Review

Purinergic Signaling Is a Novel Mechanism of the Cellular Response to Ionizing Radiation

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Recent studies suggest the effect of radiation is observed not only in irradiated cells but also in adjacent non-irradiated cells (bystander effect), although the mechanism has not yet been fully revealed. This bystander effect may be caused by intercellular communication via a gap junction or by messengers released from irradiated cells, such as reactive oxygen species, nitric oxide, or cytokines. However, an unknown mechanism is also possible in the bystander effect. On the other hand, it is known that extracellular ATP, ADP, uridine 5'-triphosphate (UTP), and uridine 5'-diphosphate (UDP), which are released from cells, act as intercellular signaling molecules by activating purinergic P2X and P2Y receptors (purinergic signaling). Recently, I have suggested these extracellular nucleotides may be novel mediators of a radiation-induced bystander effect, because our recent studies indicated that purinergic signaling is involved in important cellular responses to radiation. Our data indicate that ionizing irradiation causes activation of the transient receptor potential melastatin type 2 (TRPM2) channel, and then ATP is released from cells through the anion channel or connexin43 hemichannel mediated by the activation of a P2X7 receptor. The released nucleotides activate P2Y6 and P2Y12 receptors, which are involved in the DNA damage response after irradiation. Activation of the P2Y6 receptor is also involved in radiation-induced activation of the epithelial growth factor receptor-extracellular signal regulated protein kinase (EGFR-ERK)1/2 pathway and subsequent nuclear translocation of EGFR, which plays a role in DNA repair. Further, the induction of an antioxidant after irradiation is also mediated by the activation of the P2Y receptor. In conclusion, purinergic signaling could play an important role in the protective cellular response to ionizing irradiation.

Key words radiation; ATP; P2 receptor; DNA repair; bystander effect

1. INTRODUCTION

Exposure of cells to ionizing irradiation causes the generation of reactive oxygen species (ROS), leading to DNA damage with subsequent rapid activation of p53, ataxia telangiectasia mutated kinase (ATM), and Rad3-related protein, as well as the activation of growth factor receptors in the plasma membrane. Recently, an irradiation-induced bystander effect has been reported (Fig. 1). This refers to responses occurring in cells that were not the targets of energy deposition events following ionizing irradiation. Bystander signals may be transmitted to neighbors of irradiated cells either by direct intercellular communication through gap junctions, or by soluble extracellular factors, such as ROS, nitric oxide (NO), or transforming growth factor-β1 (TGF-β1) released from irradiated cells (Fig. 1). The bystander effect is manifested as the expression of a wide range of endpoints, such as mutation, cell death, micronucleus formation, the appearance of phosphorylated histone variant H2AX (γH2AX) foci and activation of mitogen-activated protein kinase (MAPK). Multiple intercellular and intracellular signal transduction pathways have been implicated in the bystander effect.

Intracellular ATP is a universal source of energy for cellular function. However, ATP is also known to act as an extracellular signaling molecule, which activates purinergic receptors (P2 receptors) on the cellular membrane (Fig. 2). Nucleotides such as ATP and uridine 5'-triphosphate (UTP) are released into the extracellular space in response to a variety of physiological and pathophysiological stimuli, including hypoxia, ischemia or ischemic pre-conditioning, and mechanical stretching. The nucleotides are released through various transport mechanisms including ATP-binding cassette transporters, connexin, pannexin hemichannels or possibly plasmalemmal voltage-dependent anion channels, as well as maxi-anion channels, or exocytosis. The released ATP acts as an autocrine and/or paracrine molecule to regulate cell function via interaction with P2 receptors (Fig. 2). Purinergic receptors (P2 receptors) are expressed in a number of cell types and are a family of transmembrane receptors that have been classified into two subfamilies (P2X and P2Y) according to the mechanisms of signal transduction. The P2X receptors are extracellular ATP-gated nonselective cation channels that are permeable to calcium. The P2Y receptors are metabotropic G protein-coupled receptors that possess seven transmembrane hydrophobic domains with short extra amino and intracellular carboxyl terminals. Though ATP released in response to stress stimuli is an intercellular signaling molecule, the involvement of extracellular nucleotides (ATP and/or UTP) and P2 receptors in the bystander effect of ionizing irradiation has not yet been reported.

Our recent studies have revealed that purinergic signaling plays an important role in radiation-induced biological effects (Fig. 3). In this review, I show here that ionizing radiation causes nucleotide (ATP, UTP, ADP, and uridine 5'-diphosphate (UDP)) release and the activation of P2Y receptors, which are involved in epithelial growth factor receptor (EGFR) activation, DNA damage response, and the induction of antioxidants (Fig. 3).
2. THE INVOLVEMENT OF PURINERGIC SIGNAL-ING IN γ-IRRADIATION-INDUCED EGFR ACTIVATION AND THE SUBSEQUENT ACTIVATION OF ERK1/2

First, we investigated whether the activation of P2 receptors is involved in radiation-induced biological effects. It was well known that radiation causes EGFR activation and the subsequent activation of extracellular signal regulated protein kinase 1/2 (ERK1/2). On the other hand, the activation of P2Y receptors induces the trans-activation of EGFR via a non-ligand mechanism. Therefore, we have investigated the involvement of P2 receptors in the activation of the EGFR-ERK1/2 pathway after γ-irradiation.

It was not clear whether the activation of ERK1/2 induced by ionizing irradiation is also mediated by bystander factors, although irradiation-induced bystander effects have been reported in various irradiation models. The activation of ERK1/2 induced by irradiation is mediated by trans-activation (ligand-independent) of the EGF receptor. Activation of ERK1/2 is also mediated by the activation of P2 receptors in various cell types. Activation of the P2Y2 receptor induces the activation of ERK1/2 through trans-activation of the EGF receptor. If ATP is released in response to irradiation, it is possible that irradiation causes the activation of ERK1/2 through the release of nucleotides followed by the activation of P2 receptors.

First, we showed that ionizing irradiation causes ATP release from human keratinocyte HaCaT cells. We confirmed that hydrogen peroxide as an oxidant induces ATP release from cells, suggesting that oxidative stress could cause the release of ATP from cells. Then, we showed that irradiation at a dose of more than 0.1 Gy causes ATP-release from the cells. It is known that ATP is released from cells through both non-lytic pathways, such as anion transporters, anion channels, hemichannels, or exocytosis, and by a lytic mechanism (cell death). Though irradiation can cause damage to cells, our data indicated the involvement of non-lytic mechanism(s), including anion transporters and gap junction hemichannels, in ATP release induced by irradiation, because several inhibitors of non-lytic pathways suppressed ATP release after irradiation. The increase in ATP concentration in the culture medium of irradiated cells was low (nM order). Released ATP would be rapidly metabolized by ecto-nucleotidase expressed on the cell membrane and diluted in the culture supernatant. Therefore, the pericellular concentration of ATP actually released from cells in response to γ-irradiation could be substantially high enough to activate P2 receptors. The release...
Fig. 2. Extracellular Stress-Induced Purinergic Signaling

When cells are stimulated by extracellular stresses, cells release nucleotides such as ATP or UTP into extracellular spaces through anion channels, anion transporters, hemichannels, or exocytosis. The increased extracellular ATP, UTP, and their metabolites (ADP or UDP) can activate P2X and P2Y receptors on the cellular membrane, leading to various intracellular signaling.

Fig. 3. Radiation-Induced Purinergic Signaling

First, ionizing irradiation causes activation of the TRPM2 channel, then ATP is released from cells through an anion channel or connexin43 hemichannel mediated by activation of the P2X7 receptor. The released nucleotides and their metabolites activate P2Y6 and P2Y12 receptors. Activation of the P2Y6 receptor is involved in the radiation-induced activation of the EGFR-ERK1/2 pathway and subsequent nuclear translocation of EGFR, which plays a role in DNA repair. The DNA damage response is facilitated by the activation of P2Y6 and P2Y12 receptors. Further, the induction of antioxidants after irradiation is also mediated by the activation of P2Y receptors. Thus, purinergic signaling plays an important role in the cellular response to ionizing irradiation.
of ATP in response to irradiation would serve to activate P2 receptors. Other nucleotides, such as UTP, might also be released from cells in response to irradiation.\textsuperscript{19} Our data suggest that both ATP and UTP activate ERK1/2 through P2Y receptors, but their underlying mechanisms in the activation of ERK1/2 differ.\textsuperscript{19} The UTP-induced activation of ERK1/2 is mediated by the activation of P2Y\textsubscript{2} and/or P2Y\textsubscript{6} receptors, which are also activated by UDP, and Src and EGF receptors also serve as mediators. On the other hand, ATP-induced activation of ERK1/2 is mediated by P2 receptors and the EGF receptor, but not by Src. Therefore, it is possible that released nucleotides can activate ERK1/2 through the activation of EGFR after irradiation.

Irradiation-induced activation of ERK1/2 mediated by EGF receptor has already been reported.\textsuperscript{2,26,33} We also found that γ-irradiation caused the activation of ERK1/2 in HaCaT cells. When we examined the effect of inhibitors of P2 receptors on irradiation-induced activation of ERK1/2, we found that ectonucleotidase apyrase, the P2Y receptor inhibitor suramin, the P2Y\textsubscript{6} receptor antagonist MRS2578 and the EGF inhibitor AG1478, but not the P2X inhibitor PPADS, blocked irradiation-induced activation of ERK1/2. Suppression by apyrase suggests the involvement of extracellular nucleotides in irradiation-induced ERK1/2 activation, and blockade by MRS2578 and suramin suggests the involvement of P2Y6 and other P2Y receptors as well. Considering the release of ATP induced by γ-irradiation, it is possible that irradiation induces the release of ATP and/or UTP, which act as autocrine and/or paracrine signaling molecules, inducing the activation of P2Y receptors (at least the P2Y6 receptor) in irradiated and/or non-irradiated bystander cells. However, these inhibitors and apyrase could not completely block the activation of ERK1/2, suggesting that activation of the P2Y receptor is only one of the mechanisms involved in the irradiation-induced activation of ERK1/2. On the other hand, AG1478 completely blocked the activation, indicating that activation of the EGF receptor plays a critical role in the irradiation-induced activation of ERK1/2. Moreover, these results also suggest that activation of a P2Y receptor might be involved in the irradiation-induced activation of EGFR. Thus, it appears that extracellular nucleotides are among the mediators of biological responses induced by irradiation, as well as NO, ROS and cytokines.\textsuperscript{19}

We also investigated the involvement of P2 receptors in EGFR focus formation (nuclear-translocation), which is associated with EGFR activation in response to irradiation in human lung cancer A549 cells.\textsuperscript{20} Our data suggest that extracellular nucleotides (ATP, ADP, UTP, and UDP) induce focus formation of EGFR via the activation of P2Y receptors in A549 cells. To examine the involvement of P2 receptors in the γ-irradiation-induced focus formation of EGFR, we investigated the effects of ecto-nucleotidase (apyrase) and P2 receptor antagonists (suramin, MRS2578, and PPADS). Apyrase suppressed the focus formation, suggesting the involvement of extracellular nucleotides, such as ATP, ADP, UTP and UDP in the phenomenon. Suramin and MRS2578 significantly blocked the focus formation, suggesting the involvement of P2Y receptors, at least the P2Y6 receptor. These results are consistent with the observations of nucleotide-induced EGFR focus formation and the expression of P2Y receptors in A549 cells. Moreover, knockdown of the P2Y6 receptor significantly suppressed the radiation-induced focus formation of EGFR and activation of ERK1/2, supporting the involvement of a P2Y6 receptor in these events induced by irradiation in A549 cells.\textsuperscript{20}

We investigated the involvement of intracellular Ca\textsuperscript{2+} and phosphatidyl inositol 3-kinase (PI3K) by the use of BAPTA-AM and LY294002. Focus formation in response to irradiation was almost completely blocked by BAPTA-AM, suggesting that intracellular Ca\textsuperscript{2+} elevation plays a critical role. The PI3K inhibitor also blocked focus formation. Since the activation of several P2Y receptors induces a PI3K-dependent pathway, followed by intracellular Ca\textsuperscript{2+} elevation, these results support the idea that the activation of Gq-protein-coupled P2Y receptors, such as the P2Y6 receptor, contributes to the focus formation of EGFR in response to radiation.\textsuperscript{20}

These data indicated that γ-irradiation-induced EGFR focus formation in A549 cells is mediated via the activation of P2Y receptors, including the P2Y6 receptor. This study in A549 cells,\textsuperscript{20} and previous findings in HaCaT cells,\textsuperscript{19} suggest that the release of nucleotides (ligands of P2 receptors) from cells in response to radiation, as well as the activation of the P2Y6 receptor, play important roles in radiation-induced biological effects. We have designated these responses as radiation-induced purinergic signaling. It is also important to investigate the involvement of an intercellular purinergic signaling pathway in other radiation-induced bystander effects.

3. IRRADIATION-INDUCED ATP RELEASE FROM MELANOMA IS MEDITED BY P2X7 RECEPTORS AND THE CONNEXIN43 HEMICHANNEL

ATP is released into extracellular space from various cells in response to stress stimuli, and acts as an intercellular signaling molecule, evoking the activation of P2 receptors on the cell surface in an autocrine/paracrine manner. It is widely known that various stresses induce ATP release from cells. However, the mechanism of ATP release from cells after irradiation had not been established, because the ATP release from HaCaT cells after irradiation was a first finding.\textsuperscript{19} To reveal the mechanism of ATP release from irradiated cells, we have investigated it in B16 melanoma cells.\textsuperscript{38}

We found that the concentration of ATP in culture medium of irradiated B16 melanoma cells was elevated after γ-irradiation, reaching a peak at 5 min, and then declining to the basal level within 30 min. These results are consistent with γ-irradiation-induced ATP release from the cells, followed by degradation of released ATP by ecto-nucleotidases on the cell surface. A dose of 0.5 Gy irradiation induced the greatest release of ATP. We speculate that oxidative damage to membrane proteins by higher doses of γ-irradiation might lead to the impairment of ATP-release mechanisms, such as anion channel, P2X7 receptor or hemicannels. Endogenous ATP is released through various pathways, such as the maxi-anion channel, the volume-sensitive outwardly rectifying chloride channel, and P2X7 receptors.\textsuperscript{11–15} Treatment with inhibitors of anion channels or transporters did not abrogate radiation-induced ATP release. However, we observed a significant blockade of radiation-induced ATP release by a highly selective antagonist of the P2X7 receptor, A438079, suggesting that the P2X7 receptor would be involved in the release.\textsuperscript{18}

The P2X7 receptor is the seventh member of the P2X receptor subfamily, and its expression is increased in human
melanoma. Activation of the P2X7 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. Gap junction hemichannel pannexin1 (Panx1) is part of the pore-forming unit of the P2X7 receptor, and its activation is thought to be regulated by Src tyrosine kinase, which acts downstream of the P2X7 receptor. Since several reports have shown that Panx1 mediates the release of cytoplasmic ATP, the involvement of Panx1 in P2X7 receptor-dependent ATP release has been suggested. On the other hand, although the mechanisms are not yet fully understood, a recent study has indicated a possible interaction between connexin43 (Cx43), which forms gap junction hemichannels, and the P2X7 receptor in dye uptake (pore formation) through Cx43 hemichannels.

To examine the involvement of the P2X7 receptor in radiation-induced ATP release in more detail, we established a stable P2X7 receptor-knockdown B16 melanoma cell line (P2X7-KD (1C)). We investigated whether P2X7-KD (1C) cells were suitable for the investigation of ATP release mediated by the P2X7 receptor. Since the involvement of the P2X7 receptor in hypotonic stress-induced ATP release has been reported, we examined the ATP release induced by hypotonic stress in P2X7-KD (1C) cells. The rapid phase of ATP release by hypotonic stress was blocked by pretreatment with A438079, and was decreased in P2X7-KD (1C) cells, indicating that P2X7-KD (1C) cells are available to determine the involvement of the P2X7 receptor in irradiation-induced ATP release. The γ-irradiation-induced ATP release was significantly reduced in P2X7-KD (1C) compared with wild type or mock versions. Moreover, blockade of the ATP release by irradiation was also confirmed in another P2X7-shRNA-transfected polyclonal cell (P2X7-KD (3A)). These results strongly support the involvement of the P2X7 receptor in ionizing radiation-induced ATP release from melanoma cells.

Increased expression of the P2X7 receptor has been reported in thyroid papillary cancer cells and melanoma. Since the P2X7 receptor appears to be involved in the mechanisms of radiation-induced ATP release, irradiation might induce greater ATP release from highly P2X7 receptor-expressing cancer cells during radiotherapy. Thus, nucleotides and their metabolites generated following radiotherapy might induce the enhancement of cancer progression or radioresistance through purinergic and adenosinergic signaling pathways.

In further study, we investigated in detail the mechanism of P2X7 receptor-dependent ATP release after γ-irradiation in B16 melanoma cells, focusing on the Cx43 hemichannel. Cx43 is ubiquitously expressed in various tissues and mediates the release of cytoplasmic ATP. Gap junction hemichannels formed by connexins, including Cx43, freely allow the passage of molecules smaller than 1.2 kDa. The selectivity of permeant molecules through hemichannels varies between connexins, and Cx43 favors ATP permeation. The permeability of Cx43 hemichannels is regulated by various intracellular signaling pathways, such as an increase in intracellular Ca²⁺ and actin polymerization. In radiation biology, Cx43 is known to regulate the bystander effects of ionizing radiation through gap junction intercellular communication. In addition, a recent study has suggested the involvement of both Cx43 and ATP in long-range bystander radiation damage and oncogenesis in vivo. However, the relationship between Cx43 and ATP in the bystander effect was unknown.

Thus, 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 significantly blocked the ATP release induced by 0.5 Gy γ-irradiation. To clarify the involvement of Panx1 or Cx43, we examined the effect of mimetic inhibitory peptides for Panx1 and Cx43 on the ATP release. The radiation-induced ATP release was not affected by the application of a mimetic inhibitory peptide for Panx1, however, treatment with a mimetic inhibitory peptide for Cx43, gap26, significantly inhibited the radiation-induced ATP release. We confirmed the expression of Panx1 and Cx43 mRNA in B16 melanoma cells, and detected the low expression of Panx1 mRNA and high expression of Cx43 mRNA in B16 melanoma cells. These data suggest that Cx43 hemichannels are involved in the radiation-induced ATP release in B16 melanoma cells.

Our further study indicated that 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. Our results provide a novel insight into the mechanisms of ATP release induced by irradiation. Since a radiation-induced bystander effect mediated by both ATP and Cx43 has been suggested in vivo, recognition of the importance of ATP in this radiation-induced biological effect is now increasing.

4. PURINERGIC SIGNALING PLAYS AN IMPORTANT ROLE IN DNA DAMAGE RESPONSE AFTER IRRADIATION

It is well known that ionizing radiation directly causes DNA damage and induces a DNA damage response, including the accumulation of DNA-repair factors at the sites of damage. However, the mechanism of recruitment of DNA-repair factors is not well understood. In radiation-induced biological events, we found that nucleotides, such as ATP or UTP, were released from irradiated cells via the activation of P2 receptors in an autocrine/paracrine manner. We also showed that γ-irradiation-induced translocation of an EGFR receptor into nuclei, which plays a role in DNA repair, is mediated by activation of the P2Y6 receptor in A549 cells. However, it had not been known whether nucleotide-mediated autocrine signaling contributes to the regulation of DNA-repair signaling after irradiation. In the next study, we investigated the involvement of autocrine signaling mediated by nucleotides and P2 receptors in the repair mechanisms of irradiation-induced DNA damage.

We confirmed that γ-irradiation caused the formation of γH2AX foci, which are markers of DNA damage, and induced DNA repair. The increase in γH2AX foci was completely blocked by ecto-nucleotidase, and was significantly enhanced by an ecto-nucleotidase inhibitor, suggesting the involvement of extracellular nucleotides released from cells after irradiation. This idea was supported by the finding that post-treatment with nucleotides facilitated the induction of γH2AX foci. To identify the subtype(s) of P2 receptor involved in the
response to DNA damage, we examined the effects of various P2 receptor subtype inhibitors. Among them, antagonists of P2X7 receptors, P2Y6 receptors, and P2Y12 receptors significantly suppressed the activation of ATM, the formation of γH2AX foci, and the accumulation of tumor suppressor protein 53-binding protein 1 (53BP1) at the sites of DNA damage.22) We have already reported P2X7 receptor-dependent ATP release after irradiation of B16 melanoma cells.58,22) Here, we found that the P2X7 receptor is involved in ATP release after irradiation in A549 cells, too.22) On the other hand, we examined the involvement of P2Y6 and P2Y12 receptors in the activation of ATM, the formation of γH2AX foci, and the accumulation of 53BP1 at sites of DNA damage by transfection with short interfering RNA (siRNA) for the P2Y12 receptor. These data indicate that autocrine/paracrine signaling through the activation of P2Y6 and P2Y12 receptors is involved in regulating the activation of ATM followed by the induction of γH2AX foci and accumulation of 53BP1 at the sites of DNA damage.22) This is a novel and interesting mechanism of response to DNA damage.

The P2Y6 receptor is linked to the activation of phospholipase C, leading to the generation of inositol (1,4,5)-trisphosphate and diacylglycerol, which in turn induces the elevation of cytosolic free Ca2+ and the activation of protein kinase C isoforms.53) As described above, the P2Y6 receptor is involved in radiation-induced activation of EGFR, followed by ERK1/2 activation.59) The activation of EGFR is known to contribute to DNA repair (non-homologous end-joining) via the activation of ERK1/2.50) Pretreatment of A549 cells with the MEK1/2 inhibitor U0126 suppressed the increase in γH2AX foci caused by 2.0 Gy of γ-irradiation.25) In view of these observations, we considered that the irradiation-induced activation of the P2Y6 receptor might mediate the formation of γH2AX foci via the trans-activation of EGFR followed by the activation of ERK1/2. On the other hand, the P2Y12 receptor is a G protein-coupled receptor, which induces a decrease in cAMP.52) Although the mechanism of γH2AX focus formation mediated by the P2Y12 receptor is unknown, this is the first evidence of the involvement of a P2Y12 receptor in a radiation-induced biological effect.

Whereas ATP is a ligand of the P2X7 receptor, the ligands of the P2Y6 and P2Y12 receptors are UDP and ADP, respectively.53,54) We detected ATP release from A549 cells after irradiation. However, released ATP and UTP would be readily metabolized by ecto-nucleotidases on the cellular membrane to ADP and UDP, respectively. Therefore, these nucleotides may be involved in autocrine/paracrine signaling to modulate the DNA repair response after irradiation.

As described above, bystander signals may be transmitted to neighbors of irradiated cells either by direct intercellular communication through gap junctions or via soluble extracellular factors, such reactive oxygen species, nitric oxide and cytokines, released from the irradiated cells.2) Our results support the idea that the release of nucleotides from irradiated cells is a candidate mechanism for inducing γH2AX foci via the radiation-induced bystander effect.

Thus, we have found that the activation of P2Y6 and P2Y12 receptors mediates, at least in part, the activation of ATM at the sites of DNA damage, followed by the induction of γH2AX and the accumulation of 53BP1 in γ-irradiated A549 cells.21) The P2X7 receptor is also involved in DNA repair via the mediation of ATP release after irradiation. Since pre-treatment with apyrase almost completely suppressed the focus formation, this autocrine/paracrine signaling would seem to play a significant role in the DNA repair mechanism. We also reported the enhancement of radiation-induced cytotoxicity in A549 cells by blockade of the P2Y6 receptor.25) Thus, we suggest that not only intracellular signaling, but also autocrine/paracrine signaling mediated by nucleotides and P2 receptors, contributes to the regulation of DNA repair after γ-irradiation.

In other words, we propose that the release of ATP and autocrine/paracrine positive feedback through P2Y receptors serve to amplify the cellular repair response to radiation-induced DNA damage.

5. INVOLVEMENT OF TRANSIENT RECEPTOR POTENTIAL MELASTATIN TYPE 2 (TRPM2) AND TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 (TRPV1) CHANNELS IN THE DNA DAMAGE RESPONSE, AND THE INVOLVEMENT OF A TRPM2 CHANNEL IN ATP RELEASE FROM CELLS IN RESPONSE TO IRRADIATION

Low linear energy transfer ionizing radiation, such as γ-rays, causes DNA damage via the generation of ROS. It is well known that transient receptor potential (TRP) channels are extracellular stress-sensitive cation channels.55) Recent findings indicate that TRP channels, including TRPM2 and TRPV1, mediate oxidative stress-induced Ca2+ influx.56) DNA damage-associated poly(ADP-ribose) polymerase (PARP) activation induces the activation of TRPM2 via the generation of adenine 5′-diphosphoribose (ADPR).57) However, the role of TRP channels in radiation-induced biological effects, such as the DNA damage response, has remained unclear. Therefore, we investigated the involvement of TRPM2 and TRPV1 channels in various cellular responses to radiation-induced DNA damage.24)

We used cells in which TRPM2 had been knocked down with siRNA to confirm the participation of the TRPM2 channel in γ-irradiation-induced γH2AX focus formation.24) Indeed, γ-irradiation-induced γH2AX focus formation was inhibited by knockdown of the TRPM2 channel. The formation of γH2AX foci is mediated by the activation of ATM at the sites of DNA damage. ATM activation after irradiation was also suppressed in TRPM2-knockdown cells. These results strongly support the involvement of TRPM2 channels in ATM activation leading to γH2AX focus formation. Moreover, the accumulation of 53BP1 at the sites of DNA damage was also suppressed by knockdown of TRPM2. Further, the abrogation of Ca2+ influx by the removal of extracellular Ca2+ resulted in the suppression of γH2AX focus formation. These data are consistent with our hypothesis that the TRPM2 channel plays an important role in the DNA damage response, including ATM activation and the accumulation of DNA repair proteins at the sites of DNA damage.24)

Concerning the pathway of TRPM2 channel activation after irradiation, oxidative stress generates intracellular ADPR via the DNA damage-associated activation of PARP,58) which is required for single strand break repair. It was reported that γ-ray-induced DNA damage caused PARP activation, leading to an increase in poly-ADPR, which is a precursor of ADPR,
followed by a decrease in nicotinamide adenine dinucleotide (NAD).\textsuperscript{59} Although the activation of PARP might be a pathway of TRPM2 activation after irradiation, it is important to clarify precisely how TRPM2 is activated by \(\gamma\)-irradiation.

As mentioned above, ATP release and the subsequent activation of P2Y6 and P2Y12 receptors are involved in the DNA damage response after irradiation.\textsuperscript{22} However, it is not known how cells sense radiation stress and thus induce ATP release. Our results suggest that TRPM2 is involved in \(\gamma\)-irradiation-induced ATP release, because the ATP release was suppressed by knockdown of TRPM2.\textsuperscript{24} Additionally, radiation-induced ATP release was suppressed by catalase or by the removal of extracellular Ca\textsuperscript{2+}. Treatment with H\textsubscript{2}O\textsubscript{2}, an activator of TRPM2, also caused ATP release, which was suppressed by the TRPM2 inhibitor DPQ. All of these results strongly support the involvement of TRPM2 in the mechanism of ATP release after \(\gamma\)-irradiation.\textsuperscript{20} Therefore, we conclude that the activation of TRPM2 leads to ATP release, followed by the activation of P2Y receptors, which are involved in the DNA damage response.\textsuperscript{20}

Concerning ATP release after \(\gamma\)-irradiation, we have reported that the \(\gamma\)-irradiation-induced ATP release from B16 melanoma cells is mediated by the P2X7 receptor and Cx43 hemichannel.\textsuperscript{18,23} The ATP release was dependent on intracellular Ca\textsuperscript{2+} elevation,\textsuperscript{23} which is also consistent with the involvement of Ca\textsuperscript{2+} channels in the ATP release. We have also reported that a P2X7 receptor antagonist blocks ATP release from A549 cells, and also blocks \(\gamma\)H2AX focus formation.\textsuperscript{25} These observations suggest that activation of the TRPM2 channel would lead to P2X7 receptor-dependent ATP release, which causes the activation of P2Y6 and P2Y12 receptors to induce the DNA damage response.\textsuperscript{18-22,24} Thus, our results suggest that the autocrine DNA damage response mediated by extracellular ATP starts with the activation of TRPM2 channels via ADPR generation after DNA damage-associated PARP activation. This is the first evidence of the involvement of TRPM2 channels in the radiation-induced DNA damage response via ATP release. Our results indicate that TRPM2 may serve as a radiation sensor protein mediating radiation-induced biological effects, such as ATP release and DNA repair. Further, the role of TRPM2 in the DNA damage response appears to involve not only the induction of ATP release, but also the induction of other signaling via Ca\textsuperscript{2+} influx. Thus, the TRPM2 cation channel may play a key role in radiation-induced biological effects.

In addition, we have found that TRPV1 is also involved in the DNA damage response.\textsuperscript{20} We found that pretreatment with the TRPV1 inhibitor capsazepine (CPZ), which directly blocks the TRPV1 channel, or knockdown of TRPV1, suppressed the activation of ATM and the accumulation of 53BP1 at the sites of DNA damage. Further, the TRPV1 agonist capsaicin (CAP) facilitated the formation of \(\gamma\)H2AX foci in \(\gamma\)-irradiated cells (DNA-damaged cells), although CAP did not induce the focus formation in non-irradiated cells, suggesting that activation of the TRPV1 channel would induce the DNA repair response but not DNA damage. A report that CAP induces ATM activation is consistent with our results.\textsuperscript{60} Although it was reported that stretch- or hypoosmolality-evoked ATP release was diminished in urothelial cells from TRPV1-knockout mice,\textsuperscript{61} radiation-induced ATP release was not suppressed either by pretreatment with CPZ or in TRPV1-knockdown cells.\textsuperscript{24} Though our results at least indicate that irradiation-induced ATP release is not mediated by the TRPV1 channel, the contribution of the TRPV1 channel to ATP release is still unclear. Since the TRPV1 channel is activated by extracellular ATP,\textsuperscript{62} activation of the TRPV1 channel might play a role in the DNA damage response downstream of ATP release. This is the first evidence of the involvement of the TRPV1 channel in the ionizing irradiation-induced cellular response.

As mentioned above, ionizing irradiation causes the activation of EGFR and induces nuclear translocation of EGFR, which is involved in DNA repair. We have previously reported that the translocation of EGFR after irradiation is mediated by activation of the P2Y6 receptor in A549 cells.\textsuperscript{20} We also showed that both the TRPM2 and TRPV1 channels are involved in the nuclear translocation of EGFR.\textsuperscript{24} Thus, TRPM2 channel-mediated ATP release would cause the translocation of EGFR by activation of the P2Y6 receptor in A549 cells. It has been reported that the activation of TRPV1 by extracellular ATP is involved in mouse skin tumorigenesis mediated through EGFR signaling.\textsuperscript{63} Thus, both the TRPM2 and TRPV1 channels appear to mediate the radiation-induced nuclear translocation of EGFR to induce effective DNA repair.

We found that the activation of TRPM2 and TRPV1 cation channels is involved in \(\gamma\)H2AX focus formation, ATM activation, and 53BP1 accumulation at the sites of \(\gamma\)-radiation-induced DNA damage, as well as in the nuclear translocation of EGFR.\textsuperscript{24} We also found that activation of the TRPM2 channel, but not the TRPV1 channel, mediates radiation-induced ATP release,\textsuperscript{24} which induces the DNA damage response via P2Y6 and P2Y12 receptors.\textsuperscript{22,25}

This is the first report of the involvement of TRPM2 and TRPV1 channels in ionizing irradiation-induced biological effects. Further work is needed to establish how the TRPM2 and TRPV1 channels are activated after irradiation, as well as to establish precisely which radiation-induced biological effects are mediated by the activation of TRPM2, TRPV1, or other TRP channel subtypes. Our results could lead to new insights into the function of TRP channels from the viewpoint of radiation biology.

6. CONCLUSION

In another report, we also reported the involvement of purinergic signaling in the induction of antioxidants by low (small)-dose \(\gamma\)-irradiation in mouse macrophage RAW264.7 cells, which would contribute to an adaptive response.\textsuperscript{24} Taken all together, our studies revealed the involvement of purinergic signaling in ionizing radiation-induced biological effects.\textsuperscript{18-25} We found that P2X7, P2Y6, and P2Y12 receptors play an important role in the DNA damage response, suggesting that inhibitors of these P2 receptors might enhance radiotherapy. We should investigate whether treatment with P2 antagonists enhances the suppression of tumor growth in vivo caused by irradiation. Our results provide a new insight into the function of P2 receptors and TRP channels from the viewpoint of radiation biology. Radiation-induced purinergic signaling may represent a novel paradigm for radiation-induced biological effects. We suggest here that extracellular ATP, UTP, ADP and UDP act as novel intercellular signaling molecules between cancer cells and adjacent cells when cancer cells are exposed to ionizing radiation in radiotherapy. Of course,
further investigation of radiation-induced purinergic signaling is needed. In further studies, we should examine the involvement of purinergic signaling in the biological effect caused not only by γ-rays but also other ionizing irradiation, such as α-particle, β-ray, or neutron irradiation.

Conflict of Interest The author declares no conflict of interest.

REFERENCES

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