

## Review Article

**ATP Released from Low-dose Gamma Ray-irradiated Cells Activates Intracellular Antioxidant Systems via Purine Receptors**

Shuji Kojima, Erina Takai, Mitsutoshi Tsukimoto

Faculty of Pharmaceutical Sciences, Tokyo University of Science (TUS)

**Abstract**

Antioxidants are known to prevent oxidative damage in cells caused by radiation. We have previously reported that whole-body irradiation with low doses of gamma rays to mice elevates the antioxidant thioredoxin-1 (Trx-1) levels in several organs. Furthermore, gamma ray irradiation also causes ATP release from the exposed cells. Extracellular ATP release in response to various stimuli has been reported to regulate the expression of intracellular antioxidants through activation of purinergic P2 receptors.

Here, we review the relation between gamma-radiation-induced ATP release and the induction of cellular Trx-1 via purinergic signaling. Irradiation with gamma rays or exogenously adding ATP cause an increase in Trx-1 expression, and these phenomena disappear in the presence of an ecto-nucleotidase. Further, it is revealed that ATP generates intracellular reactive oxygen species (ROS), and thereby increases Trx-1 expression as an adaptive response to ROS.

These findings suggest that gamma-radiation-induced release of extracellular ATP may contribute to the production of ROS via purinergic signaling, thereby leading to the promotion of intracellular antioxidants in response to an oxidative stress.

**KEY WORDS:** low-dose  $\gamma$ -rays, ATP release, thioredoxin-1, purinergic signaling, RAW264.7 cells

**Introduction**

Radiation toxicity is thought to be elicited from reactive oxygen species (ROS) generated by the interaction between water molecules and ionizing radiation in living cells<sup>1-4</sup>. ROS include  $O_2^-$ ,  $H_2O_2$ , and OH, *et al.*, and are readily generated in cells through metabolic processes such as respiration, ischemia/reperfusion, and oxidation of fatty acids, other than a radiolysis of the water. Even though oxygen and water are both indispensable for living cells, ROS generated from these molecules are highly toxic, damaging DNA, lipids and enzymes, and ultimately leading to the onset and progression of adult diseases such as arteriosclerosis and cancer<sup>5</sup>, when their generation overwhelms the cellular defense systems.

Cells are equipped with several defense systems which protect them from ROS attack, including enzymatic mechanisms such as superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic mechanisms involving the reduced forms of molecules such as glutathione (GSH), thioredoxin-1 (Trx-1), vitamin C (V.C), and vitamin E. Among these antioxidants, Trx-1 is a multifunctional, low-molecular-weight (12 kDa) protein containing an active thiol/disulfide site with oxidoreductase activity. Trx-1 potently protects cells from oxidative damage by enhancing the catalytic activity of peroxiredoxin and glutathione peroxidase, which decompose hydroperoxides and hydrogen peroxide, respectively<sup>6,7</sup>. It also directly reduces levels of hydrogen peroxide, glutathione disulfide, and hydroxyl radicals<sup>8</sup>, positioning it as a key protein controlling the cellular reductive/oxidative (redox) balance. Through this and other defense systems, intracellular

ROS levels are controlled, and prevented from becoming superabundant.

Since the antioxidant levels are generally considered to decrease with age, blockage of this phenomenon could lead to the development of anti-aging methods. Here, we review the slight production of ROS by extracellular ATP via purinergic receptors on cell membranes, and thereby the induction of Trx-1 as the adaptive response of living cells against low doses of ionizing radiation.

## Low-dose Gamma Ray-irradiation Activates Antioxidant Systems in Living Cells

Living cells are well known to possess adaptive functions that activate antioxidant systems in response to a small dose of ROS induced by various substances such as a quinone-derivative anticancer agents<sup>9,10</sup>, metals<sup>11</sup>, ultraviolet rays<sup>12</sup>, and hydrogen peroxide<sup>13</sup>. An adaptive response can also be expected in cases of ionizing radiation. We previously reported that whole-body low-dose gamma-irradiation induces the production of intracellular antioxidants such as GSH and Trx-1 in mice<sup>14,15</sup>. Hoshi *et al.*<sup>16</sup> had also reported that Trx-1 is induced by low-dose gamma ray irradiation in human lymphocytes. Our later studies revealed that the increase of GSH levels in immune cells subsequently activates proliferation of lymphocytes and natural killer (NK) cells, thereby leading to the suppression and/or delay of the solid-tumor growth and colony formation observed in metastatic cancer<sup>17-20</sup>.

## What Is Purinergic Signaling?

In recent years, a substantial amount of research has focused on the “radiation-induced bystander effect”<sup>21</sup>, in which cells not directly exposed to radiation display irradiated characteristics resulting from signal exchange between irradiated cells and nearby non-irradiated cells. Such signaling is believed to occur through direct physical connections such as gap junctions, direct interaction between ligands and their specific receptors, interaction between cytokines and growth factors with specific receptors, and in response to diffusible factors released into the culture medium<sup>22-24</sup>. Several recent studies have suggested that ROS and reactive nitrogen species (RNS) are the most probable molecules forming the links between the radioadaptive and bystander responses<sup>22,25,26</sup>. Overall, these findings implied that ROS and RNS were the molecules involved, but that this is no longer considered the case.

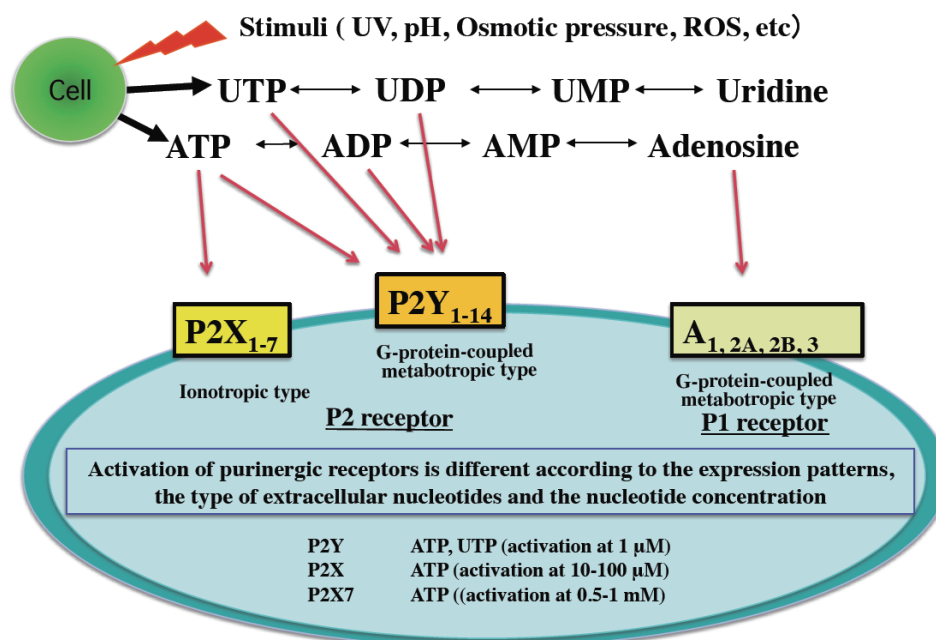
Cytoplasmic ATP is released into the extracellular space in response to several types of stimuli and then activates purinergic P2 receptors (*Fig. 1*)<sup>27</sup>. P2 receptors are classified into two major subtypes: ionotropic P2X and metabotropic P2Y receptors, both of which regulate a number of physiological functions<sup>28</sup>. ATP directly activates P2X<sub>1-7</sub> and P2Y<sub>1-14</sub>, and after being rapidly degraded by ecto-nucleotidases, also indirectly activates P2Y<sub>1-14</sub> through the byproduct ADP<sup>27, 29</sup>. Thus, cytosolic ATP causes activation of purinergic and adenosinergic signaling pathways in an autocrine/paracrine manner.

Several recent studies have shown that purinergic signaling via extracellular ATP stimulates DNA repair and antioxidant activity<sup>30-33</sup>. Our previous studies of the effect of low dose gamma- and UVB-radiation on ATP release from HaCaT cells<sup>34,35</sup> have suggested that a small dose of ROS induced by the extracellular ATP may lead to the promotion of intracellular antioxidant via purinergic signaling pathways. However, little is known about whether extracellular ATP released by gamma ray irradiation influences cellular antioxidant systems.

## Gamma Ray-irradiation Induces ATP Release from RAW264.7 via Multiple Pathways

While a number of reports have indicated that ATP is released into the interstitial spaces in response to various physiological and pathophysiological stimuli<sup>27</sup>, we have only recently demonstrated this release by stimulation with gamma ray ionizing radiation<sup>34</sup>.

In our previous studies on changes in the cellular antioxidant induction in various cells exposed to gamma rays, it was found that RAW264.7 mouse macrophage-like cells were the most sensitive to gamma rays<sup>36</sup>. We therefore examined ATP release in response to gamma-irradiation using this cell line. Concentration of extracellular ATP increased soon after the onset of gamma-irradiation, and the mechanism involved in



*Fig. 1.* Release of nucleotides by various stimuli and purinergic receptors.

this phenomenon was examined. Inhibitors of gap junction hemichannels such as flufenamic acid, lindane, and 18GA almost completely blocked ATP release, and a P2X<sub>7</sub>-receptor antagonist (carbenoxolone) and an exocytosis blocker (brefeldin A) also blocked release to a lesser degree. Similarly, a maxi-anion channel blocker (arachidonic acid), a potent chloride-channel inhibitor (glibenclamide), a vesicular-H<sup>+</sup>-ATPase inhibitor (bafilomycin A1), and GdCl<sub>3</sub> significantly suppressed ATP release. Taken together, these results indicate that gamma-irradiation-induced ATP release occurs via multiple pathways, including gap junction hemichannels, anion channels/transporters, the P2X<sub>7</sub> receptor, as well as via exocytosis<sup>37</sup>.

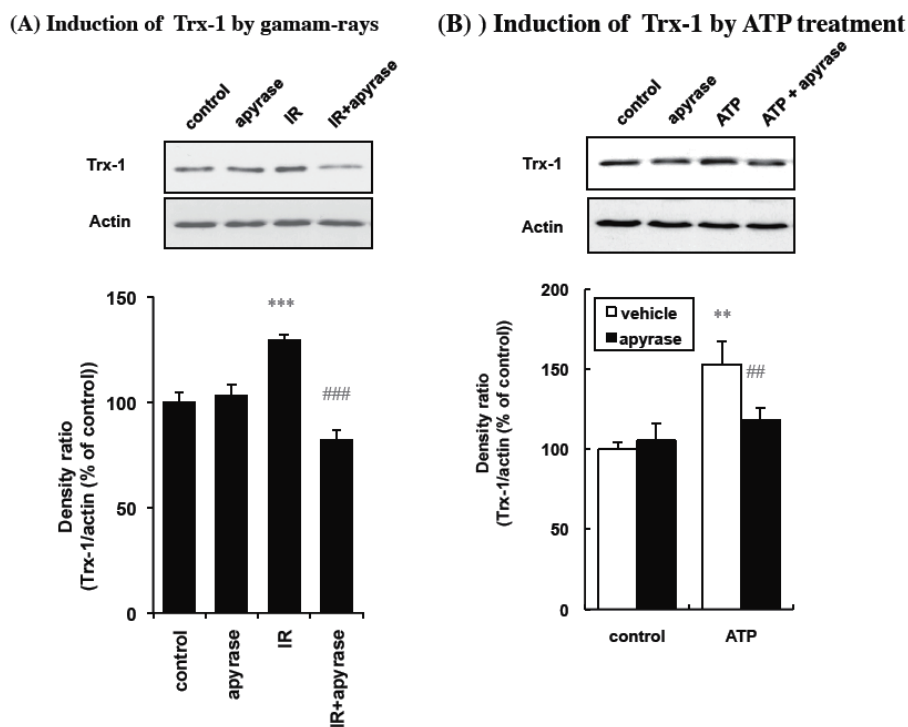
### **Trx-1 Expression is Induced by Gamma Ray Irradiation and ATP Treatment and Its Blockage by Apyrase**

The increase in Trx-1 expression induced by exposing RAW264.7 cells to gamma ray irradiation was assessed by immunoblotting. Trx-1 expression was increased time-dependently starting 1 h after irradiation, peaking 3 to 6 h later. The elevation remained until 24 h after irradiation. This peak increase was almost completely blocked by pretreatment with the ecto-nucleotidase apyrase (Fig. 2A), suggesting that the gamma ray irradiation-induced release of the extracellular ATP is involved in mediating the increase of Trx-1 expression.

Exogenously added ATP, in place of gamma-irradiation, also increased Trx-1 expression both time- and dose-dependently, reaching a maximum at approximately 6 h post-treatment. This increase was likewise suppressed by pretreatment with apyrase (Fig. 2B). Taken together, these results suggest that the irradiation-induced Trx-1 arises via the extracellular ATP and activation of purinergic signaling.

### **Evaluation of Purinergic Receptors Involved in Trx-1 Expression Induced by Gamma Ray Irradiation and ATP Treatment**

Selective purinergic-receptor antagonists were used to determine the specific pathway through which extracellular ATP induces an increase in Trx-1 expression. Suramin, MRS2578, and A438079 successfully block increases in Trx-1 expression induced by both the gamma-irradiation (Fig. 3A) and the exogenously added ATP (Fig. 3B), suggesting the involvement of both P2Y<sub>6</sub> and P2X<sub>7</sub> receptors in these phenomena.



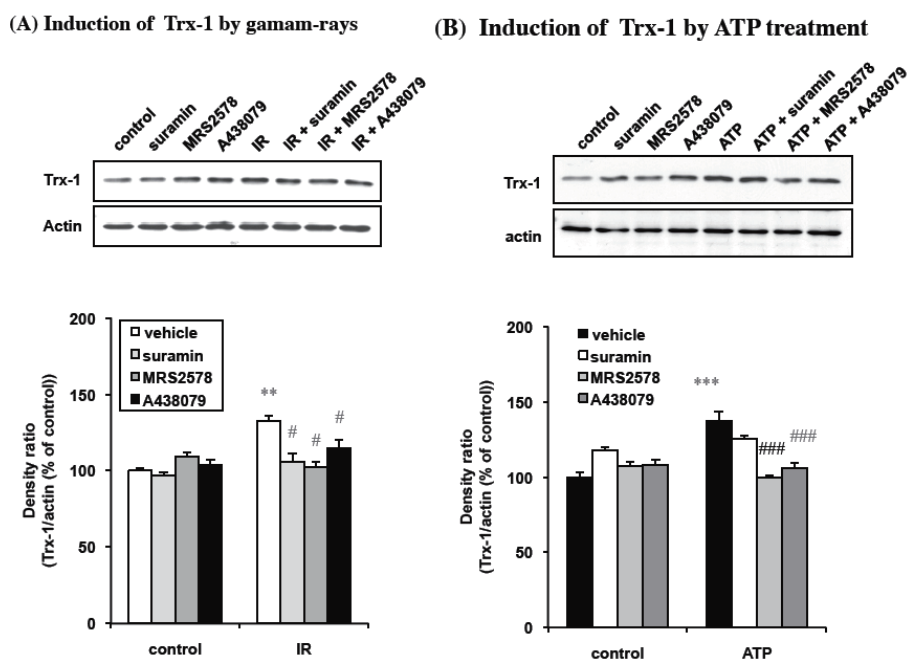
**Fig. 2.** Induction of Trx-1 by gamma rays (A) or ATP (B) and their suppression with apyrase in RAW264.7 cells.

Each value indicates the mean  $\pm$  SD (n=4).

IR: gamma ray irradiation (0.5 Gy), ATP treatment (100  $\mu$ M)

A: \*\*\*,  $p < 0.001$  vs. control group, ###,  $p < 0.001$  vs. IR alone group

Adopted from Ohshima et al. (2011)<sup>37</sup>.



**Fig. 3.** Induction of Trx-1 by gamma rays (A) or ATP (B) and their suppression by antagonist of P2 receptors in RAW264.7 cells. Each value indicates the mean  $\pm$  SD (n=4). IR: gamma ray irradiation (0.5 Gy), ATP treatment (100  $\mu$ M). A: \*\*:  $p < 0.01$  vs. control group, #:  $p < 0.05$  vs. IR alone group. B: \*\*\*:  $p < 0.001$  vs. control group, ###:  $p < 0.001$  vs. ATP alone treated group. Adopted from Ohshima et al. (2011)<sup>37</sup>.

### ***Trx-1 Expression in Response to ROS via Activation of NADPH Oxidase by ATP***

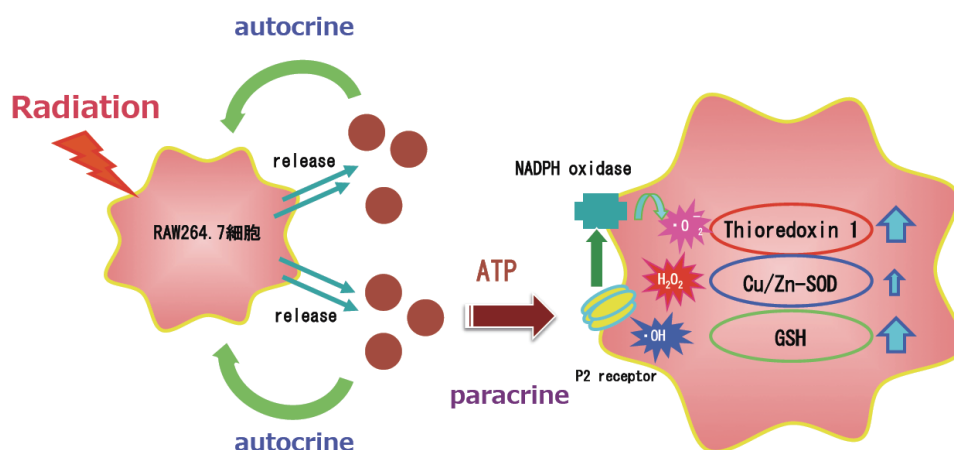
Following several studies which reported that extracellular ATP stimulation promotes intracellular ROS generation<sup>33,38-40</sup>, we also confirmed the involvement of ATP in increasing ROS. We then investigated changes in ROS level induced by ATP decrease in the presence of antioxidants such as DMSO, NAC, and V.C. Pre-treatment with these antioxidants significantly suppressed the elevation of Trx-1 expression induced by ATP, suggesting that ATP-induced ROS mediates the elevation of Trx-1 expression, at least in part.

Finally, we examined the mechanism of ROS generation induced by ATP treatment. ATP-induced ROS generation was significantly suppressed by pretreatment with diphenyliodonium chloride, apocynin, and SOD, supporting the theory that NADPH oxidase plays a major role in ATP-induced ROS generation, as previously reported<sup>41-43</sup>.

### **Conclusion**

While the interaction between water molecules and radiation has been suggested to cause ROS generation in cells exposed to ionizing radiation, our findings in the present review demonstrate that this is not all the only outcome of this exposure. In addition, ATP is released from the irradiated cells and subsequently may produce ROS by the activation of cell membrane NADPH oxidase via purinergic signaling. Antioxidants such as Trx-1 are thought to be induced as

an adaptive response to ROS produced via the previous mechanism and purinergic signaling in living cells (Fig. 4). However, the details of the signaling pathways by which ATP activates NADPH oxidase through purinergic receptors are still unknown. The mechanism by which radiation induces ATP release also appears unknown. Studies are now underway to clarify these issues by determining the link between purine receptor P2-activation and factors known to be involved in the activation of NADPH oxidase, such as  $Ca^{++}$  ions, protein kinase C, and p38MAP kinase<sup>44-47</sup>.



**Fig. 4.** Expected scheme of radiation-induced ATP release and antioxidant induction as an adaptive response to ROS generated via the activation of purinergic receptor and NADPH oxidase.

## References

- Petkau A, Chelack WS: Radioprotective effect of superoxide dismutase on model phospholipid membranes. *Biochim Biophys Acta* 433; 445-456: 1976
- Szekely JG, Perry KA, Petkau A: Simulated responses to lognormally distributed continuous low radiation doses. *Health Phys* 45; 699-711: 1983
- Quintilian M: The oxygen effect in radiation inactivation of DNA and enzymes. *Int J Radiat Res* 50; 573-594: 1986
- Feinendegen LE, Pollycove M, Sondhouse CA: Responses to low doses of ionizing radiation in biological systems. *Nonlinearity Biol Toxicol Med* 2; 143-171: 2004.
- Oberly LY, Oberly TD: Free radicals, cancer and aging, *In: Johnson JE Jr, Miquel J eds. Free radicals aging and degenerative diseases.* Alan R Liss, Inc, New York, 325-371: 1986
- Zoccarato F, Cavallini L, Allexandre A: Respiration-dependent removal of exogenous H<sub>2</sub>O<sub>2</sub> in brain mitochondria. *J Biol Chem* 279; 4166-4174: 2004
- Drechsel DA, Patel M: Respiration-dependent removal in brain mitochondria via the thioredoxin/peroxiredoxin system. *J Biol Chem* 285; 27850-27858: 2010
- Kalinina EV, Chernov NN, Saprin AN: Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry* 73; 1493-1510: 1997
- Yin X, Wu H, Chen Y, Kang YJ: Induction of antioxidants by adriamycin in mouse heart. *Biochem Pharmacol* 56; 87-93: 1998
- Shi MM, Kugelman A, Iwamoto T, et al: Quinone-induced oxidative stress elevates glutathione and induces gamma-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J Biol Chem* 269; 26512-26517: 1994
- Ochi T: Arsenic compound-induced increases in glutathione levels in cultured Chinese hamster V79 cells and mechanisms associated with changes in gamma-glutamylcysteine synthetase activity, cystine uptake and utilization of cysteine. *Arch Toxicol* 71; 730-740: 1997
- Applegate LA, Scaletta C, Panizzon R, et al: Evidence that ferritin is UV inducible in human skin: part of a putative defense mechanism. *J Invest Dermatol* 111; 159-163: 1998
- Haendeler J, Tischler V, Hoffmann J, et al: Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett* 577; 427-433: 2004
- Kojima S, Matsuki O, Nomura T, et al: Kubodera, A.; Induction of mRNAs for glutathione synthesis-related proteins in mouse liver by low doses of  $\gamma$ -rays. *Biochim Biophys Acta* 1381; 312-318: 1998
- Kojima S, Matsuki O, Nomura T, et al: Elevation of antioxidant potency in the brain of mice by low-dose  $\gamma$ -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: 1999
- Hoshi Y, Tanooka H, Miyazaki K, et al: Induction of thioredoxin in human lymphocytes with low-dose ionizing radiation. *Biochim Biophys Acta* 1359; 65-70: 1997
- Kojima S, Matsumori S, Ishida H, et al: Possible role of elevation of glutathione in the acquisition of enhanced proliferation of mouse splenocytes exposed to small-dose  $\gamma$ -rays. *Int J Radiat Biol* 76; 1641-1647: 2000
- Kojima S, Ishida H, Takahashi M, et al: Elevation of glutathione induced by low-dose  $\gamma$  rays and its involvement in increased natural killer activity. *Radiat Res* 157; 275-280: 2002
- Ohshima Y, Tsukimoto M, Kojima S: The novel mechanism of metastasis inhibition by low-dose whole-body irradiation with gamma-rays. *Int J Low Radiat* 5; 156-167: 2008
- Hayase N, Oshima Y, Takahashi M, et al: Enhancement of Th1 immunity and suppression of tumor growth by low dose  $\gamma$ -radiation. *Int J Low Radiat* 5; 275-289: 2008
- Nagasawa H, Little JB: Induction of sister chromatid exchanges by extremely low doses of alpha-particle. *Cancer Res* 52; 6394-6396
- Matsumoto H, Takahashi A, Ohnishi T: Radiation-induced adaptive responses and bystander effects. *Biol Sci Space* 18; 247-254: 2004
- Shao C, Folkard M, Michael BD, et al: Bystander signaling between glioma cells and fibroblasts targeted with counted particles. *Int J Cancer* 116; 45-51: 2005

- 24) Little JB: Cellular radiation effects and the bystander response. *Mut Res* 597; 113-118: 2006
- 25) Kadhim MA, Moore SR, Goodwin EH: Interrelationship amongst radiation-induced genomic instability, bystander effect, and the adaptive response. *Mut Res* 568; 21-32: 2004
- 26) Brooks AL.: Paradigm shifts in radiation biology: their impact on intervention for radiation-induced disease. *Radiat Res* 164; 454-461. 2005
- 27) Yegutkin GG: Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signaling cascade. *Biochim Biophys Acta* 1783; 673-694: 2008
- 28) Burnstock G. Purinergic signaling. *Br J Pharmacol* 147; S172-S181: 2006
- 29) Zimmermann H, Braum N: Ecto-nucleotidase – molecular structure, catalytic properties, and functional roles in the nervous system. *Prog Brain Res* 120; 371-385: 1999
- 30) Suh BC, Kim JS, Namgung U, et al: P2X<sub>7</sub> nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *J Immunol* 166; 6754-6763: 2001
- 31) Parvathenani LK, Tertysnikova S, Greco CR, et al: P2X<sub>7</sub> mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's diseases. *J Biol Chem* 278; 13309-13317: 2003
- 32) Pines A, Perrone L, Bivi N, et al: Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Nucleic Acids Res* 33; 4379-4394: 2005
- 33) Shinozaki Y, Koizumi S, Ishida S, et al: Cytoprotection against oxidative stress-induced damage of astrocytes by extracellular ATP via P2Y<sub>1</sub> receptors. *Glia* 49; 288-300: 2005
- 34) Tsukimoto M, Homma T, Ohshima Y, et al: Involvement of purinergic signaling in cellular response to  $\gamma$ -irradiation. *Radiat Res* 173; 298-309: 2010
- 35) Takai E, Tsukimoto M, Harada H, et al: Involvement of P2Y<sub>6</sub> receptor in p38 MAPK-mediated COX-2 expression in response to UVB irradiation of human keratinocytes. *Radiat Res* 175; 56-66: 2011
- 36) Kojima S, Matsumori S, Ono H, et al: Elevation of glutathione in RAW 264.7 cells by low-dose  $\gamma$ -ray irradiation and its responsibility for the appearances of radioresistance. *Anticancer Res* 19; 5271-5276: 1999
- 37) Ohshima Y, Tsukimoto M, Kawano A, et al: Induction of extracellular ATP mediates increase of intracellular thioredoxin in RAW264.7 cells exposed to low-dose  $\gamma$ -radiation. *Free Radic Biol Med* 51; 1240-1248: 2011
- 38) Suh BC, Kim JS, Namgung U, et al: P2X<sub>7</sub> nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *J Immunol* 166; 6754-6763: 2001
- 39) Parvathenani LK, Tertysnikova S, Greco CR, et al: P2X<sub>7</sub> mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's diseases. *J Biol Chem* 278; 13309-13317: 2003
- 40) Pines A, Perrone L, Bivi N, et al: Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Nucleic Acids Res* 33; 4379-4394: 2005
- 41) Pfeiffer ZA, Guerra AN, Hill GL, et al: Nucleotide receptor signaling in murine macrophages is linked to reactive oxygen species generation. *Free Radic Biol Med* 42; 1506-1516: 2007
- 42) Guerra AN, Gavala ML, Chung HS, et al: Nucleotide receptor signalling and the generation of reactive oxygen species. *Purinergic Signalling* 3; 39-51: 2007
- 43) Noguchi T, Ishi K, Fukumoto H, et al: 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: 2008
- 44) Guerra AN, Gavala ML, Chung HS, et al: Nucleotide receptor signalling and the generation of reactive oxygen species. *Purinergic Signalling* 3; 39-51: 2007
- 45) Lin JM, Shah AM: Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol* 287; R1014-R1030: 2004
- 46) Quinn MT, Gauss KA: Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nanophagocyte oxidases. *J Leukocyte Biol* 76; 760-781: 2004
- 47) DeCoursey TE, Ligeti E: Regulation and termination of NADPH oxidase activity. *Cell Mol Life Sci* 62; 2173-2193: 2005