Involvement of connexin43 hemichannel in ATP release after γ-irradiation

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Ionizing radiation induces biological effects not only in irradiated cells but also in non-irradiated cells, which is called the bystander effect. Recently, in vivo and in vitro experiments have suggested that both gap junction hemichannel connexin43 (Cx43) and extracellular adenosine triphosphate (ATP) released from cells play a role in the bystander effect. We have reported that γ-irradiation induces ATP release from B16 melanoma cells, which is dependent on the P2X7 receptor. However, the mechanism of ATP release caused by irradiation remains unclear. We here show the involvement of Cx43 in P2X7 receptor-dependent ATP release after 0.5 Gy γ-irradiation. Inhibitors of gap junction hemichannels and an inhibitory peptide for Cx43 (gap26), but not an inhibitory peptide for pannexin1 (Panx1), significantly blocked γ-irradiation-induced ATP release from B16 melanoma cells. We confirmed high expression of Cx43 mRNA in B16 melanoma cells. These results suggest involvement of Cx43 in radiation-induced ATP release. We found that after 0.5 Gy γ-irradiation tyrosine phosphorylation was significantly blocked by P2X7 receptor antagonist, but not gap26, suggesting that tyrosine phosphorylation is a downstream event from the P2X7 receptor. Since tyrosine kinase inhibitor significantly suppressed radiation-induced ATP release, tyrosine phosphorylation appears to play an important role in the Cx43-mediated ATP release downstream of the P2X7 receptor. In conclusion, the Cx43 hemichannel, which lies downstream of the P2X7 receptor, is involved in ATP release in response to radiation. Our results suggest a novel mechanism for radiation-induced biological effects mediated by both ATP and Cx43.

Keywords: γ-ray; ATP release; P2X7 receptor; connexin43; tyrosine kinase

INTRODUCTION

Ionizing radiation induces various biological effects not only in irradiated cells but also in non-irradiated cells or tissues [1]. This phenomenon is called the radiation-induced bystander effect, and is critically dependent on intercellular communication by gap junctions or by soluble factors released from the irradiated cells such as reactive oxygen species (ROS), cytokines or nitric oxide [1].

Cytoplasmic adenosine triphosphate (ATP) is released into the extracellular space in response to various stress stimuli, including shear stress, hypoxia, ischemia, inflammation, mechanical stretching and cell death [2].
Extracellular ATP and other nucleotides activate ionotropic P2X<sub>1,7</sub> and metabotropic P2Y<sub>1,14</sub> receptors expressed in the cell membrane in an autocrine/paracrine manner [2]. We have reported that γ-irradiation also induces ATP release and activation of the P2Y receptor in an autocrine/paracrine manner [3–5].

Usually, activation of P2 receptors by extracellular nucleotides leads to intracellular signaling and causes various physiological events, such as cell proliferation or cell death [6]. However, among P2 receptor subtypes only the P2X<sub>7</sub> receptor is known to contribute to the mechanism of ATP release from cells, because of interaction with hemichannels [7]. Recently, we have also shown that γ-irradiation induces P2X<sub>7</sub> receptor-dependent ATP release in B16 melanoma cells [8]. Several mechanisms of ATP release including P2X<sub>7</sub> receptor-dependent release have been reported [9–10]. However, the mechanism through which the P2X<sub>7</sub> receptor contributes to ionizing radiation-induced ATP release remains unclear.

The P2X<sub>7</sub> receptor is the seventh member of the P2X receptor subfamily, and its expression is increased in human melanoma cells [11]. Activation of the P2X<sub>7</sub> receptor induces an increase in cationic permeability, followed by plasma membrane depolarization; intense or prolonged activation leads to intracellular signaling cascades and the opening of large non-selective pores, allowing the passage of hydrophilic molecules of up to 900 Da in size, such as ethidium bromide (EtBr) [12]. Gap junction hemichannel connexin 43 (Cx43) is part of the pore-forming unit of the P2X<sub>7</sub> receptor and its activation is thought to be regulated by Src tyrosine kinase, which acts downstream of the P2X<sub>7</sub> receptor [13, 14]. Since several reports have shown that Panx1 mediates the release of cytoplasmic ATP, the involvement of Panx1 in P2X<sub>7</sub> receptor-dependent ATP release has been suggested [15]. On the other hand, although the mechanisms are not yet fully understood, a recent study has indicated a possible interaction between connexin 43 (Cx43), which forms gap junction hemichannels, and the P2X<sub>7</sub> receptor in dye uptake (pore formation) through Cx43 hemichannels [16].

Cx43 is ubiquitously expressed in various tissues and mediates the release of cytoplasmic ATP [9, 17, 18]. Gap junction hemichannels formed by connexins, including Cx43, freely allow the passage of molecules smaller than 1.2 kDa [17]. Selectivity of permeant molecules through hemichannels is different between connexins, and Cx43 favors ATP permeation [17]. Permeability of Cx43 hemichannels is regulated by various intracellular signaling pathways, such as increase of intracellular Ca<sup>2+</sup> and actin polymerization [19, 20]. In radiation biology, Cx43 is known to regulate the bystander effects of ionizing radiation through gap junction intercellular communication [21–23]. In addition, a recent study has suggested the involvement of both Cx43 and ATP in long-range bystander radiation damage and oncogenesis in vivo [24]. However, the relationship between Cx43 and ATP in their role on the bystander effect is unknown.

In this study, we investigated in detail the mechanism of P2X<sub>7</sub> receptor-dependent ATP release after γ-irradiation in B16 melanoma cells, focusing on the Cx43 hemichannel. Our results indicate involvement of the Cx43 hemichannel in radiation-induced ATP release in B16 melanoma cells downstream of the P2X<sub>7</sub> receptor. Furthermore, tyrosine phosphorylation, Rho-kinase, actin cytoskeleton, intracellular Ca<sup>2+</sup> and ROS also appear to contribute to radiation-induced ATP release. In this work, we have uncovered a novel mechanism of ATP release in γ-irradiated B16 melanoma cells. Our results suggest a relationship between Cx43 and ATP in the mechanism of radiation-induced biological effects.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), RPMI1640 medium (phenol red free), penicillin, streptomycin, dithiothreitol (DTT), L-ascorbic acid (vitamin C) and vitamin E were purchased from Wako Pure Chemical Industries (Osaka, Japan). A selective inhibitor of the P2X<sub>7</sub> receptor, 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl] pyridine hydrochloride (A438079) was purchased from Toeris Bioscience (Ellisville, MO, USA). Genistein, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), AG1478, 1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), 18α-glycyrrhetinic acid (18GA), carbenoxolone (CBX), 1,2,3,4,5,6-hexachlorocyclohexane (lindane), Y27632, diphenylene iodonium (DPI), apocynin, ATP and EtBr were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France). *Clostridium botulinum* C3 exoenzyme (C3 toxin) was purchased from BioAcademia (Osaka, Japan). Mimetic inhibitory peptides for Panx1 (10panx1) and Cx43 (gap26) were synthesized at Operon Biotechnologies (Tokyo, Japan). The sequences of 10panx1 and gap26 were WRQAAFVDSY and VCYDKSFPISHVR, respectively. All other chemicals used were of the highest purity available.

**Cell culture**

B16 murine melanoma was routinely maintained in DMEM containing 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) at 37°C in 5% CO<sub>2</sub>, 95% air.
Measurement of extracellular ATP
Extracellular ATP concentration was measured using ENLITEN® rLuciferase/Luciferin Reagent (Promega, Madison, WI, USA). Cells (3.0 × 10^4 cells/well) were incubated in 500 μl of RPMI1640 medium containing 1% FBS for 16 h in a 12-well culture plate. An aliquot (40 μl) of the conditioned medium was collected as a control sample for background ATP release. The cells were then irradiated with γ-rays from a GammaCell 40 (137Cs source) (Nordion International, Inc., Ontario, Canada, 0.88 Gy/min) at room temperature for a suitable time. To irradiate 0.5 Gy of γ-rays, cells were exposed to γ-rays for 34 s at room temperature. After γ-irradiation, 40 μl of conditioned medium was collected at the indicated time points. Each sample was centrifuged at 600 g for 5 min and 10 μl of the supernatant was used for ATP determination. The concentration of ATP was determined by measuring chemiluminescence with a TR717™ Microplate Luminometer (Applied Biosystems, Foster City, CA) 1.0 s after adding 100 μl of rLuciferin-Luciferase Reagent to 10 μl of sample solution.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)
Total RNA was isolated from cells using a Fast Pure RNA kit (Takara Bio, Shiga, Japan). The first-strand cDNA was synthesized from 0.5 μg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio). The sequences of specific primers for Panx1 were 5'-ACTTTGGCCACGGAGTATGTGTTTCT-3' (sense) and 5'-TGTTACAGGCAGCCCAGCAGTTAAGGAA-3' (antisense) and those for Cx43 were 5'-TGCTCTGTACTTGGCTCACGCTGTT-3' (sense) and 5'-AGGAACACCCACACATGAAGATGA-3' (antisense). The sequences of primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-GATATGAACGAGCTAGAAGATGA-3' (antisense). The sequences of primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-TGATGACATCAAGAAAGGTTGGAAG-3' (sense) and 5'-TCCCTGAGAGGCCAAGCAGTGCCT-3' (antisense). PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), Blend Taq polymerase (1.25 U; Toyobo, Osaka, Japan) and deoxynucleotide mix (0.2 mM each; Toyobo). Amplification was carried out for 35 cycles (95°C for 30 s, annealing at 55°C for 30 s, 72°C for 1 min). The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with EtBr and photographed.

Immunoblotting
After cells were irradiated with γ-rays, they were incubated at 37°C for the indicated times. Cells were dissolved in sample buffer (25% glycerol, 1% SDS, 62.5 mM Tris-Cl, 10 mM DTT) and boiled for 5 min. Aliquots of samples containing 20 μg of protein were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were incubated at 4°C overnight in Tris-Buffered Saline-Tween 20 (TBST: 10 mM Tris-Cl, 100 mM NaCl, 0.1% Tween 20, pH 7.5) with 1% bovine serum albumin, and then with mouse anti-phosphotyrosine antibody (1:1000) (Cell Signaling Technology, Beverly, MA, USA) or mouse anti-actin antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After having been washed with TBST, blots were incubated with goat horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:20,000) (Santa Cruz Biotechnology) for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA).

Statistics
Results are expressed as mean ± SEM. The statistical significance of differences between control and other groups was calculated by using Dunnett’s test. Comparisons between multiple groups were performed using Bonferroni’s test. The criterion of significance was P < 0.05. Calculations were done with the Instat version 3.0 statistical package (Graph Pad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION
Involvement of Cx43 in radiation-induced ATP release in B16 melanoma cells
According to our previous report, radiation-induced ATP release was examined at 5 min after 0.5 Gy γ-irradiation [8]. We first investigated whether gap junction hemichannels, including Panx1 or Cx43, are involved in ATP release induced by γ-irradiation. Treatment with inhibitors of gap junction hemichannels (50 μM 18GA, 20 μM CBX and 100 μM lindane) significantly blocked the ATP release induced by 0.5 Gy γ-irradiation (Fig. 1A). To clarify the involvement of Panx1 or Cx43, we examined the effect of mimetic inhibitory peptides for Panx1 and Cx43 on ATP release. The radiation-induced ATP release was not affected by application of a mimetic inhibitory peptide for Panx1,10α-panx1 (100 μM). However, treatment with a mimetic inhibitory peptide for Cx43, gap26 (300 μM), significantly inhibited the radiation-induced ATP release (Fig. 1B). We confirmed the expression of Panx1 and Cx43 mRNA in B16 melanoma cells, and detected low expression of Panx1 mRNA and high expression of Cx43 mRNA in B16 melanoma cells (Fig. 1C). Another study has also reported the expression of Cx43 hemichannels in B16 melanoma cells [25]. These results suggest that Cx43 hemichannels are involved in radiation-induced ATP release in B16 melanoma cells.
P2X7 receptor-dependent activation of tyrosine kinase is involved in radiation-induced ATP release

Since the involvement of tyrosine kinase in ATP release induced by hypotonic stress has been reported [26], we examined the phosphorylation of tyrosine in B16 melanoma cells after \( \gamma \)-irradiation. Figure 2A showed that tyrosine phosphorylation of a protein of approximately 160 kDa was induced after 0.5 Gy \( \gamma \)-irradiation. The phosphorylation peaked at 1 min, and then gradually decreased to the basal level (Fig. 2B). The early transient increase coincides with the release of ATP after irradiation, which was reported in our previous study [8]. We reported that \( \gamma \)-irradiation-induced ATP release from B16 melanoma cells was detected from 2.5 min after irradiation and ATP release peaked at 5 min after irradiation [8]. Therefore, we focused on the tyrosine phosphorylation of 160 kDa protein, which was observed at 1 min after irradiation.

We next investigated the involvement of the P2X7 receptor in the tyrosine phosphorylation of 160 kDa protein after 0.5 Gy \( \gamma \)-irradiation. The tyrosine phosphorylation at 1 min after 0.5 Gy \( \gamma \)-irradiation was significantly inhibited by pretreatment with a non-specific tyrosine kinase inhibitor, genistein (10 \( \mu \)M) and a P2X7 receptor antagonist, A438079 (100 \( \mu \)M) (Fig. 2C). This result indicated that tyrosine phosphorylation is a downstream event of the activation of the P2X7 receptor after irradiation. On the other hand, tyrosine phosphorylation was not affected by gap26 (300 \( \mu \)M) or an ecto-nucleotidase, apyrase (5 U/ml), suggesting that it lies upstream of Cx43-mediated ATP release.

To determine the contribution of tyrosine kinase to radiation-induced ATP release, we examined the effect of tyrosine kinase inhibitor. Treatment with genistein (10 \( \mu \)M) significantly blocked radiation-induced ATP release (Fig. 2D), supporting the involvement of tyrosine kinase in ATP release. Furthermore, as Src kinase and epidermal growth factor receptor (EGFR) may be activated after exposure of cells to ionizing irradiation, we examined the effects of an Src kinase inhibitor, PP2, and an EGFR inhibitor, AG1478 [27]. Treatment with PP2 (10 \( \mu \)M) or AG1478 (10 \( \mu \)M) did not block the radiation-induced ATP release (Fig. 2D), indicating that Src kinase and EGFR are not involved in ATP release. Considering the results in Fig. 2, we conclude that tyrosine phosphorylation plays an important role in the pathway of radiation-induced ATP release at a point between the activation of the P2X7 receptor and the opening of the Cx43 hemichannel. Our results also suggest that the P2X7 receptor could be a key molecule to initiate signal transduction for radiation-induced ATP release because tyrosine phosphorylation was dependent on the P2X7 receptor at a very early stage after \( \gamma \)-irradiation.

Involvement of Rho-kinase, actin cytoskeleton, intracellular Ca\(^{2+}\) and ROS in radiation-induced ATP release

We further examined whether intracellular signaling molecules, which are downstream of the P2X7 receptor and regulate the permeability of Cx43, mediate radiation-induced ATP release. Activation of Rho/Rho-kinase, a regulator of actin cytoskeleton, is rapidly induced by activation of the P2X7 receptor, and is involved in hypotonic stress-induced ATP release [26, 28–31]. The actin cytoskeleton directly interacts with the P2X7 receptor, and regulates membrane...
It has been also reported that actin polymerization regulates permeability of Cx43 hemichannels [19]. Therefore, we investigated the involvement of Rho/Rho-kinase and actin cytoskeleton in radiation-induced ATP release. As shown in Fig. 3A, radiation-induced ATP release was significantly inhibited by pretreatment with a Rho-kinase inhibitor, Y27632 (10 μM), and an actin polymerization inhibitor, cytochalasin D (10 μM), but not an inhibitor of Rho-GTPase, C3 toxin (1 μg/ml). These results indicate that Rho-kinase and actin cytoskeleton are involved in radiation-induced ATP release.

The permeability of Cx43 hemichannels is regulated by an increase in intracellular Ca²⁺ [20], and stimulation of the P2X7 receptor increases intracellular Ca²⁺ through the opening of cation channels and non-selective large pores [12]. We examined the involvement of intracellular Ca²⁺ in radiation-induced ATP release, and found that ATP release was significantly suppressed by pretreatment with an
intracellular Ca²⁺ chelator, BAPTA-AM (Fig. 3A). This supports the involvement of intracellular Ca²⁺ in ATP release through Cx43.

Ionizing radiation, including γ-rays, generates ROS through ionization of water molecules and activation of oxidases [33]. It has been reported that the P2X₇ receptor mediates ROS generation through NADPH oxidase (Nox) [34]. Therefore, we further examined the involvement of ROS in radiation-induced ATP release. Application of a ROS scavenger, vitamin C (1 mM) and vitamin E (100 μM), and Nox inhibitors, DPI (10 μM) and apocynin (100 μM), significantly blocked radiation-induced ATP release (Fig. 3A), suggesting the involvement of ROS produced by Nox and ionization of water in radiation-induced ATP release. Considering the results in Fig. 3, these intracellular signaling molecules might mediate signal transduction between the P2X₇ receptor and Cx43 in radiation-induced ATP release, though further investigation is needed to clarify the mechanism in detail.

CONCLUSION

In the present study, we investigated in detail the mechanism of radiation-induced ATP release, which is dependent on the P2X₇ receptor. Our results indicate the involvement of the Cx43 hemichannel in radiation-induced ATP release in B16 melanoma cells. P2X₇ receptor-dependent tyrosine phosphorylation plays an important role in the pathway of ATP release at a point before the opening of Cx43 hemichannels. Moreover, Rho/Rho-kinase, actin cytoskeleton, intracellular Ca²⁺ and ROS also contribute to radiation-induced ATP release (Fig. 3B). Our results provide a novel insight into the mechanisms of ATP release induced by irradiation. Since the existence of a radiation-induced bystander effect mediated by both ATP and Cx43 has been suggested in vivo [24], recognition of the importance of ATP in radiation-induced biological effects is now increasing.

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