nonmetal haptens DNCB, 4-NBB, and DPCP, but not cell death caused by the metal hapten $NiCl_2$ or by irritants, was abrogated by NAC. The fact that NAC is a thiol-containing compound that interferes with thiol redox transitions (Parasassi *et al.*, 2010), and that haptens exhibit a strong affinity toward thiol groups (Becker *et al.*, 2003), suggests that nonmetal haptens kill keratinocytes via reactivity to thiol residues in keratinocytes. In contrast, the mechanism of cell death induced by $NiCl_2$ or irritants was not dependent on this.

Next, we demonstrated that only nonmetal haptens induced ROS production by HaCaT cells that was significantly attenuated by NAC treatment. Again, this suggests that thiol modification by haptens has a crucial role in ROS production. Apart from NAC, MnTBAP, TEMPOL, and apocynin significantly suppressed ROS production by hapten-treated HaCaT cells. However, the three antioxidants did not decrease cell death as evaluated by PI exclusion that suggests that ROS generated by hapten-treated HaCaT cells does not cause membrane disruption.

On the other hand, the three antioxidants suppressed ATP and LDH release from hapten-treated HaCaT cells. We found that ATP and LDH release from hapten-treated HaCaT cells

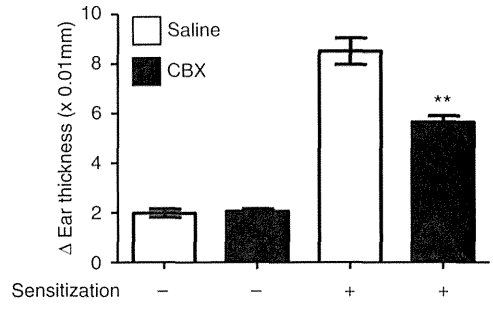

Figure 6. Inhibition of pannexin-1 (Panx1) by carbenoxolone (CBX) significantly reduces contact hypersensitivity (CHS) induced by dinitrochlorobenzene (DNCB). To examine the role of extracellular adenosine 5′-triphosphate (eATP) in sensitization, C57Bl/6 mice were given an intraperitoneal injection of CBX or saline on day 0 and then sensitized with 1% DNCB (or without sensitization) on days 0, 1, and 2, followed by epicutaneous application of 20 μl of 0.5% DNCB on the dorsum of both ears on day 4. Ear measurement was taken on days 4 and 6. The data represent the mean increase in ear thickness for groups of seven mice ± SD. Representative data from three independent experiments are shown. $^{**}P<0.01$ (vs. saline control, Student's *t*-test).

was evident 6 hours after hapten treatment, whereas they started to incorporate PI from 1 hour after exposure. As the molecular weights of LDH and PI are ~140,000 and 688 Da, respectively, we speculate that considerable time is required for sufficient membrane disruption to occur that permits the passage of large molecules. However, molecular size alone cannot fully explain the delay in ATP release as the molecular weight of ATP is lower than that of PI.

It has been reported that ischemia-related oxidative stress culminating in the cell death of oligodendrocytes induced ATP release through the opening of Panx hemichannels (Domercq *et al.*, 2010). As Panx1 is ubiquitously expressed in human tissues including the skin (Baranova *et al.*, 2004), we hypothesized that ROS produced by hapten-treated HaCaT cells may open Panx hemichannels. Indeed, Panx inhibitors as well as Panx1 siRNA significantly attenuated ATP release from HaCaT cells exposed to DNCB, 4-NBB, or DPCP. In addition, significant suppression of ATP release by antioxidants in a dose-dependent manner suggests that ROS production by hapten-treated HaCaT cells has a role in the opening of Panx hemichannels. Combined, our findings suggest that irritants induce ATP release from keratinocytes by disrupting cell membranes, whereas nonmetal haptens such as DNCB, 4-NBB, and DPCP induce ATP release from keratinocytes via ROS-mediated opening of Panx1 channels. Therefore, it is conceivable that Panx hemichannels have a crucial role in sensitization, just as eATP and $P2X_7$ are essential in the mouse CHS model (Weber *et al.*, 2010). This is supported by observation of the attenuated CHS response after CBX pretreatment in mice.

This study also demonstrated differences in the mechanism of ATP release among haptens. ATP release from HaCaT cells treated with the metal hapten $NiCl_2$ was independent of thiol reactivity of $NiCl_2$, whereas nonmetal hapten-induced ATP release from HaCaT cells was dependent on reactivity to thiol residues and ROS production. It has been reported that Ni can stimulate human TLR4 (Schmidt *et al.*, 2010) that suggests that Ni utilizes the TLR4 pathway to activate the innate immune response instead of generating ROS in allergic sensitization. In addition, there was quantitative difference in ROS and superoxide anion production among nonmetal haptens. It is conceivable that different nonmetal haptens generate ROS by different mechanisms dependent on their own chemical properties. Further studies are required to examine the precise mechanism by which nonmetal haptens generate ROS and/or superoxide anion and open Panx channels.

In this study, we attempted to determine the source of ROS in keratinocytes after hapten exposure. Although superoxide anion production by mitochondria occurred after hapten exposure, antioxidants such as TEMPOL and MnTBAP did not attenuate mitochondrial superoxide anion production despite their suppression of ROS production, LDH activity, and ATP release. This suggests that ROS production by mitochondria does not have a significant role in ATP release from hapten-treated keratinocytes, consistent with the observation by Mehrotra *et al.* (2005). However, our study could not determine which cytosolic compartment or enzyme was responsible for ROS production that led to the release of ATP. Although Kim *et al.* (2012) and Esser *et al.* (2012) demonstrated ROS production and mitochondrial superoxide anion production by hapten-treated keratinocytes, neither group succeeded in identifying the source of ROS production that influenced IL-1α production, ICAM-1 expression, or induction of hyaluronidase activity.

Our study provides an insight into the mechanism by which haptens kill keratinocytes and cause a large release of ATP. These findings provide additional evidence of the crucial role of keratinocytes in the sensitization of CHS. In addition, the results of this study suggest that Panx1 may be targeted to protect humans from sensitization by haptens. The Panx1 inhibitor CBX has already been approved as a cosmetic ingredient and may be useful as a topical agent in inflammatory or immune skin diseases by modulating innate immunity.

MATERIALS AND METHODS

Test chemicals and preparation of chemicals

Four contact sensitizers (DNCB, 4-NBB, $NiCl_2$, and DPCP) and two irritants (SDS and LA) were used. The following antioxidants were used in experiments: NAC, allopurinol, MnTBAP, and apocynin. Panx was inhibited using carbenoxolone disodium salt (CBX), probenecid, or Panx-1 mimetic blocking peptide. Full details are available in the Supplementary Methods online.

Keratinocyte culture

HaCaT cells, a gift from Norbert Fusenig in Heidelberg, Germany, and neonatal foreskin NHEKs purchased from Kurabo (Osaka, Japan) were used in this study. Full details regarding cell culture are available in the Supplementary Methods online.

Chemicals exposure of keratinocytes

HaCaT cells or NHEKs were cultured in 24-well plates, washed twice 48 hours later, and incubated with DMEM without phenol red at

37 °C in 10% CO_2 for 1 hour. Afterwards, they were pretreated with or without graded concentrations of antioxidants or Panx inhibitors for 30 minutes, followed by treatment with graded concentrations of haptens or irritants for various time periods at 37 °C in 10% CO_2.

Knockdown of Panx1 by stealth siRNA

In some experiments, HaCaT cells were treated with siRNA against Panx1 as described previously (Hirakawa *et al.*, 2011), followed by hapten exposure. Full details are available in the Supplementary Methods online.

Cell viability

Cell viability was determined by either a PI exclusion assay using flow cytometry or LDH release. Full details are available in the Supplementary Methods online.

Measurement of intracellular ROS

Intracellular ROS were measured fluorometrically using a CM-H_2DCFDA probe (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Full details are available in the Supplementary Methods online.

In vitro detection of mitochondrial superoxide anion

Mitochondrial superoxide anion was detected by MitoSOX RED (Invitrogen). Full details are available in the Supplementary Methods online.

Measurement of ATP

The extracellular ATP level was measured with a commercially available kit (ENLITEN, rLuciferase/Luciferin Reagent; Promega, Madison, WI). Full details are available in the Supplementary Methods online.

Murine model of CHS

Female C57Bl/6 mice were sensitized by painting the shaved abdominal skin with 100 μl of 1% DNCB in 4:1 (v/v) acetone/olive oil on days 0, 1, and 2. For elicitation, 20 μl of 0.5% DNCB was applied to the dorsum of both ears on day 4. To examine the role of eATP in sensitization, we injected 20 mg kg^{-1} of CBX into the peritoneum on day 0. Full details are available in the Supplementary Methods online.

Statistical analysis

At least three independent experiments were performed for each analysis and representative data from one experiment are shown. A one-way or two-way analysis of variance test was used to evaluate statistical significance. The *P*-values of <0.05 were considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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ORIGINAL ARTICLE

Effects on asthma and induction of interleukin-8 caused by Asian dust particles collected in western Japan

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Abstract

Objective: Asian dust storms (ADS) contain various airborne particles that may augment airway inflammation by increasing the level of interleukin-8. The objective of the study was to investigate the association of exposure to an ADS with worsening of symptoms of adult asthma and the effect of ADS particles on interleukin-8 transcriptional activity. *Methods*: The subjects were 112 patients with mild to moderate asthma who recorded scores for their daily upper and lower respiratory tract symptoms and measured morning peak expiratory flow (PEF) from March to May 2011. Interleukin-8 transcriptional activity was assessed in THP-G8 cells that were exposed to airborne particles collected during days of ADS exposure. *Results*: Of the 112 patients, 31 had comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS), and had worsened scores for upper respiratory tract symptoms on ADS days compared to non-ADS days. Scores for lower respiratory tract symptoms during ADS days were higher than non-ADS days in all patients. Three patients also had unscheduled hospital visits for exacerbation of asthma on ADS days. However, there was no significant difference in daily morning PEF between ADS and non-ADS days. Airborne particles collected on ADS days induced interleukin-8 transcriptional activity in THP-G8 cells compared to the original soil of the ADS. *Conclusion*: Exposure to an ADS aggravates upper and lower tract respiratory symptoms in patients with adult asthma. ADS airborne particles may increase airway inflammation through enhancement of interleukin-8 transcriptional activity.

Keywords

Asian dust storms, interleukin-8, luciferase assay, respiratory tract, THP-G8 cells

History

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Introduction

Exposure to particulate matter is associated with respiratory and cardiovascular morbidity or mortality [1,2]. In pediatric asthma patients, Strickland et al. found that short-term exposure to air pollutants increased the number of emergency department visits, even with exposure at relatively low concentrations [3]. Desert dust, which is considered to be harmless, can increase the prevalence of asthma and the occurrence of asthma symptoms [4–6]. Asian dust storms (ADS) originating in the deserts of Mongolia, northern China, and Kazakhstan often disperse dust over East Asia from spring until late autumn. In Japan, the highest frequency of ADSs has occurred from March to May in recent years, based on information from the Japan Meteorological Agency. An ADS can occasionally be large enough to reach the west coast of the United States [7,8] and Uno et al. [9] showed that an ADS can spread around the globe.

Recent studies have shown an association of ADSs with an increased risk of exacerbation of asthma [10–13]. Our previous telephone surveys also showed that an ADS can aggravate symptoms and pulmonary dysfunction in adult patients with asthma [14,15]. However, it has been suggested that an ADS is not significantly associated with the risk of hospitalization for asthma or the incidence of asthma attacks in Taipei [16,17]. Min et al. showed that yellow sand aggravated upper respiratory tract symptoms in adult patients

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