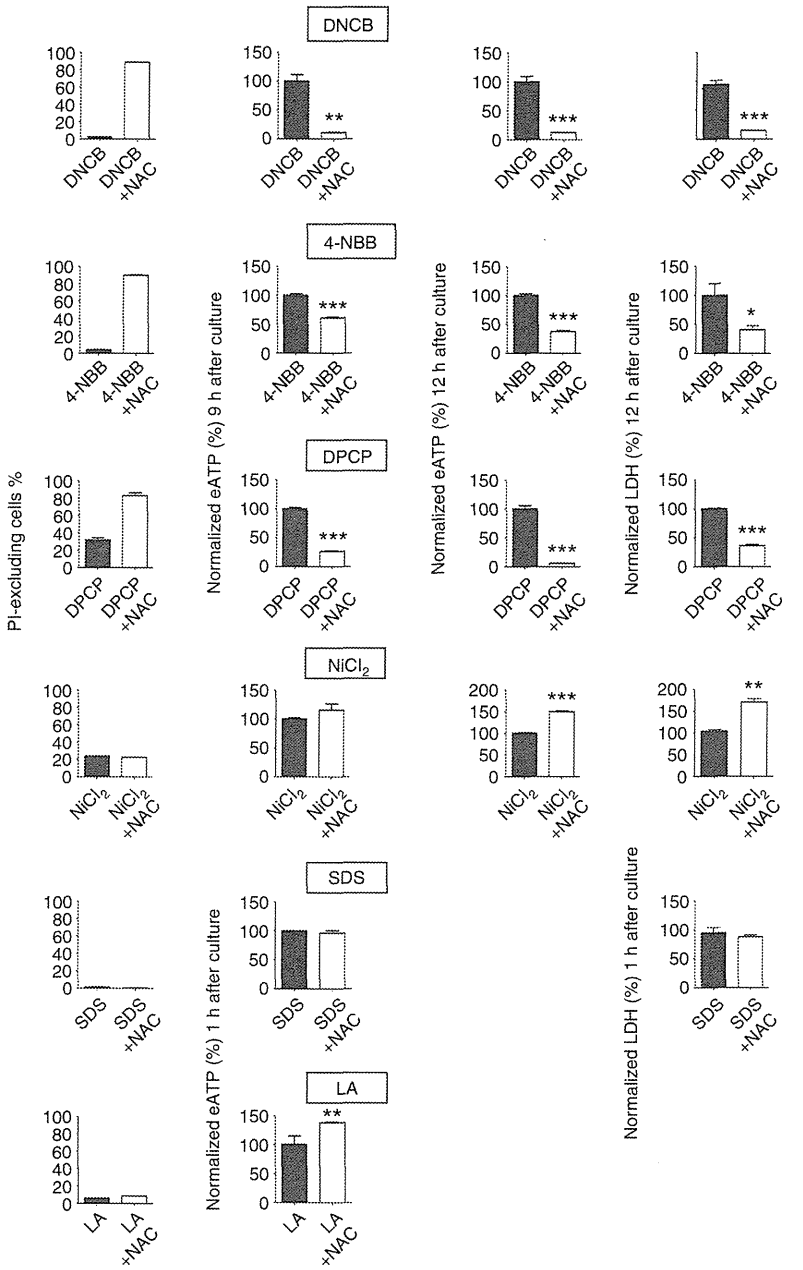

Figure 2. *N*-acetylcysteine (NAC) significantly attenuates cell death and adenosine 5′-triphosphate (ATP) and lactate dehydrogenase (LDH) release by HaCaT cells exposed to dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), or diphenylcyclopropenone (DPCP) but does not affect the death or ATP 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

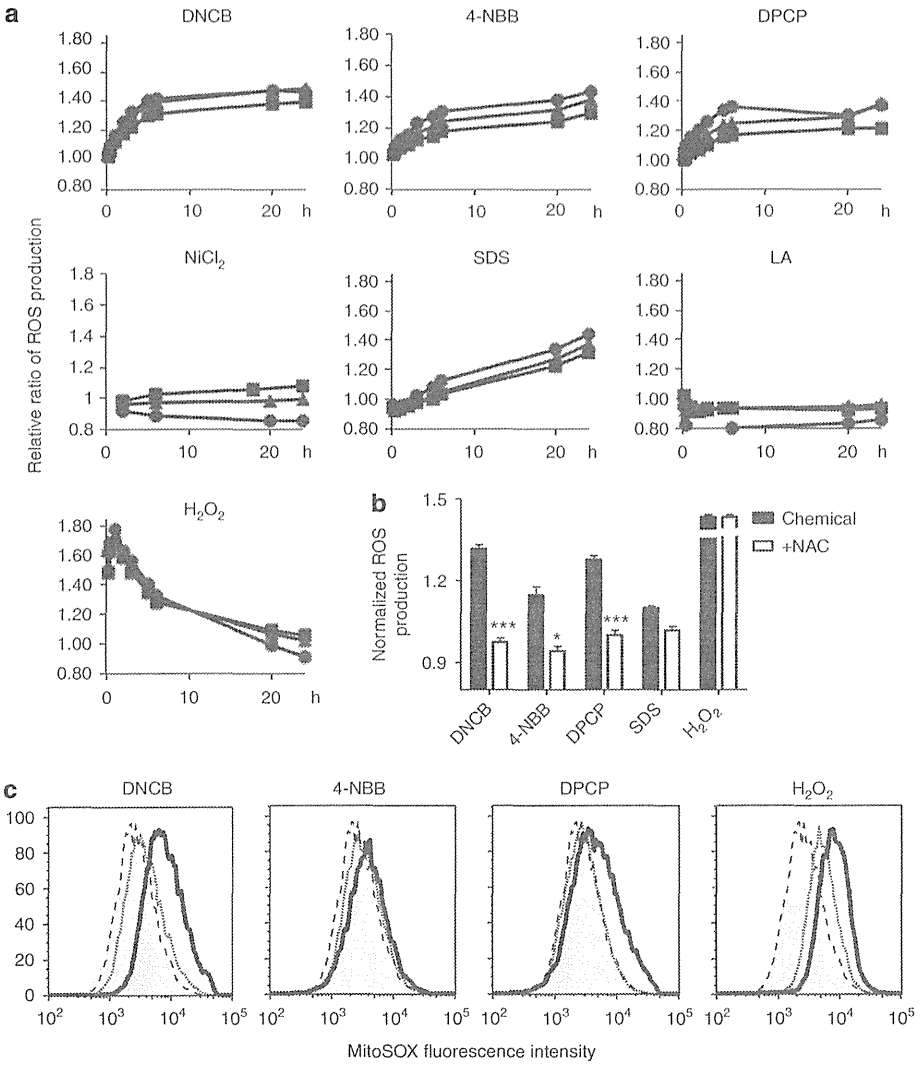

Figure 3. HaCaT cells treated with haptens generate reactive oxygen species (ROS) and mitochondrial superoxide anion in a dose-dependent manner that are significantly reduced by *N*-acetylcysteine (NAC). (**a**) HaCaT cells were treated with graded concentrations of haptens, irritants, or H_2O_2 as a 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

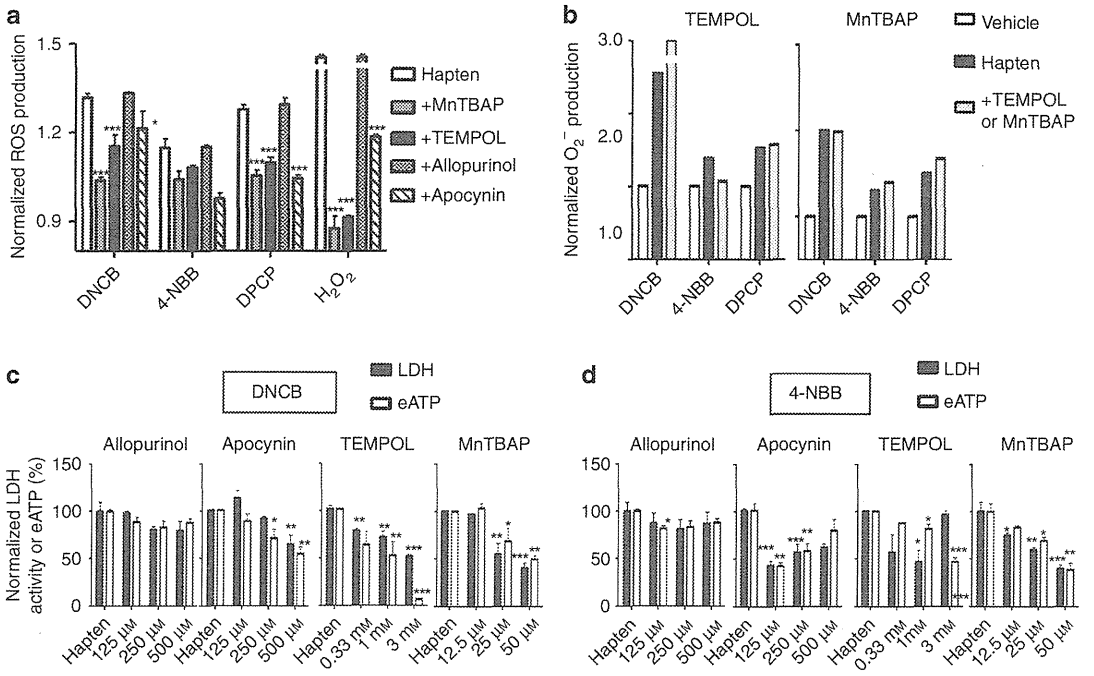

Figure 4. Antioxidants significantly suppress reactive oxygen species (ROS) production by hapten-treated HaCaT cells, and reduce lactate dehydrogenase (LDH) activity and adenosine 5′-triphosphate (ATP) release without decreasing the number of propidium iodide (PI)-positive cells. (**a**) HaCaT cells 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.

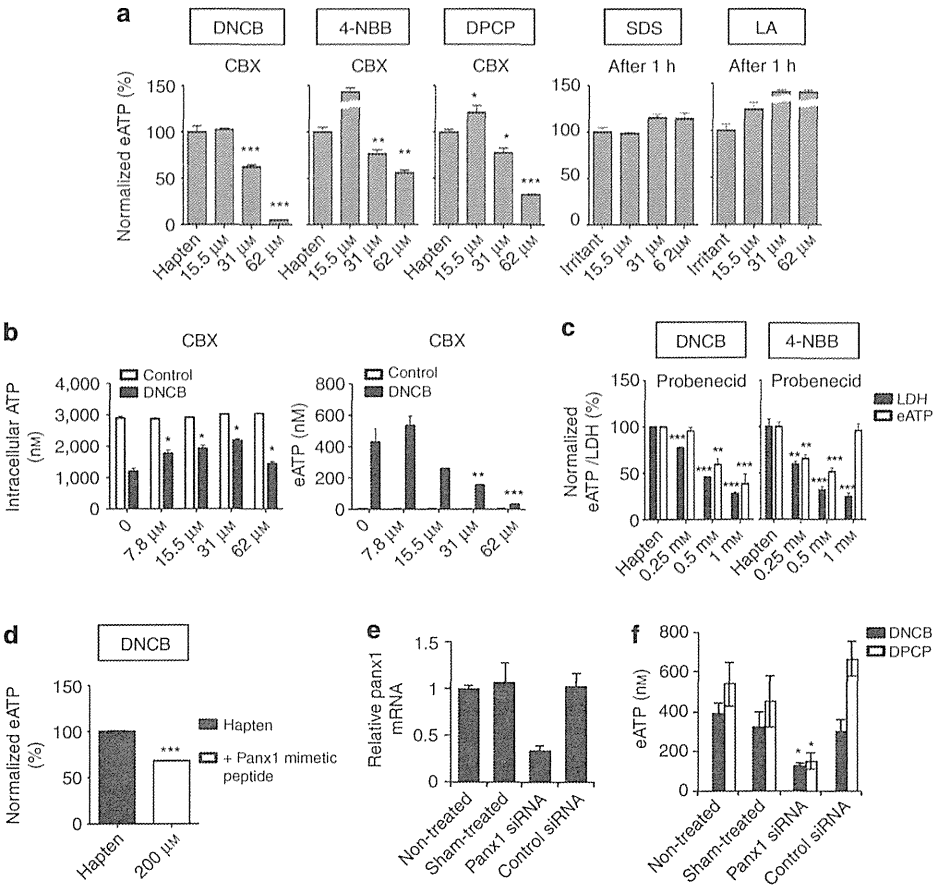

Figure 5. Carbenoxolone (CBX), probenecid, and a pannexin-1 (Panx1) mimetic blocking peptide suppress adenosine 5′-triphosphate (ATP) release from hapten-treated HaCaT cells. (**a**) HaCaT cells were either pretreated or not pretreated with graded concentrations of CBX, followed by exposure to haptens 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

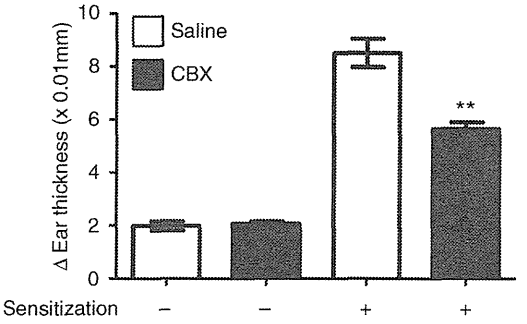

Figure 6. Inhibition of pannexin-1 (Panx1) by carbenoxolone (CBX) significantly reduces contact hypersensitivity (CHS) induced by dinitrochlorobenzene (DNCB). To examine the role of extracellular adenosine 5′-triphosphate (eATP) in sensitization, C57Bl/6 mice were given an intraperitoneal injection of 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.

ACKNOWLEDGMENTS

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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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特集

自己免疫性疾患としての水疱症

ゲスト編集
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医薬ジャーナル社

序
～臓器特異的自己免疫性疾患のプロトタイプとしての自己免疫性水疱症の最新の知見～

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Summary
自己免疫性水疱症は，その自己抗体が病原性を有していることが証明されている数少ない臓器特異的自己免疫性疾患である。最近の生化学的・分子生物学的検討によりその自己抗原が明らかとなり，新しい分類が提唱されている。また，ELISA 法や免疫ブロット法などの診断法の開発も進んでいる。さらに，天疱瘡と後天性表皮水疱症を中心に各種の疾患モデルが開発されており，今後，自己免疫性疾患の研究のプロトタイプとなることが期待される。今回の特集では，10 名の新進の皮膚科研究者が自己免疫性水疱症の最新のトピックを紹介する。

Key Words
自己抗原／デスモソーム／ヘミデスモソーム／ ELISA 法／疾患モデル

はじめに

現在，多くの全身性および臓器特異的自己免疫性疾患が知られており，その多くは重篤・難治性で，患者の QOL の低下あるいは生命を脅かすことが多い。自己免疫性水疱症は皮膚の抗原に対する自己抗体を示す臓器特異的自己性免疫疾患であり，現在多種の疾患が知られている。多くの疾患は非常に難治性であり，致死的な経過をとることも少なくない。

自己免疫性水疱症は，天疱瘡群と類天疱瘡群に分類される。最近の生化学的・分子生物学的手法を用いた研究により多くの自己免疫性水疱症の抗原が明らかとなった。その自己抗原の多くは，天疱瘡群はデスモソーム構成蛋白であり，類天疱瘡群は，ヘミデスモソームから表皮基底膜部－真皮上部の構成蛋白である。これらの自己抗原とその分布を**図 1** にまとめた。さらに，その抗原解析の結果から新しい疾患概念も提唱され，新しい分類法も提案されている。

自己免疫性水疱症はその自己抗体が実際に病原性を有していることが証明された数少ない自己免疫性疾患である。現在，各種の *in vivo* あるいは *in vitro* の疾患モデルが開発され，その疾患モデルを用いて，その病因の検討も進んでいる。そのため，自己免疫性水疱症はまだその病態がほとんど不明

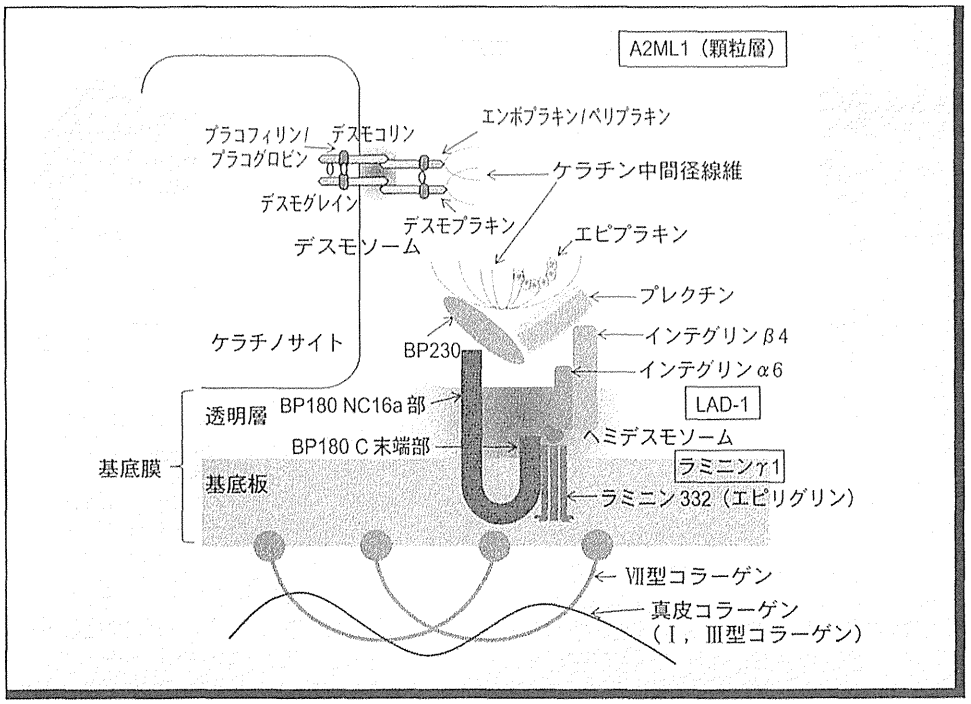

図1　デスモソームとヘミデスモソームの構造と構成蛋白の模式図

自己免疫性水疱症は，天疱瘡群と類天疱瘡群に分類される．天疱瘡群の自己抗原の多くはデスモソーム構成蛋白，類天疱瘡群はヘミデスモソームから表皮基底膜部－真皮上部の構成蛋白である．

（筆者作成）

である自己免疫性疾患の発症機序を研究する上でプロトタイプとなる可能性が高い。

Ⅰ．特集について

本特集では，まず，久留米大学皮膚科の石井文人先生が，主に自己抗原の検出による新しい自己免疫性水疱症の分類について述べる。次に，久留米大学皮膚科の古村南夫先生が今までの組織・蛍光抗体法から新しい免疫ブロット法・ELISA (enzyme-linked immunosorbent assay) 法などの抗原解析を含めた自己免疫性水疱症の診断法について詳細に述べ，慶應義塾大学皮膚科の谷川瑛子先生が自己免疫性水疱症の最新の治療について述べる。さらに，久留米大学皮膚細胞生物学研究所の上坂享成先生がいまだ未知の皮膚抗原の同定法について述べる。慶應義塾大学皮膚科の高橋隼人先生には最新の動物モデルを用いた天疱瘡の病態に関するに研究成果について，久留米大学皮膚科の古賀浩嗣先生には表皮下水疱症，特に後天性表皮水疱症について，最近の動物モデルを用いた病態に関するに研究成果について述べていただいた。次に，慶應義塾大学皮膚科の山上 淳先生が最近開発された多くの ELISA 法についてその有用性を，久留米大学皮膚科の大園綾花先生が久留米

ELISA (enzyme-linked immunosorbent assay)
AIRE (autoimmune regulator)
mTEC (medullary thymic epithelial cell)