Original Article

Involvement of P2Y receptors in the protective effect of ATP towards the cell damage in HaCaT cells exposed to H₂O₂

Emi Satoh, Mitsutoshi Tsukimoto and Shuji Kojima

Faculty of Pharmaceutical Sciences, Tokyo University of Science (TUS), 2641 Yamazaki,
Noda-shi, Chiba 278-8510, Japan

(Received August 11, 2011; Accepted September 16, 2011)

ABSTRACT — It has recently been reported that activation of P2Y₁ receptor, one of the purine receptors, by extracellular nucleotides induces cytoprotection against oxidative stress. In this study, we examined the protective effect of ATP on the cell damage in human epidermal keratinocyte HaCaT cells exposed to H₂O₂ via the P2Y receptor-mediated induction of intracellular antioxidants. The cells were damaged by exposure to H₂O₂ in a dose- and time-dependent manner. The damage induced by 7.5 mM H₂O₂ was blocked by pretreatment of the cells with ATP (1-10 μM). The protective effect of ATP was significantly reduced by P2Y receptor antagonists. Exogenously added ATP induced various intracellular antioxidants, including thiol-containing proteins, Cu/Zn superoxide dismutase (SOD) and thioredoxin-1, in HaCaT cells. In conclusion, it was found that ATP protected the cells from the H₂O₂-induced cell damages via the P2Y receptor-mediated induction of intracellular antioxidants.

Key words: ATP, Cytoprotection, P2Y receptor, Antioxidants, Hydrogen peroxide

INTRODUCTION

The skin is the largest organ of the body and is exposed to multiple external stimuli. It functions as a physical barrier and protects tissues and internal organs from harmful stimuli. It is well known that UV radiation, in particular UVB (290-320 nm), has adverse effects on human skin, inducing DNA damage (Matsunaga *et al.*, 1991; Matsumura and Ananthaswamy, 2004), skin cancer (Fisher and Kripke, 1982; Gloster and Brodland, 1996; Gerbaudo and Dinisio de Cabalier, 2010), inflammation (Yoshihisa *et al.*, 2010), and apoptosis (Kulms and Schwarz, 2000), which are mediated by generation of reactive oxygen species (ROS). Since skin is frequently exposed to oxidative stress, it is important to know what mechanisms serve to protect the skin cells.

Cellular production of ROS occurs in a wide variety of cells and plays a role in various biological processes. ROS, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (·OH) (Blokina *et al.*, 2003), are generated in cells via ROS-producing enzymes, such as NADPH oxidase. There is growing evidence that plasma membrane-bound NADPH oxidase is a major source of ROS production induced by UV radiation in keratino-

cytes (Beak *et al.*, 2004; Park *et al.*, 2006) and that ROS generation requires elevation of intracellular Ca²⁺ level in keratinocytes (Goldman *et al.*, 1997; Masaki *et al.*, 2009). ROS are highly reactive and are capable of causing oxidative DNA damage (Ehlers *et al.*, 1999), altering membrane potential (Ehlers *et al.*, 1999), peroxidizing proteins (Perluigi *et al.*, 2010) and lipids (Punnonen *et al.*, 1991), and inducing apoptosis (Clayton *et al.*, 1974). Production of ROS contributes to several patho-physiological processes, such as cancer (Lopez-Torres *et al.*, 1994), aging (Wallace *et al.*, 1998), neurodegenerative diseases (Albers and Beal, 2000) and inflammatory disorders (Trenam *et al.*, 1991). As an intracellular second messenger, ROS also activates transcription factors, such as NF-kappaB (Park *et al.*, 2006; Shimosawa, 2005), and intracellular signaling systems, such as MAPK (Ding *et al.*, 2002; Méndez-Samperio *et al.*, 2010).

ROS is scavenged by various antioxidants, including low-molecular-mass antioxidants such as reduced glutathione (GSH), thioredoxin (Trx), Vitamin C and Vitamin E, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Blokina *et al.*, 2003). Antioxidants act as a cooperative network, undergoing a series of redox reactions. The level of the molecules involved

are regulated to maintain an appropriate redox state, and are increased by exposure to exogenous oxidative stress. Even a slight increase of intracellular ROS generation stimulates intracellular antioxidant systems (Kojima *et al.*, 1998, 1999, 2000 and 2002; Haendeler *et al.*, 2004), though the mechanisms involved remain to be fully established.

Nucleotides, such as ATP and UTP, are physiologically released from cytoplasm into the extracellular space in various types of cells both under basal conditions and in response to various stimuli (Borst and Schrader, 1991; Guthrie *et al.*, 1999; Bodin and Burnstock, 2001; Schwiebert and Zsembery, 2003; Arthur *et al.*, 2007; Yegutkin, 2008). Extracellular ATP, in particular, is known to play an important role in rapid intracellular signaling. These nucleotides bind to specific plasma membrane receptors called purinergic (P2) receptors, and immediately activate these receptors (Bodin and Burnstock, 2001; Yegutkin *et al.*, 2008). Two large families of P2 receptors are known, ligand-gated ionotropic P2X₁₋₇ receptors and G-protein-coupled metabotropic P2Y_{1,2,4,6,11-14} receptors (Abbracchio *et al.*, 2006; Burnstock, 2007). The released ATP is rapidly degraded to ADP, AMP and adenosine by ecto-nucleotidases (Yegutkin, 2008). Activation of P2 receptors of epidermal keratinocytes plays a significant role in proliferation, differentiation, and apoptosis (Pillai and Bikle, 1992; Greig *et al.*, 2003; Inoue *et al.*, 2005). Recently, it has been reported that extracellular ATP-mediated activation of P2 receptors (ATP signaling) attenuates oxidative damage in several types of cells, including astrocytes (Shinozaki *et al.*, 2005, 2006) and human lung microvascular endothelial cells (Ahmad *et al.*, 2004). The protective effect of ATP was mediated primarily by P2Y receptors in most cases. We also previously found that the ATP-induced Trx-1 expression in mouse macrophage-like RAW264.7 cells was significantly inhibited by treatment with MRS2578, a specific antagonist of P2Y₆, suggesting the involvement of P2Y₆ receptors in this phenomenon (Ohshima *et al.*, 2011). Human epidermal keratinocyte HaCaT cell has been extensively used for studies on the effects of UV radiation and oxidative stresses, and is also known to highly express P2Y receptors and to release ATP in response to various stimuli (Burrell *et al.*, 2003, 2005). We previously also examined the effect of H₂O₂ and ionizing radiation on ATP release from HaCaT cells and confirmed that these stimuli induced ATP release from cells and that the release was induced via non-lytic pathways including anion transporter and gap junction hemichannels (Takai *et al.*, 2011; Tsukimoto *et al.*, 2010). It would be suggested that oxidative stresses induced ATP release may be an adaptive

response, leading to promotion of intracellular antioxidants, via purinergic signalling. However, the involvement of purinergic signaling in the cytoprotective effect of ATP is still unclear.

Here, we examined whether ATP ameliorates H₂O₂-induced cell damage via the P2Y receptor-mediated induction of intracellular antioxidants in human epidermal keratinocyte HaCaT cells.

MATERIALS AND METHODS

Reagents

ATP and UTP were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), H₂O₂, and DTT were from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Suramin, A438079, MRS2179, MRS2578, NF157, clopidogrel hydrochloride and MRS2211 were from Tocris Bioscience (Bristol, UK). Fetal bovine serum (FBS) was from Biowest (Nuaille, France). All other chemicals used were of the highest purity available.

Cell culture

Since we have already reported that HaCaT cells highly express P2Y_{1,2,6,12,13} receptors and weakly express P2X_{5,7} and P2Y₁₄ receptors (Tsukimoto *et al.*, 2010; Takai *et al.*, 2011), the cells were chosen in this study. The cells (kindly provided by Dr. K. Takeda) were cultured in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 mg/ml streptomycin (Wako Pure Chemicals Co. Ltd., Osaka, Japan) with 5 % CO₂ at 37°C. For experiments, the cells were seeded on a 60 mm dish and a 96-well plate.

H₂O₂-induced cell damage of HaCaT cells

HaCaT cells were exposed to various concentrations of H₂O₂ (0.5-10 mM) for 6 hr, or 7.5 mM H₂O₂ for 1-24 hr. The effects of ATP and UTP on the cell damage were examined at the fixed concentration of 7.5 mM H₂O₂. Cells were pretreated with various concentrations (1, 10, and 100 μM) of ATP or UTP for 6 hr, and then exposed to H₂O₂ at a concentration of 7.5 mM for 6 hr, and the culture supernatant was collected and subjected to LDH assay.

Involvement of P2 receptor subtypes in the protective effect of ATP and UTP was examined by using selective inhibitors; Cells were incubated with ATP or UTP at a concentration of 10 μM for 6 hr in the presence or absence of MRS2179 (100 μM), MRS2578 (10 μM), NF157 (50 μM), clopidogrel (30 μM), MRS2211 (100 μM), or suramin (100 μM). After incubation, cells were

exposed to H_2O_2 at a dose of 7.5 mM for another 6 hr and cell damage was evaluated by means of LDH assay.

LDH activity was determined colorimetrically with a Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN, USA). The activity was calculated in accordance with the manufacturer's instructions.

Assay of intracellular thiol contents

Intracellular thiol levels were measured using ThiolTracker Violet (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were incubated for 24 hr in a 96-well plate and treated with ATP or UTP for 12 hr. After the incubation, the supernatant was removed and the cells were washed once with PBS then incubated in HBSS containing 20 μ M (final concentration) ThiolTracker Violet for 30 min at 37°C. The supernatant was replaced with HBSS, and fluorescence was measured using a Wallac 1420 ARVO-SX multilabel counter, Perkin Elmer Life Sciences, Tokyo, Japan (excitation/emission = 355 nm/535 nm).

Detection of catalase mRNA

Total RNA was extracted from HaCaT cells and the first-strand cDNA was synthesized as described above. The cDNA was used as template for real-time PCR analysis: reactions were performed in an Stratagene Mx3000P® QPCR system (Agilent Technologies, La Jolla, CA, USA). The sequences for specific primers for hCatalase were 5'-CGTGCTGAATGAGGAACAGA -3' (sense), 5'-TTGTCCAGAAGAGCCTGGAT -3' (antisense) and for hActin as a positive control were 5'-GAACGGTGAAGGTGACAGCAG -3' (sense), 5'-GTGGACTTGGGAGAGGACTGG -3' (antisense). Each sample was assayed in a 20 μ l amplification reaction, containing cDNA, primers mixture (0.4 μ M each of sense and antisense primers) and 2 x GoTaq® qPCR Master Mix (Promega, Madison, WI, USA). The amplification program included 40 cycles of two steps, each comprising heating to 95°C and to 60°C, respectively. Fluorescent products were detected at the last step of each cycle. The obtained values were within the linear range of a standard curve and were normalized to yield the same amount of β -actin mRNA expression.

Immunoblotting

Cells were washed with cold PBS twice and lysed in cold lysis buffer (10 mM HEPES-NaOH pH 7.4, 1% Triton X-100, 5 mM 2Na-EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Proteins

were dissolved in sample buffer (25% glycerin, 1% SDS, 62.5 mM Tris-Cl, 10 mM DTT) and boiled for 5 min. Aliquots of samples (10 μ g per lane) were separated by means of 10% SDS-PAGE and transferred onto a PVDF membrane. Blots were incubated at 4°C overnight in TBST (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5) with 1% BSA, and then with rabbit anti-thioredoxin-1 antibody (1:1,000) (Cell Signaling Technology, Inc., Beverly, MA, USA), and mouse anti- β -actin antibody (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, and with rabbit anti-Cu/Zn SOD antibody (1:1,000) (StressGen, Victoria, Canada) at room temperature for 1.5 hr. After having been washed with TBST, blots were incubated with goat HRP-conjugated anti-rabbit IgG antibody (1:20,000) (Cell Signaling Technology, Beverly, MA, USA) or goat HRP-conjugated anti-mouse IgG antibody (1:20,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 hr at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Equal loading was confirmed using anti- β -actin antibody to normalize the amount of total protein.

Statistical analysis

Data are presented as mean $\pm$ S.E. The significance of differences was evaluated by means of ANOVA, and P values of less than 0.05 were considered statistically significant.

RESULTS

H_2O_2 -induced cell damage to HaCaT cells

The cell-damaging effect of H_2O_2 on HaCaT cells was evaluated in terms of LDH release. The release ratio of LDH at 6 hr post-treatment was significantly increased by exposure of the cells to H_2O_2 at concentrations of more than 5 mM (Fig. 1A). The time-dependence of the effect of H_2O_2 at the fixed concentration of 7.5 mM was then examined. The release was significantly increased at 3 hr post-treatment with H_2O_2 , and reached a plateau after 6 hr (Fig. 1B). In subsequent experiments, the cell damage was assessed at 6 hr post-treatment with H_2O_2 at the fixed concentration of 7.5 mM.

Effect of exogenously added nucleotides on H_2O_2 -induced cell damage to HaCaT cells

The effect of exogenously added ATP on the H_2O_2 -induced cell damage was first investigated. Cells were preincubated with various concentrations of ATP for 6 hr

Fig. 1. Effect of H₂O₂ on cell damage to HaCaT cells. (A) Dose-dependence of cell damage due to H₂O₂. Cells were incubated with different concentrations of H₂O₂ (0.5–10 mM) for 6 hr and a part of the culture medium was collected for LDH assay (n = 4). (B) Time-dependence of cell damage due to H₂O₂. Cells were incubated for different times (1–24 hr) with H₂O₂ at a dose of 7.5 mM and LDH assay was performed. The activity is expressed as a percentage of the total content determined by lysing an equal amount of cells with 1% Triton X-100. Each value represents the mean ± S.E. (n = 4). *, **, and *** Indicate that experimental values are significantly different (p < 0.05, p < 0.01, and p < 0.001, respectively) compared to the non-H₂O₂-exposed control group. #, ##, and ### Indicate that experimental values are significantly different (p < 0.05, p < 0.01, and p < 0.001, respectively) compared to the time 0 group.

and then exposed to H₂O₂ at a concentration of 7.5 mM for 6 hr. Pretreatment with ATP at concentrations between 1 and 10 μM significantly attenuated the H₂O₂-induced cell damage. However the efficacy of ATP at concentrations more than 50 μM appeared to be somewhat less (Fig. 2 A). Next, the effect of UTP, another ligand of P2Y, on the H₂O₂-induced cell damage was examined. UTP also significantly inhibited the cell damage at concentrations between 1 and 100 μM (Fig. 2 B). In addition, neither ATP nor UTP alone had any effect on HaCaT cell viability (data not shown).

Fig. 2. Effect of pretreatment with ATP and UTP on H₂O₂-induced cell damage to HaCaT cells. Cells were pretreated with various concentrations (1, 5, 10, 50 and 100 μM) of ATP (A) or UTP (B) for 6 hr. Then, the cells were exposed to H₂O₂ at a concentration of 7.5 mM for 6 hr and cell damage was analyzed by LDH assay (n = 4). The data represent the mean ± S.E. (n = 4). ### Indicates that experimental value is significantly different (p < 0.005) compared to the non-H₂O₂-exposed control group. *, **, and *** Indicate that experimental values are significantly different (p < 0.05, p < 0.01, and p < 0.001, respectively) compared to the H₂O₂-alone-treated group.

Estimation of P2Y receptor subtypes involved in the protective effect of ATP

Since we have already reported that HaCaT cells highly express P2Y_{1, 2, 6, 12, 13} receptors and weakly express P2X_{5, 7} and P2Y₁₄ receptors (Tsukimoto *et al.*, 2010; Takai *et al.*, 2011), the involvement of P2 receptor subtypes in the protective effect of ATP and UTP was examined by using P2Y inhibitors: MRS2179 (a selective P2Y₁ receptor antagonist), MRS2578 (a selective P2Y₆ receptor antagonist), NF157 (a selective P2Y₁₁ receptor antagonist), clopidogrel (a selective P2Y₁₂ receptor

Involvement of purine (P2Y) receptors in the protective effect of ATP

Fig. 3. Effect of P2Y receptor inhibitors on ATP- and UTP-induced cytoprotection against H₂O₂-induced cell damage to HaCaT cells. Cells were incubated with ATP (A) or UTP (B) at a concentration of 10 μ M for 6 hr in the presence or absence of MRS2179 (100 μ M), MRS2578 (10 μ M), NF157 (50 μ M), clopidogrel (30 μ M), MRS2211 (100 μ M), or suramin (100 μ M). After incubation, cells were exposed to H₂O₂ at a dose of 7.5 mM for 6 hr and cell damage was evaluated by means of LDH assay. The data represent the mean $\pm$ S.E. (n = 4). ### Indicates that experimental value is significantly different (p < 0.005) compared to the non- H₂O₂-exposed control group. *, **, and *** Indicate that experimental values are significantly different (p < 0.05, p < 0.01, and p < 0.001, respectively) compared to the H₂O₂-alone-treated group. \$ Indicates that experimental value is significantly different (p < 0.05) compared to the H₂O₂ plus ATP- or UTP-treated group.

antagonist), MRS2211 (a selective P2Y₁₃ receptor antagonist), and suramin (a non-selective P2Y receptor antagonist). The protective effect of ATP was significantly reduced by pretreatment with P2Y receptor antagonists, such as MRS2179, MRS2578, and MRS2211, suggesting the involvement of P2Y_{1,6,13} receptors (Fig. 3A). The effect of UTP was significantly blocked by treatment with MRS2578 and MRS2211, suggesting the involvement of P2Y_{6,13} receptors (Fig. 3B).

Induction of intracellular antioxidants by ATP in HaCaT cells

It is generally accepted that various antioxidants, including GSH, Trx-1, and SOD, participate in cellular protection against oxidative stress (Maître *et al.*, 1993; Holmgren, 2000; Blokhina *et al.*, 2003). We previously showed that extracellular nucleotides such as ATP and UTP increase intracellular thiol-containing proteins and expression of cytosolic thioredoxin (Trx-1) in RAW264.7 cells via activation of P2Y₆ receptor, and the inductions were observed in the same concentration range (1-100 μ M) of nucleotides (Ohshima *et al.*, 2011). Here, induction of intracellular antioxidants in HaCat cells treated with ATP and UTP was examined at the protein expression levels. We found that the intracellular thiol content was significantly increased in a dose-dependent manner at 12 hr post-treatment with ATP and UTP at concentrations between 1 and 100 μ M (Figs. 4A and B). The protein expression levels of Cu/Zn SOD and thioredoxin-1 were both significantly increased at 12 hr after treatment with ATP and UTP in the same concentration range (Figs. 4 C and D).

Induction of catalase mRNA expression

Since the protein expression of catalase, a specific scavenger of H₂O₂, was not induced at 12 hr post-treatment at the protein expression level, induction of the catalase mRNA was examined. The expression was elevated from 3 hr, got to a maximum at 6 hr, and returned to almost the time 0 level at 24 hr in HaCat cells after treatment with 10 μ M ATP (Fig. 5).

DISCUSSION

It has recently been reported that pre-incubation of cells with ATP has a cytoprotective effect against oxidative stress induced by H₂O₂ (Shinozaki *et al.*, 2005, 2006) or hypoxia (Ahmad *et al.*, 2004). However, little is known about the mechanisms involved. Here, we examined the involvement of ATP released from cells exposed to H₂O₂ in cytoprotection via induction of antioxidant enzymes in human keratinocyte HaCaT cells, which have been reported to highly express P2 receptors and to release ATP in response to various stimuli (Burrell *et al.*, 2003, 2005; Takai *et al.*, 2011; Tsukimoto *et al.*, 2010).

First, we examined whether or not exogenously added ATP has a protective effect on H₂O₂-induced cell damage in HaCaT cells. The damage induced by H₂O₂ exposure was significantly reduced by pretreatment with exogenously added ATP. Interestingly, low concentrations of ATP between 1 and 10 μ M significantly attenuated the

Fig. 4. Effects of exogenously added ATP and UTP on intracellular thiol content and the protein expression of thioredoxin-1 and Cu/Zn SOD in HaCaT cells. Thiol content; cells were incubated with ATP (A) or UTP (B) at concentrations of 1, 10, 100 and 1,000 μM . After 12 hr incubation, the thiol content was assayed with ThiolTracker Violet. Protein expression levels of Trx-1 and Cu/Zn SOD induced by ATP (C) and UTP (D) at concentrations of 1, 10, and 100 μM were determined by Western blotting. β -Actin was simultaneously measured as a control for equivalent protein loading in Western blotting. Each thiol content indicated is the mean $\pm$ S.E. ($n = 4$). * Indicates that experimental value is significantly different compared to the non-ATP/UTP-treated 0 group.

Fig. 5. Increased catalase mRNA expression in HaCaT cells treated with ATP. Cells were treated with ATP (10 μM) and incubated for the indicated times (0-24). After incubation, expression of catalase mRNA was determined by real time RT-PCR. The data represent the mean $\pm$ S.E. ($n = 3$). * and *** Indicate that experimental values are significantly different ($p < 0.05$ and $p < 0.001$, respectively) compared to the time 0 group.

H_2O_2 -induced cell damage, while the efficacy more than 50 μM appeared to be somewhat less. These results are consistent with the finding that the protein expression levels of Trx-1 and SOD induced by ATP at the concentration of 100 μM were less than those at the lower concentrations. Similar results were obtained with UTP, which is also reported to be released from cells in response to various stimuli, disease, or injury (Lazarowski *et al.*, 1997, 2003; Lazarowski and Boucher, 2001; Erlinge *et al.*, 2005) and recognizes P2Y receptors. These results suggest involvement of P2Y receptors in the protective effect of ATP against H_2O_2 -induced cell damage in HaCaT cells as well as in RAW264.7 cells.

Next, the P2Y receptors involved in the nucleotide-mediated protection against H_2O_2 -induced cell damage were investigated by using selective inhibitors. It was suggested that P2Y_{1, 6, 13} receptors are involved. Similarly, the effect of UTP was significantly blocked by the P2Y_{6, 13} inhibitors. ADP, a metabolite of ATP, activates

P2Y₁, P2Y₁₂, and P2Y₁₃ and was reported to be equipotent or more potent than ATP towards P2Y₁. In addition, it is considered that UTP is more potent than ATP against P2Y₆ receptor. It has been reported that exogenously applied ATP is rapidly metabolized by ubiquitously expressed ecto-nucleotidases on the cell membrane (Shinozaki *et al.*, 2005; Dwyer *et al.*, 2007)). Thus, the protective effect of ATP appears likely to be induced through activation of P2Y₁ and P2Y₁₃ receptors by not ATP, but rather its metabolite ADP.

Interesting, P2Y₁₃ receptor was recently reported to play a role in induction of the antioxidant HO-1, thereby protecting neuronal cells from oxidative stress (Espada *et al.*, 2010). It is well established that adenosine receptors play a role in cytoprotection against oxidative stress and inflammation (Maggirwar *et al.*, 1994; Zhang *et al.*, 2005), but little is known about the involvement of P2Y receptors. Our results are consistent with an important role of P2Y receptors in ATP- and UTP-mediated protection against oxidative stresses.

As described above, antioxidants such as GSH, Trx, catalase and SOD participate in cellular protection against oxidative stress (Maître *et al.*, 1993; Holmgren, 2000). Shinozaki *et al.* (2005) reported that ATP induced a dramatic up-regulation of various oxidoreductase genes in astrocytes, based on DNA microarray analysis. We also found that extracellular nucleotides, ATP and UTP, in the same concentration range (1-100 μ M) induced intracellular antioxidants, such as reduced glutathione and thioredoxin-1, in RAW264.7 cells via activation of P2X₇ and P2Y₆ and that these inductions continued for up to 24 hr after the treatment (Ohshima *et al.*, 2011). Here, we examined the induction of intracellular antioxidant proteins in HaCat cells treated with ATP and UTP. The intracellular thiol content was increased dose-dependently at 12 hr post-treatment with ATP or UTP in the concentration range between 1 and 100 μ M. The protein expression levels of Cu/Zn SOD and thioredoxin-1 were both significantly increased as well, though the extent of the increase was somewhat less at concentrations more than 50 μ M, compared with those at lower concentrations. These results seem consistent with the finding that ATP also significantly attenuated the H₂O₂-induced cell damage at 1 and 10 μ M, but weakly did at concentrations more than 50 μ M, and the report that a local extracellular ATP concentrations of 1 to 10 μ M is sufficient to activate Ca²⁺ responses mediated by high-affinity P2 receptors in neighboring cells (Bulanova and Bulfone-Paus, 2010).

Trx-1 potentially protects cells from oxidative damage due to its ability to enhance the catalytic activities of peroxiredoxin and glutathione peroxidase, which decompose

hydroperoxides and hydrogen peroxide, respectively. Trx-1 can also directly reduce hydrogen peroxide, glutathione disulfide, and hydroxyl radical (Kalinina *et al.*, 2008; Dalton *et al.*, 1999). Therefore, Trx-1 is a key protein controlling the cellular reductive/oxidative (redox) balance. Moreover, protein expression of catalase, a specific scavenger of H₂O₂, was not induced at 12 hr post-treatment with 10 μ M ATP, but induction of the mRNA at an earlier time of 6 hr was observed, expecting that catalase in protein expression level may be induced within 12 hr post-treatment with ATP. Overall, these results suggest that extracellular ATP and UTP induce intracellular antioxidants in HaCaT keratinocyte cells via activating P2Y receptors, as well as RAW264.7 cells.

It is generally recognized that high levels of ROS directly cause oxidative damage of DNA, lipids, and proteins and may impair cellular functions, even inducing apoptosis, while low concentrations of ROS stimulate signal transduction and modulate gene expression, leading to induction of various proteins, including antioxidants (Kojima *et al.*, 1998, 1999, 2000 and 2002; Haendeler *et al.*, 2004). We have already found that the elevation of Trx-1 expression induced by ATP in RAW264.7 cells was significantly lowered almost to the control level by pre-treatment with antioxidants, suggesting that ATP-induced ROS are involved, at least in part, in the elevation of Trx-1 expression as an adaptive response (Ohshima *et al.*, 2011). Haendeler *et al.* (2004) reported that low concentrations of H₂O₂ (10 to 50 μ M) have an anti-apoptotic effect in endothelial cells, as well as up-regulating Trx-1 protein expression. In this case, it is considered that H₂O₂ caused induction of Trx-1 mRNA, leading to protein expression. Since Trx-1 directly reacts with H₂O₂ to protect cells from oxidative damage, it seems reasonable to expect feedback regulation, in which H₂O₂ regulates the expression of this antioxidant. Various types of ROS, such as hydroxyl radical, superoxide anion radical and so on, may be produced from H₂O₂ via the Fenton reaction and Haber-Weiss reaction. Therefore, similar induction of other antioxidants would also be expected.

Another point of interest is the role of nucleotide-dependent ROS generation. It has already been reported that ROS generation in RAW264.7 cells in the presence of ATP was significantly suppressed by pretreatment with the NADPH oxidase inhibitor DPI and apocynin, supporting the idea that NADPH oxidase is a major mediator of ATP-induced ROS generation (Noguchi *et al.*, 2008; Ohshima *et al.*, 2011). Similar ROS production might occur in the present case, though we did not confirm the fact. From another point of view, it is reported that ATP stimulates release of interleukin (IL)-6 through activation

of P2Y receptor in HaCaT cells (Yoshida *et al.*, 2006). Fujita *et al.* (2009) also reported that IL-6 released via the same receptor plays a significant role in ATP-induced cytoprotection against oxidative stress in astrocytes. IL-6 is a pleiotropic cytokine that is produced at sites of tissue inflammation. Interestingly, it has potent anti-inflammatory and protective properties (Tilg *et al.*, 1994; Xing *et al.*, 1998), and moreover inhibits apoptosis (Rollwagen *et al.*, 1998). Furthermore, IL-6 activates multiple signal transduction pathways, such as the JAK/STAT pathway, the Ras/ERK pathway and the PI3K/Akt pathway (Hirano *et al.*, 1997, 2000; Horvath, 2004). More recently, it has been further demonstrated that IL-6 has a cytoprotective effect against oxidative stress though PI3K/Akt-mediated Bax phosphorylation, which inhibits apoptosis by blocking translocation of Bax to mitochondria (Kolliputi and Waxman, 2009). However, the possible involvement of IL-6 in the cytoprotective effect of nucleotides in keratinocytes has not yet been explored. Further investigation focused on intracellular signaling will be needed to understand in detail the mechanisms of nucleotide-induced cytoprotection against oxidative stress.

Our results indicate that ATP released from keratinocyte-derived HaCaT cells exposed to H₂O₂ protects the cells from H₂O₂-induced cell damage, at least in part, by induction of antioxidants in response to slight ROS generation as a result of activation of P2Y receptors. These findings suggest that the processes underlying extracellular nucleotide-mediated cytoprotection against oxidative stress in epidermal keratinocytes may be rather indirect.

ACKNOWLEDGMENTS

Parts of this work were supported by Grant-in-Aid for Scientific Research "KAKENHI" (C) (No. 20591506) (to SK) and (HOGAKENKYU) (No. 23651055) (to SK) from the Ministry of Education, Culture, Sport, Science, and Technology of Japan, and also by a Grant-in-Aid for the private University Science Research Upgrade Promotion Business Academic Frontier Project.

REFERENCES

- Abbracchio, M.P., Burnstock, G., Boeynaems, J.M., Barnard, E.A., Boyer, J.L., Kennedy, C., Knight, G.E., Fumagalli, M., Gachet, C., Jacobson, K.A. and Weisman, G.A. (2006): International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol. Rev.*, **58**, 281-341.
- Ahmad, S., Ahmad, A., Ghosh, M., Leslie, C.C. and White, C.W. (2004): Extracellular ATP-mediated signaling for survival in hyperoxia-induced oxidative stress. *J. Biol. Chem.*, **279**, 16317-16325.
- Albers, D.S. and Beal, M.F. (2000): Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease. *J. Neural Transm., Suppl.* **59**, 133-154.
- Arthur, D.B., Taupenot, L. and Insel, P.A. (2007): Nerve growth factor-stimulated neuronal differentiation induces changes in P2 receptor expression and nucleotide-stimulated catecholamine release. *J. Neurochem.*, **100**, 1257-1264.
- Beak, S.M., Lee, Y.S. and Kim, J.A. (2004): NADPH oxidase and cyclooxygenase mediate the ultraviolet B-induced generation of reactive oxygen species and activation of nuclear factor-kappaB in HaCaT human keratinocytes. *Biochimie*, **86**, 425-429.
- Blokhina, O., Virolainen, E. and Fagerstedt, K.V. (2003): Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.*, **91**, 179-194.
- Bodin, P. and Burnstock, G. (2001): Purinergic signalling: ATP release. *Neurochem. Res.*, **26**, 959-969.
- Borst, M.M. and Schrader, J. (1991): Adenine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine. *Circ. Res.*, **68**, 797-806.
- Bulanova, E. and Bulfone-Paus, S. (2010): P2 receptor-mediated signaling in mast cell biology. *Purinergic Signalling*, **6**, 3-17.
- Burnstock, G. (2007): Physiology and pathophysiology of purinergic neurotransmission. *Physiol. Rev.*, **87**, 659-797.
- Burrell, H.E., Bowler, W.B., Gallagher, J.A. and Sharpe, G.R. (2003): Human keratinocytes express multiple P2Y-receptors: evidence for functional P2Y1, P2Y2, and P2Y4 receptors. *J. Invest. Dermatol.*, **120**, 440-447.
- Burrell, H.E., Wlodarski, B., Foster, B.J., Buckley, K.A., Sharpe, G.R., Quayle, J.M., Simpson, A.W. and Gallagher, J.A. (2005): Human keratinocytes release ATP and utilize three mechanisms for nucleotide interconversion at the cell surface. *J. Biol. Chem.*, **280**, 29667-29676.
- Clayton, D.A., Doda, J.N. and Friedberg, E.C. (1974): The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. *Proc. Natl. Acad. Sci. USA*, **71**, 2777-2781.
- Dalton, T.P., Shertzer, H.G. and Puga, A. (1999): Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol.*, **39**, 67-101.
- Ding, M., Li, J., Leonard, S.S., Shi, X., Costa, M., Castranova, V., Vallyathan, V. and Huang, C. (2002): Differential role of hydrogen peroxide in UV-induced signal transduction. *Mol. Cell Biochem.*, **234-235**, 81-90.
- Dwyer, K.M., Deaglio, S., Gao, W., Friedman, D., Strom, T.B. and Robson, S.C. (2007): CD39 and control of cellular immune responses. *Purinergic Signalling*, **3**, 171-180.
- Ehlers, R.A., Hernandez, A., Bloemendal, L.S., Ethridge, R.T., Farrow, B. and Evers, B.M. (1999): Mitochondrial DNA damage and altered membrane potential (delta psi) in pancreatic acinar cells induced by reactive oxygen species. *Surgery*, **126**, 148-155.
- Erlinge, D., Harnek, J., van Heusden, C., Olivecrona, G., Jern, S. and Lazarowski, E. (2005): Uridine triphosphate (UTP) is released during cardiac ischemia. *Int. J. Cardiol.*, **100**, 427-433.
- Espada, S., Ortega, F., Molina-Jijón, E., Rojo, A.N., Pérez-Sen, R., Pedraza-Chaverri, J., Miras-Portugal, M.T. and Guadado, A. (2010): The purinergic P2Y13 receptor activates the Nrf1/HO-1 axis and protects against oxidative stress-induced neuronal death. *Free Radic. Biology Med.*, **49**, 416-426.
- Fisher, M.S. and Kripke, M.L. (1982): Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. *Science*, **216**, 1133-1134.
- Fujita, T., Tozaki-Saitoh, H. and Inoue, K. (2009): P2Y1 receptor

Involvement of purine (P2Y) receptors in the protective effect of ATP

- signaling enhances neuroprotection by astrocytes against oxidative stress via IL-6 release in hippocampal cultures. *Glia*, **57**, 244-257.
- Gerbaudo, M. and Dionisio de Cabalier, M.E. (2010): Effect of UV index in the skin exposition. *Rev. Fac. Cien. Med. Univ. Nac. Cordoba*, **67**, 32-39.
- Gloster, H.M.Jr. and Brodland, D.G. (1996): The epidemiology of skin cancer. *Dermatol. Surg.*, **22**, 217-226.
- Goldman, R., Moshonov, S., Chen, X., Berchansky, A., Fürstenberger, G. and Zor, U. (1997): Crosstalk between elevation of $[Ca^{2+}]_i$, reactive oxygen species generation and phospholipase A2 stimulation in a human keratinocyte cell line. *Adv. Exp. Med. Biol.*, **433**, 41-45.
- Greig, A.V., Linge, C., Cambrey, A. and Burnstock, G. (2003): Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. *J. Invest. Dermatol.*, **121**, 1145-1149.
- Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M.V., Charles, A.G. and Kater, S.B. (1999): ATP released from astrocytes mediated glial calcium waves. *J. Neurosci.*, **19**, 520-528.
- Haendeler, J., Tischler, V., Hoffmann, J., Zeiher, A.M. and Dimmeler, S. (2004): Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett.*, **577**, 427-433.
- Hirano, T., Nakajima, K. and Hibi, M. (1997): Signaling mechanisms through gp130: a model of the cytokine system. *Cytokine Growth Factor Rev.*, **8**, 241-252.
- Hirano, T., Ishihara, K. and Hibi, M. (2000): Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene*, **19**, 2548-2556.
- Holmgren, A. (2000): Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid. Redox Signal*, **2**, 811-820.
- Horvath, C.M. (2004): The Jak-STAT pathway stimulated by interleukin 6. *Sci. STKE*, **260**, tr9.
- Inoue, K., Dendam, M., Tozaki, H., Fujishita, K., Koizumi, S. and Inoue, K. (2005): Characterization of multiple P2X receptors in cultured normal human epidermal keratinocytes. *J. Invest. Dermatol.*, **124**, 756-763.
- Kalinina, E.V., Chernov, N.N. and Saprin, A.N. (2008): Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry Biokhimiia*, **73**, 1493-1510.
- Kojima, S., Matsuki, O., Numura, T., Kubodera, A., Nonda, S., Tanooka, H., Wakasugi, K. and Yamaoka, K. (1998): Induction of mRNAs for glutathione synthesis-related proteins in mouse liver by low doses of γ -rays. *Biochim. Biophys. Acta*, **1381**, 312-318.
- Kojima, S., Matsuki, O., Nomura, T., Yamaoka, K., Takahashi, M. and Niki, E. (1999): Elevation of antioxidant potency in the brain of mice by low-dose γ -ray irradiation and its effect on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced brain damage. *Free Radic. Biol. Med.*, **26**, 388-395.
- Kojima, S., Matsumori, S., Ishida, H. and Yamaoka, K. (2000): Possible role of elevation of glutathione in the acquisition of enhanced proliferation of mouse splenocytes exposed to small-dose γ -rays. *Int. J. Radiat. Biol.*, **76**, 1641-1647.
- Kojima, S., Ishida, H., Takahashi, M. and Yamaoka, K. (2002): Elevation of glutathione induced by low-dose gamma rays and its involvement in increased natural killer activity. *Radiat. Res.*, **157**, 275-280.
- Kolliputi, N. and Waxman, A.B. (2009): IL-6 cytoprotection in hyperoxic acute lung injury occurs via PI3K/Akt-mediated Bax phosphorylation. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **297**, L6-L16.
- Kulms, D. and Schwarz, T. (2000): Molecular mechanisms of UV-induced apoptosis. *Photodermatol. Photoimmunol. Photomed.*, **16**, 195-201.
- Lazarowski, E.R. and Boucher, R.C. (2001): UTP as an extracellular signaling molecule. *News Physiol. Sci.*, **16**, 1-5.
- Lazarowski, E.R., Boucher, R.C. and Harden, T.K. (2003): Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol. Pharmacol.*, **64**, 785-795.
- Lazarowski, E.R., Homolya, L., Boucher, R.C. and Harden, T.K. (1997): Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J. Biol. Chem.*, **272**, 24348-24354.
- Lopez-Torres, M., Shindo, Y. and Packer, L. (1994): Effect of age on antioxidants and molecular markers of oxidative damage in murine epidermis and dermis. *J. Invest. Dermatol.*, **102**, 476-480.
- Maggirwar, S.B., Dhanraj, D.B., Somani, S.M. and Ramkumar, V. (1994): Adenosine acts as an endogenous activator of the cellular antioxidant defense system. *Biochem. Biophys. Res. Commun.*, **201**, 508-515.
- Maître, B., Jornot, L. and Junod, A.F. (1993): Effects of inhibition of catalase and superoxide dismutase activity on antioxidant enzyme mRNA levels. *Am. J. Physiol.*, **265**, L636-L643.
- Masaki, H., Izutsu, Y., Yahagi, S. and Okano, Y. (2009): Reactive oxygen species in HaCaT keratinocytes after UVB irradiation are triggered by intracellular Ca^{2+} levels. *J. Investig. Dermatol. Symp. Proc.*, **14**, 50-52.
- Matsumura, Y. and Ananthaswamy, H.N. (2004): Toxic effects of ultraviolet radiation on the skin. *Toxicol. Appl. Pharmacol.*, **195**, 298-308.
- Matsunaga, T., Hieda, K. and Nikaido, O. (1991): Wavelength dependent formation of thymine dimers and (6-4) photoproducts in DNA by monochromatic ultraviolet light ranging from 150 to 365 nm. *Photochem. Photobiol.*, **54**, 403-410.
- Méndez-Samperio, P., Pérez, A. and Alba, L. (2010): Reactive oxygen species-activated p38/ERK 1/2 MAPK signaling pathway in the *Mycobacterium bovis* bacillus Calmette Guérin (BCG)-induced CCl_2 secretion in human monocytic cell line THP-1. *Arch. Med. Res.*, **41**, 579-585.
- Noguchi, T., Ishi, K., Fukumoto, H., Naguro, I., Matsuzawa, A., Takeda, K. and Ichijo, H. (2008): Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. *J. Biol. Chem.*, **283**, 7657-7665.
- Ohshima, Y., Tsukimoto, M. and Kojima, S. (2011): Induction of extracellular ATP mediates increase in intracellular thioredoxin in RAW264.7 cells exposed to low-dose γ -rays. *Free Radic. Biol. Med.*, **51**, 1240-1248.
- Park, L.J., Ju, S.M., Song, H.Y., Lee, J.A., Yang, M.Y., Kang, Y.H., Kwon, H.J., Kim, T.Y., Choi, S.Y. and Park, J. (2006): The enhanced monocyte adhesiveness after UVB exposure requires ROS and NF- κ B signaling in human keratinocyte. *J. Biochem. Mol. Biol.*, **39**, 618-625.
- Perluigi, M., Di Domenico, F., Blarzino, C., Foppoli, C., Cini, C., Giorgi, A., Grillo, C., De Marco, F., Butterfield, D.A., Schininà, M.E. and Coccia, R. (2010): Effects of UVB-induced oxidative stress on protein expression and specific protein oxidation in normal human epithelial keratinocytes: a proteomic approach. *Proteome Sci.*, **8**, 13.

- Pillai, S. and Bikle, D.D. (1992): Adenosine triphosphate stimulates phosphoinositide metabolism, mobilizes intracellular calcium, and inhibits terminal differentiation of human epidermal keratinocytes. *J. Clin. Invest.*, **90**, 42-51.
- Punnonen, K., Autio, P., Kiistala, U. and Ahotupa, M. (1991): In-vivo effects of solar-simulated ultraviolet irradiation on antioxidant enzymes and lipid peroxidation in human epidermis. *Br. J. Dermatol.*, **125**, 18-20.
- Rollwagen, F.M., Yu, Z.Y., Li, Y.Y. and Pacheco, N.D. (1998): IL-6 rescues enterocytes from hemorrhage induced apoptosis *in vivo* and *in vitro* by a bcl-2 mediated mechanism. *Clin. Immunol. Immunopathol.*, **89**, 205-213.
- Schwiebert, E.M. and Zsembery, A. (2003): Extracellular ATP as a signaling molecule for epithelial cells. *Biochim. Biophys. Acta*, **1615**, 7-32.
- Shimosawa, T. (2005): Increasing oxidative stress in aging. *Nippon Rinsho*, **63**, 994-999.
- Shinozaki, Y., Koizumi, S., Ishida, S., Sawada, J., Ohno, Y. and Inoue, K. (2005): Cytoprotection against oxidative stress-induced damage of astrocytes by extracellular ATP via P2Y1 receptors. *Glia*, **49**, 288-300.
- Shinozaki, Y., Koizumi, S., Ohno, Y., Nagao, T. and Inoue, K. (2006): Extracellular ATP counteracts the ERK1/2-mediated death-promoting signaling cascades in astrocytes. *Glia*, **54**, 606-618.
- Takai, E., Tsukimoto, M., Harada, H. and Kojima, S. (2011): Involvement of P2Y6 receptor in p38 MAPK-mediated COX-2 expression in response to UVB irradiation of human keratinocytes. *Radiat. Res.*, **175**, 358-366.
- Tilg, H., Trehu, E., Atkins, M.B., Dinarello, C.A. and Mier, J.W. (1994): Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood*, **83**, 113-118.
- Trenam, C.W., Dabbagh, A.J., Morris, C.J. and Blake, D.R. (1991): Skin inflammation induced by reactive oxygen species (ROS): an in-vivo model. *Br. J. Dermatol.*, **125**, 325-329.
- Tsukimoto, M., Homma, T., Ohshima, Y., Kojima, S. (2010): Involvement of purinergic signaling in cellular response to gamma radiation. *Radiat. Res.*, **173**, 298-309.
- Wallace, D.C., Brown, M.D., Melov, S., Graham, B. and Lott, M. (1998): Mitochondrial biology, degenerative diseases and aging. *Biofactors*, **7**, 187-190.
- Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.F. and Achong, M.K. (1998): IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.*, **101**, 311-320.
- Yegutkin, G.G. (2008): Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim. Biophys. Acta*, **1783**, 673-694.
- Yoshida, H., Kobayashi, D., Ohkubo, S. and Nakahata, N. (2006): ATP stimulates interleukin-6 production via P2Y receptors in human HaCaT keratinocytes. *Eur. J. Pharmacol.*, **540**, 1-9.
- Yoshihisa, Y., Honda, A., Zhao, Q.L., Makino, T., Abe, R., Matsui, K., Shimizu, H., Miyamoto, Y., Kondo, T. and Shimizu, T. (2010): Protective effects of platinum nanoparticles against UV-light-induced epidermal inflammation. *Exp. Dermatol.*, **19**, 1000-1006.
- Zhang, Y., Handy, D.E. and Loscalzo, J. (2005): Adenosine-dependent induction of glutathione peroxidase 1 in human primary endothelial cells and protection against oxidative stress. *Circ. Res.*, **96**, 831-837.