Journal of Cellular Biochemistry 97:314–326 (2006)

Elucidation of ATP-Stimulated Stress Protein Expression of RBA-2 Type-2 Astrocytes: ATP Potentiate HSP60 and Cu/Zn SOD Expression and Stimulates pI Shift of Peroxiredoxin II

Hammer B. Chen, Ya-Ting Chan, Amos C. Hung, You-Cheng Tsai, and Synthia H. Sun*

Institute of Neuroscience, National Yang Ming University and Brain Research Center, University System of Taiwan, Taipei, Taiwan, Republic of China

Abstract ATP has been shown to mediate stress responses in the brain. The present study examined the ATP-stimulated stress protein expression of RBA-2 type-2 astrocytes. Our results revealed that ATP stimulated HSP60 expression in a dose- and time-dependent manner. The stimulation requires a minimal ATP concentration of 500 μM and high concentration of extracellular ATP (1 mM) stimulated a significant increase of HSP60 expression from 2 to 24 h. In addition, the ATP-stimulated HSP60 expressions were inhibited by inhibitors for protein kinase C (PKC) and phospholipase D (PLD), and by antioxidants, resveratrol, and catalase. Furthermore, ATP stimulated the expression of Cu/Zn superoxide dismutase (SOD). In addition, ATP and $P2X_7$ receptor selective agonist BzATP also decreased mitochondria membrane potential measured by flow cytometry. To further examine the proteins involving in ATP-mediated stress responses, we conducted proteomic analysis. We found that RBA-2 astrocytes possess abundant peroxiredoxin II (Prx II), an antioxidant enzyme. ATP and exogenous H_2O_2 stimulated Prx II shifting from oxidized form to reduced form. Thus, we concluded that ATP potentiated the expression of HSP60 and Cu/Zn SOD, and decreased mitochondria membrane potential. In addition, RBA-2 astrocytes expressed Prx II that might also serve as a protective mechanism to control the concentration of reactive oxygen species. J. Cell. Biochem. 97: 314–326, 2006.

Key words: ATP; Cu/Zn SOD; HSP60; peroxiredoxin II; type-2 astrocytes

Astrocytes are a group of important glial cells involving in the neuronal regeneration and degeneration. These cells are also known to be immunocompetent cells by possessing the ability to produce a wide variety of cytokines and chemokines [Dong and Benveniste, 2001]. The high concentration of extracellular ATP (1 mM) activated $P2X_7$ receptors has been demonstrated to affect such immune responses in various types of immune cells [Ferrari et al., 1997; Hide et al., 2000; Morigiwa et al., 2000; Shigemoto-Mogami et al., 2001]. In addition, researchers also revealed that the high concentration ATP-stimulated apoptosis or necrosis is mediated through activation of $P2X_7$ receptor [Surprenant et al., 1996; Schulze-Lohoff et al., 1998; Coutinho-Silva et al., 1999; Ferrari et al., 1999]. Because high concentration ATP-stimulated cellular injury is important in immune responses [Hogquist et al., 1991], thus the ATP-activated $P2X_7$ receptors might induce stress responses, and lead to immune response and cellular injury of astrocytes.

Furthermore, we have previously demonstrated that 1 mM ATP stimulated Ca^{2+} influx and phospholipase D (PLD) activities [Sun et al., 1999], *c-fos* and *zif268* immediate early gene expressions [Hung et al., 2000], GABA release [Wang et al., 2002], and cytokine transforming growth factor (TGF)-β1 expression of RBA-2 astrocytes [Wang et al., 2003]. Therefore, ATP

Grant sponsor: National Science Council; Grant numbers: NSC92-2320-B-010-035, NSC93-2323-B010-005; Grant sponsor: Ministry of Education, Taipei, Taiwan, Republic of China; Grant number: 89-B-FA22-1-4-02.

*Correspondence to: Synthia H. Sun, Institute of Neuroscience, National Yang Ming University, Taipei, Taiwan, Republic of China. E-mail: shsun@ym.edu.tw

Received 5 November 2004; Accepted 3 May 2005

DOI 10.1002/jcb.20547

might stimulate stress-protein expression, for example, heat shock protein (HSP) and superoxide dismutase (SOD), and caused cellular injury of these astrocytes.

HSPs are expressed under normal culture conditions and functions as molecular chaperones to regulate the fundamental cellular process such as protein folding, protein sorting, protein degradation, protein assembly into larger complex, and resolublization of aggregation. These proteins were found rapidly synthesized in response to stress-mediated cellular injury and played a role in protecting cells from deleterious stresses [Welch, 1992; Becker and Craig, 1994; Ohtsuka and Suzuki, 2000]. HSP60 was found to locate in both cytosol and mitochondria [Cabiscol et al., 2000] and interacted with mitochondria DNA (mtDNA), thus plays a role in the mtDNA transmission [Kaufman et al., 2003]. Transient exposure of cell to H_2O_2 stimulated mitochondria HSP60 expression as well as the expression of other peptides involving in oxidation-reduction and protein biogenesis [Mitsumoto et al., 2002]. The mitochondrial HSP60 has been shown to interact with caspase-3 [Xanthoudakis et al., 1999], induced apoptosis and led to the activation of mitochondrial procaspase-3 and lastly resulted in a loss of mitochondrial membrane potential [Samali et al., 1999]. Both mitochondrial [Lin et al., 2001] and cytosolic [Kirchhoff et al., 2002] HSP60 have anti-apoptotic effects on cardiomyocytes. Overexpression of HSP60 increased the anti-apoptotic Bcl family and reduced the protein content of pro-apoptotic Bax of cardiomyocytes [Shan et al., 2003]. Recent study revealed that mammalian HSP60 is quickly imported into mitochondria by cytoplasmic HSP70 under severe conditions [Itoh et al., 2002]. Proinflammatory cytokines have been shown to enhance HSP60 expression in astrocytes suggesting that HSP60 might play an important role in the pathogenesis of autoimmune disease [Bajramović et al., 2000]. However, the ATP-stimulated HSP60 expression in astrocytes remained unexplored.

Peroxiredoxins (Prx), a hydroperoxide thioreductase, are highly conserved proteins with six subclasses and are a ubiquitous family of antioxidant enzymes with active cysteine sites. They play an important role to control H_2O_2 as well as to mediate signal transduction in mammalian cells [Wood et al., 2003b]. Early studies have identified Prx as antioxidants [Shau and Golob, 1993; Rhee et al., 1994], which have been cloned from a rat brain cDNA library [Chae et al., 1994]. The active site 2-Cys Prx act as floodgates by keeping the resting levels of H_2O_2 low, while permitting higher levels during signal transduction [Wood et al., 2003a]. The oxidized active site cysteine of mammalian Prx I was demonstrated to be rapidly reduced to the catalytically active reduced form suggesting that the enzyme might serve as a mechanism to repair oxidatively damaged proteins or present as a new type of cyclic modification by which the functions of various proteins is regulated [Woo et al., 2003]. Therefore Prx may play important roles in the control of cellular signaling and viability in CNS.

In the present study, we found high concentration of extracellular ATP stimulated HSP60 expression and the expression correlated with an upregulation of Cu/Zn SOD. In addition, we demonstrated that ATP and $P2X_7$ receptor selective agonist BzATP decreased mitochondria membrane potential. Using two-dimensional polyacrylamide gel elctrophoresis analysis, we observed that RBA-2 astrocytes expressed abundant Prx II. ATP and exogenous H_2O_2 caused a pI shift but did not affect the protein expression of Prx II. Therefore, Prx II is a constitutively expressed protein in RBA-2 astrocytes and may play an important role in protecting these cells against oxidative stress-induced cellular injury.

MATERIALS AND METHODS

Cell Culture of RBA-2 Astrocyte Cell Line

The RBA-2 type-2 astrocytes used in this study were isolated as described previously [Sun et al., 1999], and grown in cultured medium, F10 medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 95% air and 5% CO_2. In all experiments, the cells used had been cultured for no more than 10 passages.

Western Blot Analysis of Cellular Proteins

RBA-2 cells were cultured into 100 mm dishes in F10 media supplemented with 2.5% FBS, treated with the indicated agonists, antagonists and inhibitors, washed, scraped, centrifuged, and resuspendend by the addition of 400 μl of wash buffer (0.02 M phosphate buffer containing 0.1% glucose) and 100 μl of lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 10 mM *N*-ethylmaleimide,

0.6% sodium dodecyl sulfate, and 200 μM leupeptin, pH 8.0) in an ice-cold water bath. Cells were then homogenized with a 2-ml Dounce homogenizer. After removal of cell nuclei, protein levels were analyzed by the method of Lowry et al. [1951] using bovine serum albumin as the standard, and aliquots of homogenate protein (50 μg) were loaded onto each lane of 12.5% sodium dodecyl sulfate-poly-acrylamide gel for electrophoresis according to Laemmili [1970]. After separation, the proteins were transferred to PVDF membrane using a BIO-RAD semi-dry transfer cell (Hercules, CA). For detection of HSP 60, Cu/Zn SOD, and Prx II, nonspecific binding site were blocked by soaking the protein-loaded PVDF membranes for 30 min in a solution of PBS containing 0.05% Tween 20 and 5% dried skim milk. The membrane was then reacted with a 1:500 dilution of monoclonal anti-HSP60 or anti-Cu/Zn SOD or anti-Prx II antibody. Then after five washes with PBS containing 0.05% Tween 20, the membranes were treated with 1:10,000 dilution of a secondary antibody, rabbit anti-mouse IgG, conjugated with horseradish peroxidase (Sigma, St. Louis, MO). The blots were then washed thoroughly, dried, and reacted with ECL immunodetection reagents (Amersham Biosciences, Buckinghamshire, England), and visualized by autoradiography using Fuji medical X-ray film.

To detect the expression of β-actin of the same sample, the blots were then stripped and stained with antibody against β-actin and the secondary antibody, reacted with ECL reagent and visualized by autoradiography.

Flow Cytometric Analysis of Mitochondrial Membrane Potential

A flow cytometric analysis by a FACS system (Becton Dickenson, Bedford, MA) equipped with a 488-nm argon laser was used for the flow cytometric analysis of mitochondria membrane potential. Forward and side scatters were used to establish size gates and to exclude cellular debris from the analysis. The excitation wavelength was set at 488 nm. The observation wavelength was set at 530 nm for green fluorescence and 585 nm for red fluorescence. The emitted fluorescence was collected on the FL1 channel. For analyzing mitochondrial membrane potential, cells were loaded with 2 μM rhodamine 123 for 15 min [Vander Heiden et al., 1997; Wadia et al., 1998]. After rinsing, ten thousand (1×10^4) suspended RBA-2 cells per sample were analyzed. The results were expressed as mean fluorescence intensity of 10,000 cells.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

After treatment, cellular protein sample was prepared in sample buffer consisting of 20 mM Tris, 7 M urea (Merck, Darmastadt, Germany), 2 M thiourea (Sigma), 4% CHAPS (Sigma), 10 mM 1,4-dithioerythrtol (Merck), 1 mM EDTA (Merck), 1 mM PMSF (Sigma), and 1 μl/mg each of pepstatin A, chymostatin, leupeptin, and antipain [Lubec et al., 2003]. The samples were homogenized and centrifuged at 150,000*g* for 45 min to obtained cytosolic fraction. Quantitative precipitation of sample proteins and removal of interfering substances such as detergents, salts, lipids, phenolics, and nucleic acids were conducted using 2-D clean-up kit (Amersham). Total protein concentrations were determined by a modified version of Bradford's assay using BSA as the standard.

Aliquots of protein samples were then separated in the first dimension on the basis of differences in their net charges using an immobilized pH 4–7 gradient (IPG) strip (BioRad). Aliquot of protein (250 μg) was applied on sample cups at the basic and acidic ends and separated by iso-electric focusing using a Protein IEF set (BioRad). In the second dimension, proteins were separated according to their sizes. Proteins were negatively charged by SDS–PAGE as described by Laemmili [1970]. The gels were run at 40 mA per gel. After protein fixation for 12 h in 40% methanol, containing 5% phosphoric acid, gels were stained with colloidal commassie blue for 48 h.

Microsequencing Analysis

The protein spots were excised, destained, eluted, proteolytically digested by trypsin, microsequencing analyzed and proteins identified by quadrupole time-of-flight mass spectrometry (Q-TOF-MS). The peptide mass finger printing (PMF) was analyzed by MASCOT. The MASCOT search result is listed in Table I.

MTT Assay

RBA-2 cells were cultured into each well (2×10^4 cells per well) of a 96-well plate in cultured media for 24 h and switched to F10 medium supplemented with 1% FBS cultured

TABLE I. MASCOT Search Result

Protein	gi number	Full name	Mass (Da)	Theoretical pI value	Observed pI value	Total score	Peptide matched
A	2499469	Reduced form peroxiredoxin II	21765	5.2	4.98	182	4
B	2499469	Oxidized form peroxiredoxin II	21765	5.2	5.20	116	2

Cytosolic proteins of RBA-2 cells cultured in the presence or the absence of ATP (1 mM) were separated by two-demensional gel electrophoresis and stained with coommassie blue. The protein spots corresponding to A and B of Figure 5A were excised, destained, proteolytically digested by trypsin, extracted, microsequencing analyzed, and proteins identified by quadruple time-of-flight mass spectrometry (Q-TOF-MS). The peptide mass finger printing (PMF) was analyzed by MASCOT.

for 24 h. After treating the cells with various agonists, MTT assay was performed by adding 50 μl MTT (2 mg/ml 3-(4,5-domethylthioazol)-2,5 dophenyl tetrazolium bromide dissolved in F-10 medium) into each well and incubated in the 37°C CO_2 incubator for 2.5 h. The medium was removed and the insoluble formazan was dissolved with the addition of 100 μl DMSO. After 30 min, the absorbance of the mixture was measured by an ELISA reader (LabSystems Multiskan RC) at 490 nM wavelength.

Hoechst 33258 Staining of Nuclear Chromatin

Hoechst 33258 binds contigous A-T bases in DNA. Cells were cultured on poly-L-lysin coated coverglass in a 6-well plate in culture medium, treated with various agonists, fixed with 4% paraformaldehyde for 2 h at 4°C, and treated with 0.2% triton X-100 for 10 min. After washing with PBS, Hoechst 33258 (1 μg/ml in PBS) was added into each well and further incubated for 10 min at room temperature. The cells were then washed three times with PBS and observed by a fluorescence microscope (Nikon Eclipse E800M Fluorescence Microscope, Tokyo, Japan) using an excitation filter at wavelength 352 nm.

DNA Fragmentation

DNA fragmentation was performed using SUICIDE-TRACK™ DNA ladder isolation kit (Oncogene Research Product, San Diego, CA). Cells were cultured on 100-mm dishes, treated with various agonists, washed, scraped, and centrifuged at 1,000*g* for 5 min at room temperature. Supernatant was removed, DNA extracted, loaded onto a 1.5% agarose gel, and separated by gel electrophoresis. The separated gel was stained for 1 h in 0.5 μg/ml ethidium bromide and visualized by UV illumination.

RESULTS

ATP Stimulated HSP60 Expression of RBA-2 Type-2 Astrocytes

To examine whether ATP stimulates stress responses, RBA-2 cells were treated with various concentration of ATP (1–1000 μM) for 24 h and the expression of HSP60 analyzed by Western-blot analysis. The expression of β-actin was used as internal controls. As shown in Figure 1A, 0–100 μM ATP did not affect the expression of HSP60 whereas 500–1,000 μM ATP significantly potentiated the expression of HSP60 in these cells. Thus, stimulation of HSP60 expression required a high concentration of extracellular ATP. We then treated these cells with 1 mM ATP for 0–24 h. As shown in Figure 1B, high concentration of extracellular ATP also stimulated a time-dependent HSP60 expression of these cells. Significant increases were observed from 2 to 24 h. Thus, ATP stimulated HSP60 expression in RBA-2 astrocytes.

Protein Kinase C and Phospholipase D Signaling is Involved in ATP Enhanced HSP60 Expression

Protein kinase C (PKC) has been shown to be important in ATP-mediated signaling of RBA-2 astrocytes [Sun et al., 1999; Hung and Sun, 2002; Wang et al., 2002, 2003]. To examine the mechanism involved in the ATP-stimulated HSP60 expression, we treated the cells with two PKC inhibitors. As shown in Figure 2A, chelerythrine chloride (CL, 1 μM) but not GF109203X (GF, 1 μM) per se slightly enhanced the expression of HSP60, nevertheless both PKC inhibitors effectively inhibited the ATP-stimulated HSP60 expression.

In addition, ATP was also known to stimulate phospholipase D (PLD) signaling through activation of $P2X_7$ receptors in these cells [Sun et al., 1999; Hung and Sun, 2002]. To further elucidate

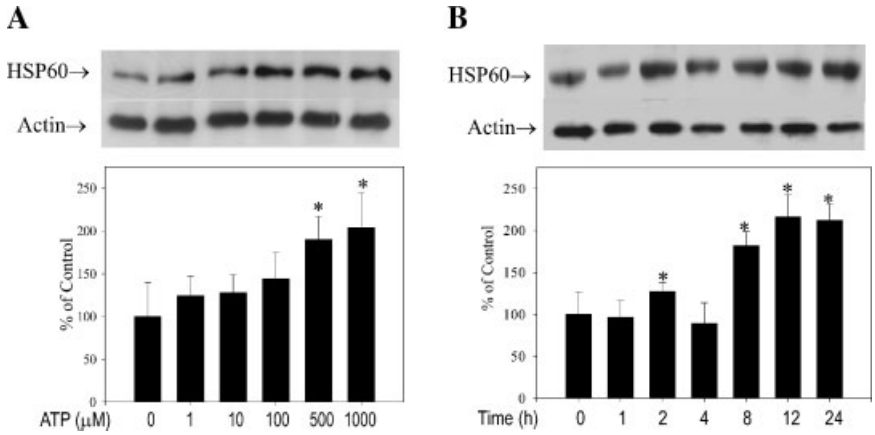

Fig. 1. ATP stimulated HSP60 expression. RBA-2 type-2 astrocytes were cultured in F10 medium, treated with **(A)** ATP (0–1000 μM) for 24 h, and **(B)** ATP (1 mM) for 0–24 h. Aliquots of proteins (40 μg) from cell lysates were separated by gel electrophoresis, transblotted to PVDF membrane. Detection of HSP60 was performed by Western-blot analysis using anti-HSP60 antibody and visualized by the ECL method. Quantitative analysis of HSP60 was determined by image-densitometry analysis and the ratio of individual HSP60/actin calculated. Data represent the mean ± SD of three separate cultures expressed as percentage of controls. The symbol * indicates significant different means compared with the zero time controls with $P < 0.05$ by non-paired Student's *t*-test.

the mechanism, cells were cultured in the presence or the absence of PLD inhibitors, D609 and neomycin, and the expression of HSP60 analyzed by Western blots. As shown in Figure 2B, PLD inhibitors, both D609 (1 μM) and neomycin (100 nM), inhibited the ATP-stimulated HSP60 expression. Thus, we concluded that if not all, but at least in part, the ATP-stimulated HSP60 expression is mediated through $P2X_7$ receptors.

Antioxidants Inhibited ATP-Stimulated HSP60 Expression

Because HSP60 is a stress protein, we then examine whether oxidative stress response is involved in the enhanced HSP60 expression. We

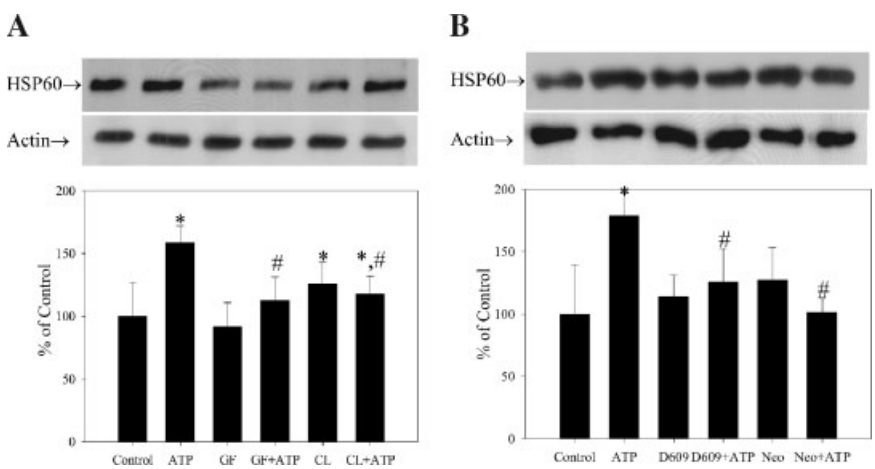

Fig. 2. Protein kinase C and phospholipase D signaling affect the ATP-stimulated HSP60 expression. RBA-2 type-2 astrocytes were cultured in F10 medium, in the presence or the absence of ATP (1 mM), and the **(A)** protein kinase C inhibitors GF109203X (GF, 1 μM) or chelerythrine chloride (CL, 1 μM), or the **(B)** phospholipase D inhibitors, D609 (1 μM) or neomycin (Neo, 1 μM) for 24 h as indicated. Aliquots of proteins (40 μg) from cell lysates were separated by gel electrophoresis, transblotted to PVDF membrane. Detection of HSP60 was performed by Western-blot analysis using anti-HSP60 antibody and visualized by the ECL method. Quantitative analysis of HSP60 was determined by image-densitometry analysis and the ratio of individual HSP60/actin calculated. Data represent the mean ± SD of three separate cultures expressed as percentage of controls. The symbol * indicates significant different means compared with the controls and the symbol # indicates significant different means compared with the ATP-stimulated HSP60 expression with $P \leq 0.05$ calculated by non-paired Student's *t*-test.

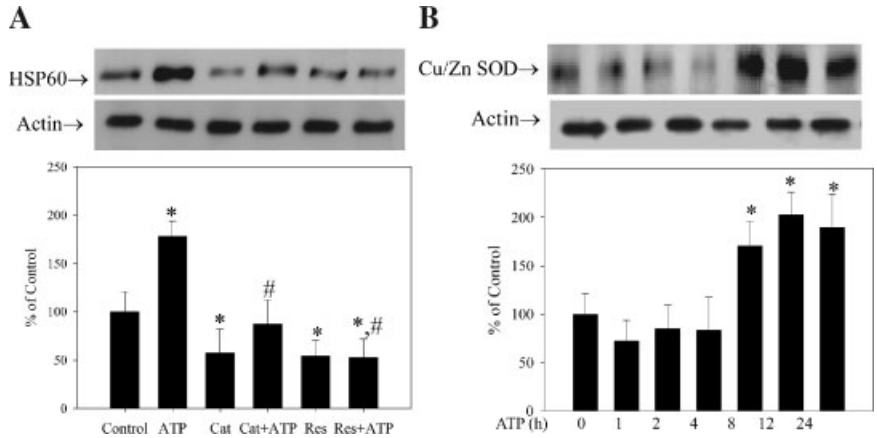

Fig. 3. The ATP-stimulated HSP60 expression involves stress response. RBA-2 type-2 astrocytes were cultured in F10 medium in the presence or the absence of ATP (1 mM) and **(A)** antioxidants, catalase (Cat, 100 μg/ml) or resveratrol (Res, 1 μM) for 24 h. Detection of HSP60 was performed by reacting with anti-HSP60 antibody and visualized by the ECL method. **(B)** RBA-2 astrocytes were cultured in F10 medium, in the presence or the absence of ATP (1 mM) for 0–24 h. Detection of Cu/Zn SOD (Cu/Zn SOD) was performed by reacting with anti-Cu/Zn SOD antibody and visualized by the ECL method. Quantitative analysis of HSP60 or Cu/Zn SOD expression was determined by image-densitometry analysis and the ratio of individual HSP60/actin or Cu/Zn SOD/actin calculated. Data represent the mean ± SD of three separate cultures expressed as percentage of controls. The symbol * indicates significant different means compared with the controls and the symbol # indicates significant different means compared with the ATP-stimulated HSP60 expression with $P < 0.05$ by non-paired Student's *t*-test.

treated the cells with antioxidants catalase and resveratrol, and analyzed the expression of HSP60 by Western blot analysis. As shown in Figure 3A, both catalase (100 μg/ml) and resveratrol (1 μM) per se slightly decreased the basal level of HSP60 expression, and effectively inhibited the ATP-stimulated HSP60 expression in these cells.

ATP Stimulated Cu/Zn Superoxide Dismutase (SOD) Expression

To further elucidate whether ATP treatment induced stress response, we then examine the effect of ATP (1 mM) on the expression of Cu/Zn SOD. As shown in Figure 3B, ATP enhanced a time-dependent Cu/Zn SOD expression and significant increases were observed from 8 to 24 h.

ATP Treatment Decreased Mitochondria Membrane Potential

Because HSP60 is a mitochondria chaperone protein, we then measured the effect of ATP on mitochondria membrane potential of RBA-2 astrocytes by flow cytometry. As shown in Figure 4A, using rhodamine 123 preloaded cells, we found that ATP rapidly decreased the mitochondrial membrane potential of these cells. An initial decrease was observed at 10 min, a ATP-stimulated maximal decrease (70% of the controls) was observed at 60 min and the low levels were maintained throughout the test periods 1–12 h (Fig. 4C). We also stimulated the cells with the $P2X_7$ receptor selective agonist BzATP. Similarly, BzATP rapidly induced a decrease (60% of controls) in 60 min and at 12 h, the BzATP-stimulated mitochondria membrane potential was 40% of the controls.

RBA-2 Express Abundant Peroxiredoxin (Prx) II, and ATP and Exogenous H_2O_2 Stimulated a Shift of Peroxiredoxin II to Acidic Form

Because ATP might induce other stress protein expression, we separated the proteins of RBA-2 cells treated with and without ATP (1 mM, 16 h) by 2D-PAGE gel electrophoresis and stained with commassie blue. As shown in Figure 5A, ATP treatment affected the expressions of two low molecular weight (22 kDa) proteins of observed pI 4.98 (A) and 5.20 (B). The protein spots were then excised, purified, proteolytically digested, extracted, and then subjected to proteomic analysis by Q-TOF. Both of the protein spots were identified to be Prx II. The MASCOT search results are shown in Table I. As shown in Figure 5A, ATP treatment caused an increase in A protein (observed pI 4.98) and a decrease in B protein (observed pI 5.20). To verify the effect of ATP on the expression of Prx II, cells were treated with ATP (1 mM) for 0–12 h and the expression of actin and Prx II were examined by Western blot

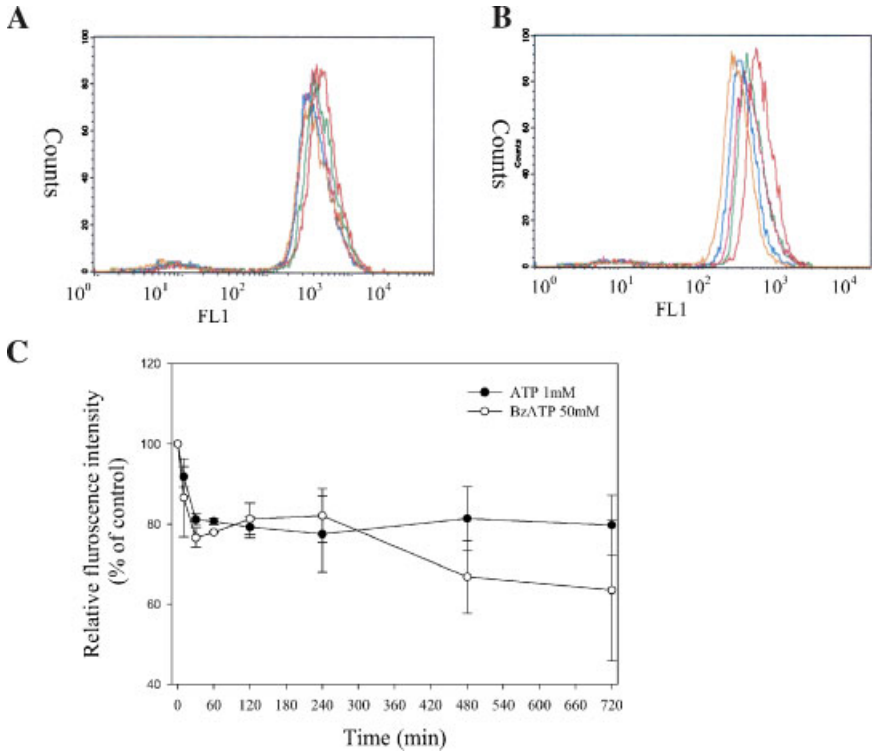

Fig. 4. ATP decreased mitochondria membrane potential of RBA-2 type-2 astrocytes. Histograms of flow cytometry measured mitochondrial membrane potential of RBA-2 astrocytes treated with **(A)** ATP (1 mM) or **(B)** BzATP (50 μM) for 0–720 min. **C**: The time-dependent statistical analysis of ATP- and BzATP-stimulated mitochondria membrane potential. RBA-2 cells were treated with ATP (1 mM) or BzATP (50 μM) for 0–720 min, scraped and loaded with fluorescent dye rhodamine123 to measure the mitochondria membrane potential. The fluorescence of the cells was then measured by FACS. Forward and side scatters were used to establish size gates and exclude cellular debris from the analysis. The excitation wavelength was set at 488 nm. The observation wavelength is set at 585 nm for red fluorescence. The emitted fluorescence was collected on FL1 channel, respectively. Ten thousand (1×10^4) RBA-2 cells per sample were analyzed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

analysis. As shown in Figure 5B, the autoradiograph and statistic analysis revealed that ATP did not affect the levels of Prx II. Thus, we concluded, ATP induced pI shift of Prx II.

Prx II is an important antioxidant in controlling H_2O_2 levels and maintaining normal functions of cells. To elucidate whether ATP might stimulate endogenous H_2O_2 production and induce pI shift of Prx II, RBA-2 cells were treated with exogenous H_2O_2 (100 μM, 1h). As shown in Figure 5C, H_2O_2 stimulated a similar pI shift of Prx II in these cells. To confirm that addition of exogenous H_2O_2 resulted in an increase in the endogenous H_2O_2, intracellular H_2O_2 concentrations were measured. As shown in Figure 5D, treatment of cells with 50–200 μM exogenous H_2O_2 resulted in a threefold increase in intracellular H_2O_2 concentrations as compared with the controls. ATP and exogenous H_2O_2 both induced a pI shift of Prx II, from oxidized form (pI 5.20) shifting to reduced form (pI 4.98). Therefore, ATP might stimulate the production of endogenous H_2O_2 and Prx II might serve as an important enzyme to protect RBA-2 astrocytes from oxidative stress responses.

Prolonged ATP Treatment Decreased Cellular Viability and Induced Apoptosis of RBA-2 Astrocytes

To elucidate whether prolonged ATP treatment affect viability of RBA-2 astrocytes, we conducted MTT, nuclei condensation and DNA fragmentation assays of these cells. As shown on Figure 6A, treatment of cells with 1 mM ATP or 50 μM BzATP for 4 to 8 h decreased viability of the cells by 20% and prolonging the treatment further decreased the viabilities of these cells. And 50% of cells remained viable when cells were treated with ATP (1 mM) for 24 h. Nevertheless, only 20% of the cells remained viable when cells were treated with BzATP (50 μM) for 24 h. In addition, treatment of cells with

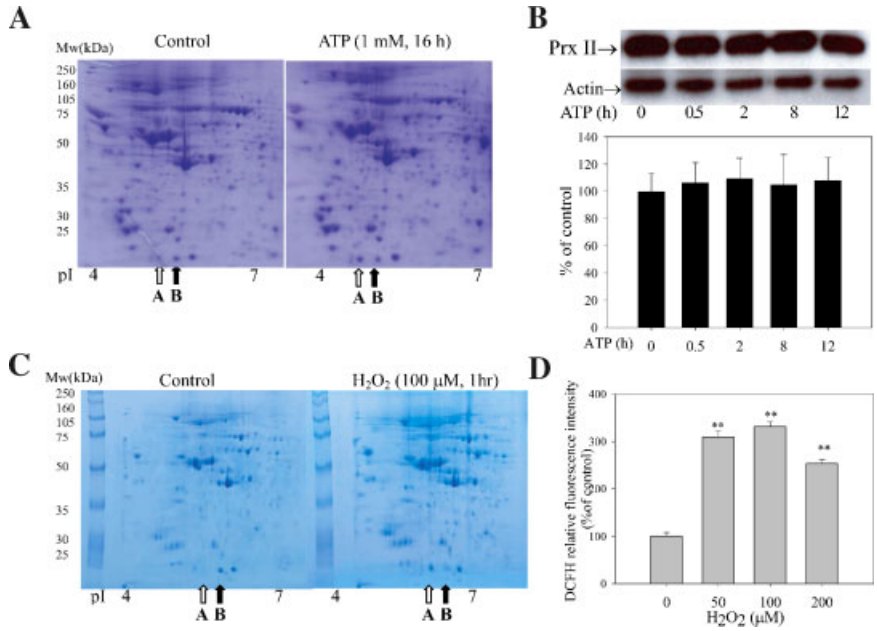

Fig. 5. RBA-2 astrocytes express peroxiredoxin II (Prx II), and ATP and H_2O_2 treatment stimulate pI shift of Prx II. **A**: The commassie blue-stained 2D-PAGE analysis of cytosolic proteins of RBA-2 astrocytes cultured in the presence or the absence of ATP (1 mM, 16 h). The two protein spots A (22 kDa, observed pI = 4.98) and B (22 kDa, observed pI = 5.20) indicated by arrows were excised, destained, eluted, proteolytically digested, microsequencing analyzed, and proteins identified by quadruple time-of-flight mass spectrometry (Q-TOF-MS). Both proteins are Prx II. Protein A is the reduced form Prx II and B is the oxidized form Prx II. **B**: Effect of ATP (1 mM, 0–12 h) on Prx II expression was performed by Western-blot analysis using anti-Prx II antibody and visualized by the ECL method. Quantitative analysis of Prx II was determined by image-densitometry analysis and the ratio of individual Prx II/actin calculated. The bar graphs are percentage of control of the ratio of Prx II/actin from three determinations. **C**: The commassie blue-stained two-dimensional gel electrophoresis analysis of cytosolic proteins of RBA-2 astrocytes cultured in the presence or the absence (control) of exogenous H_2O_2 (100 μM, 1 h). Arrows indicated reduced and oxidized forms of Prx II as described in (A). **D**: Intracellular H_2O_2 analysis of cells treated with exogenous H_2O_2 (1 h, 0–200 μM). RBA-2 cells were loaded with DCFHDA and intracellular H_2O_2 measured by FACS. The symbol ** indicates significant different means with $P < 0.01$ compared with the no H_2O_2 treated controls calculated by non-paired Student's *t*-test. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

0.5∼3 mM ATP or 50–500 μM BzATP for 12 h induced a dose-dependent decrease in the cellular viability (Fig. 6B).

To delineate whether the PKC and PLD inhibitors used might affect the viability of the cells, we conduct MTT assay on these cells. As shown in Figure 6C, treating the cells with 1 μM of neomycin, GF109203, chelerythrine chloride, or D609 for 24 h did not affect cellular viabilities of RBA-2 astrocytes.

To elucidate prolonged ATP treatment induced apoptosis of these cells, nuclei condensation, and DNA fragmentation assays were performed. As shown in Figure 7A,B, treatment of RBA-2 astrocytes with ATP or BzATP for ≤8 h did not cause nuclei condensation or DNA fragmentation of these cells. However, prolonged ATP treatment (≥24 h) induced a very obvious nuclei condensation (Fig. 7A) and DNA fragmentation (Fig. 7B) of RBA-2 astrocytes.

DISCUSSION

Our recent study has shown that high concentration of extracellular ATP (1 mM) activated $P2X_7$ receptors and stimulated cytokine, TGF-β1, expression of RBA-2 astrocytes [Wang et al., 2003]. Because high concentration of extracellular ATP (1 mM) would be provided during pathological condition in the brain [Dubyak and El-Moatassim, 1993], the cytokine induction might result from stressful insults, and HSPs or stress proteins were expressed in response to a wide variety of stressful stimulation, thus, in the present study we correlated the ATP stimulation with stress protein expression in RBA-2 astrocytes.

In the present study, 1 mM ATP stimulated HSP60 expression and antioxidants, resveratrol, and catalase, effectively inhibited the response, and also stimulated the expression of Cu/Zn

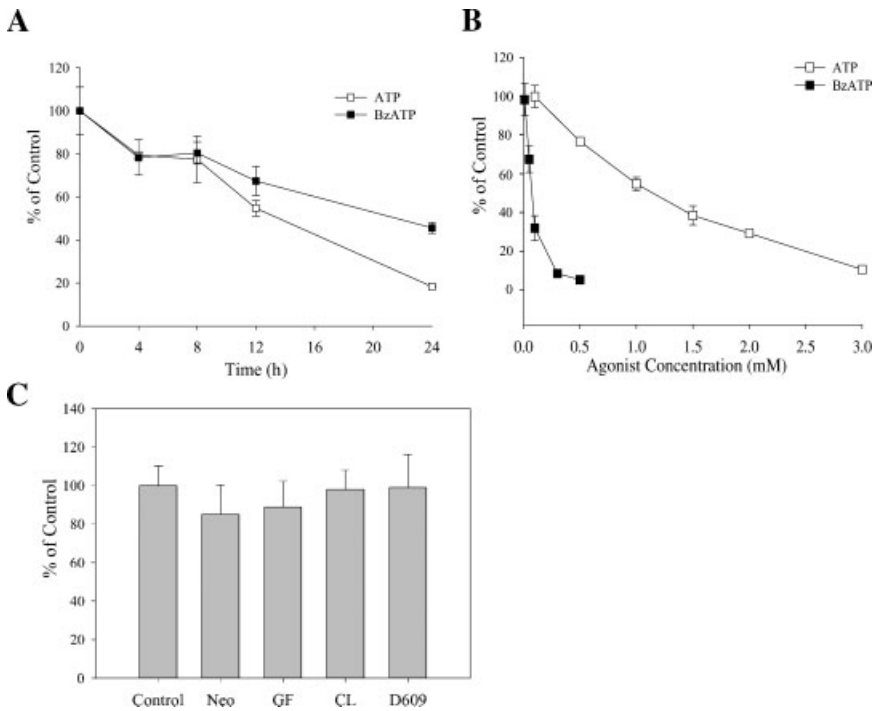

Fig. 6. ATP and BzATP but not the inhibitors decreased cellular viabilities. RBA-2 astrocytes were subcultured on 96-well plates for 2 days in culture media and then treated with **(A)** 1 mM ATP or 50 μM BzATP for 0–24 h, **(B)** 0–3 mM ATP or 0–500 μM BzATP for 12 h, and **(C)** 1 μM of various inhibitors, Neo (neomycin), GF (GF109203X), CL (chelerythrine chloride), and D609, for 24 h in F10 media supplemented with 1% FCS. MTT assays perform as described in the Materials and Methods. Data are means ± SD from three determinations using separate cultures and statistic analysis are performed by non-paired Student's *t*-test.

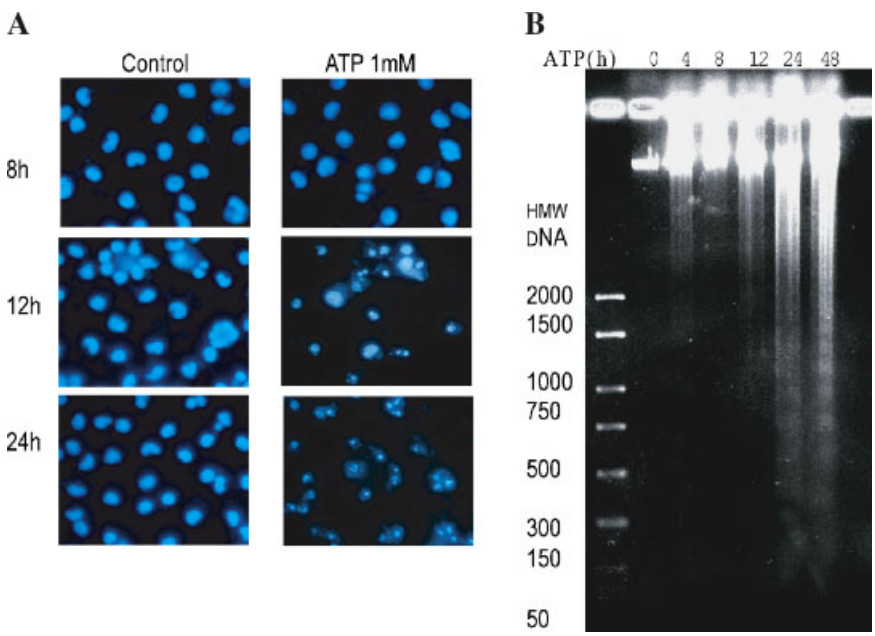

Fig. 7. Prolonged ATP and BzATP treatment induced cell death. **A**: RBA-2 astrocytes were subcultured on coverglasses for 2 days and then cultured in F10 media containing 1% FBS in the presence or the absence (control) of 1 mM ATP for 8–24 h. Cells were then fixed with 4% paraformaldehyde and subsequently stained with Hoechst 33258. Photomicrographs were taken by a Nikon fluorescent microscope. **B**: RBA-2 astrocytes were cultured on 100 mm dishes for 2 days and then cultured in F10 media containing 1% FBS in the presence or the absence (control) of 1 mM ATP for 0–48 h. DNA extracted, loaded onto a 1.5% agarose gel and separated by gel electrophoresis. The separated gel was stained for 1 h in 0.5 μg/ml ethidium bromide and visualized by UV illumination. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

SOD. Because HSP60 is a mitochondria chaperone, we subsequently measured the ATP- and $P2X_7$ receptor selective agonist BzATP-stimulated mitochondrial membrane potential by flow cytometry. Our results revealed that both ATP and BzATP decreased mitochondrial membrane potential. These results suggest that ATP stimulated a stress response of RBA-2 astrocytes.

Treating the cells with various $P2X_7$ receptor-associated intracellular signaling inhibitors, such as PKC inhibitors, chelerythrine and GF109203X, and PLD inhibitors, neomycin, and D609, all effectively decreased the ATP-stimulated HSP60 expression but without affecting cellular viabilities indicating that the ATP-stimulated HSP60 expression is associated with activation of $P2X_7$ receptor. Interestingly, chelerythrine (1 μM, 24 h) per se slightly enhanced HSP60 expression of RBA-2 astrocytes. Chelerythrine has been shown to possess effects other than inhibition of PKC. Lombardini [1995] identified that chelerythrine stimulated the phosphorylation of a mitochondria protein of retina and cerebral cortex. Recently, Yamamoto et al. [2001] revealed that chelerythrine rapidly induced apoptosis through generation of reactive oxygen species (ROS). Thus, the chelerythrine-stimulated HSP60 expression might due to its effect on mitochondria and the generation of ROS.

It is interesting to find that both ATP and BzATP caused rapid decreases in mitochondrial membrane potential. Mitochondria are the key element in oxidative stress response, apoptosis, and neurodegeneration [Finkel and Holbrook, 2000; Giasson et al., 2002]. We also found that prolonged ATP (1 mM, $\geq$24 h) treatment induced nuclei condensation and DNA fragmentation in RBA-2 astrocytes. Thus the mitochondrial dysfunction might be associated with the ATP-mediated stress responses. Similarly, the mitochondrial pathway was recently identified to be associated with H_2O_2-induced apoptotic cell death of oligodendrocytes [Mronga et al., 2004]. Therefore, production of reactive oxygen species may play a role in the ATP-stimulated mitochondrial dysfunction of these astrocytes.

In the present study, both the two-dimensional gel electrophoresis and the Western-blot analysis revealed that RBA-2 type-2 astrocytes expressed abundant Prx II. Earlier researches showed that Prx II mRNA was most abundantly expressed in rat brain as compared with Prx I, III, and IV of other tissues [Matsumoto et al., 1999]. However, immunohistochemical analysis revealed that Prx II is exclusively expressed in the neuron of human brain sections [Sarafian et al., 1999]. The exact reason for such a discrepancy in Prx II expression is not known at this moment. However, differential expression of various Prx isozymes in glial cells has been reported. Immunohistochemical analysis found intense staining of Prx I (MSP23) in oligodendrocytes [Nakaso et al., 1999; Mizusawa et al., 2000] whereas a much less obvious Prx I immunostaining was found in astrocytes [Mizusawa et al., 2000]. In contrast, Prx I expression was found in astrocytes with a co-induction of stress protein, heme oxygenase-1, during subacute and chronic phase of intracranial hemorrhage of brain [Nakaso et al., 2000] and action of the transcription factor Nrf2 at the antioxidant response element (ARF) upregulated Prx I in astrocytes [Shih et al., 2003]. The expression of Prx I of RBA-2 astrocytes is not known at this moment. RBA-2 astrocyte is a clonal type-2 astrocyte cell line [Sun et al., 1999]. In the brain, type-2 astrocytes are not as abundant as type-1 astrocytes. Thus it would be difficult to detect the specific expression of Prx II in type-2 astrocytes in brain slices. Further examination of Prx I and other Prx family of antioxidant protein expression of astrocytes is needed.

In the present study, ATP treatment induced a pI shift of Prx II but did not alter the levels of Prx II as revealed by Western-blot analysis. Furthermore, addition of exogenous H_2O_2 (50–200 μM) caused a similar threefold increase in intracellular H_2O_2 and 100 μM exogenous H_2O_2 induced a pI shift from 5.2 to 4.98 of Prx II. In addition, our results also revealed that 200 μM exogenous H_2O_2 caused a drop in the intracellular H_2O_2 concentrations as compared with 100 μM. Because H_2O_2 has been shown to be a cytotoxic agent [Trembovler et al., 2003; Cardoso et al., 2004; Huang et al., 2004; Ruffels et al., 2004], thus the drop might due to a leakage of the fluorescence from the injured cells.

Similarly, exogenous H_2O_2 has been shown to induce a structural modification of Prx. Addition of 100 μM exogenous H_2O_2 lowered 0.2–0.3 pH units of Prx in human umbilical vein endothelial cells [Mitsumoto et al., 2001]. The pI shift was due to the rapid scavenging of peroxides and reduction of redox-sensitive cysteines by Prx II [Netto et al., 1996; Chevallet

et al., 2003]. Prx II was first cloned from brain cDNA [Chae et al., 1994]. Recently the oxidized enzyme was found to reduce rapidly to its catalytically active thiol form and may act as a new type of cyclic modification to regulate the functions of many proteins [Woo et al., 2003]. In addition, recent studies revealed that guided by the Cys^{47} residue, which serves as H_2O_2 sensor, the protein structures of Prx I and Prx II shifted from peroxidase to chaperone during oxidative stress and heat shock, and the chaperone function enhanced yeast resistance to heat shock [Jang et al., 2004]. These findings suggest that Prx II is an important antioxidant that controls H_2O_2 levels and maintains the normal function of cells. Thus, Cu/Zn SOD and Prx II may play important roles to metabolize the ATP-stimulated reactive oxygen species and protect cells under stress condition.

ACKNOWLEDGMENTS

We thank Prof. Hungyi Shau, Division of Surgical Oncology, UCLA School of Medicine, Los Angeles, California, for the generous gift of Anti-Prx II antibody.

REFERENCES

Bajramović JJ, Bsibsi M, Geutskens SB, Hassankhan R, Verhulst KC, Stege GJJ, de Groot CJA, Noort JM. 2000. Differential expression of stress proteins in human adult astrocytes in response to cytokines. J Neuroimmun 106: 14–22.

Becker J, Craig EA. 1994. Heat shock proteins as molecular chaperons. Eur J Biochem 219:11–23.

Cabiscol E, Piulates E, Echave P, Herrero E, Ros J. 2000. Oxidative stress promotes specific damages in *Saccharomyces cerevisivae*. J Biol Chem 275:27393–27398.

Cardoso SM, Rego AC, Penacho N, Oliveira CR. 2004. Apoptotic cell death induced by hydrogen peroxide in NT2 parental and mitochondrial DNA depleted cells. Neurochem Int 45:693–698.

Chae HZ, Robinson K, Poole LB, Church G, Storz G, Rhee SG. 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc Natl Acad Sci USA 91:7017–7021.

Chevallet M, Wagner E, Luche S, van Dorsselaer A, Leize-Wagner E, Rabilloud T. 2003. Regeneration of peroxiredoxins during recovery after oxidative stress. J Biol Chem 278:37146–37153.

Coutinho-Silva R, Perschini PM, Da Cunha Bisaggio R, Perfettini J-L, Torres de Santo AC, Kanellopoulos JM, Mottaly I, Dautry-Varsat A, Ojcius DM. 1999. P2Z/$P2X_7$ receptor-dependent apoptosis of dendritic cells. Am J Physiol 276:C1139–C1147.

Dong Y, Benveniste EN. 2001. Immune function of astrocytes. Glia 36:180–190.

Dubyak GR, El-Moatassim A. 1993. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol 265:C577–C606.

Ferrari D, Wesselborg S, Bauer MK, Schulze-Osthoff K. 1997. Extracellular ATP activates transcription factor NF-kB through the P2Z purinoceptor by selectively targeting NF-kB p65 RelA. J Cell Biol 139:1635–1643.

Ferrari D, Los M, Bauer MKA, Vandenabeele P, Wesselborg S, Schulze-Osthoff K. 1999. P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 447:71–75.

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of aging. Nature 408:239–247.

Giasson BI, Ischiropoulos H, Lee VM-Y, Trojanowski X 2002. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. Free Rad Biol Med 32:1264–1275.

Hide I, Tanaka M, Inoue A, Nakajima K, Koshaka S, Inoue K, Nakata Y. 2000. Extracellular ATP triggers tumor necrosis factor-α release from rat microglia. J Neurochem 75:965–972.

Hogquist KA, Nett MA, Unanue ER, Chaplin DD. 1991. Interleukin 1 is processed and released during apoptosis. Proc Nat Acad Sci USA 88:8485–8489.

Huang F, Vemuri MC, Schneider JS. 2004. Modulation of ATP levels alter the mode of hydrogen peroxide-induced cell death in primary cortical cultures: Effects of putative neuroprotective agents. Brain Res 997:79–88.

Hung AC, Sun SH. 2002. Characterization of the $P2X_7$ receptor-mediated phosphatidylcholine-phospholipase D activities in a rat brain-derived type-2 astrocyte cell line, RBA-2: Involvement of PKC-dependent and PKC-independent pathways. Cell Signal 14:1–10.

Hung AC, Huang HM, Tsay HJ, Lin TN, Kuo JS, Sun SH. 2000. ATP stimulated *c-fos* and *zif268* mRNA expressions are inhibited by chemical hypoxia in a rat brain-derived type-2 astrocyte cell line, RBA-2. J Cell Biochem 77:323–332.

Itoh H, Komatsuda A, Ohtani H, Wakui H, Imai H, Sawada KI, Otaka M, Ogura M, Suzuki A, Hamada F 2002. Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration. Eur J Biochem 269:5931–5938.

Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong G-W, Yun D-J, Rhee SG, Lee SY. 2004. Two enzymes in one: Two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117:825–835.

Kaufman BA, Kolesar JE, Perlman PS, Butow RA. 2003. A function for the mitochondrial chaperon Hsp60 in the structure and transmission of mitochondrial DNA nucleotides in *Saccharomyces Cerevisiae*. J Cell Biol 163:457–461.

Kirchhoff SR, Gupta S, Knowlton AA. 2002. Cytosolic heat shock protein 60, apoptosis and myocardial injury. Circulation 105:2899–2904.

Laemmili UK 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lin KM, Lin B, Lian IY, Mestril R, Scheffler IE, Dillmann WH. 2001. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes

protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. Circulation 102:1787–1792.

Lombardini JB. 1995. Paradoxical stimulatory effect of the kinase inhibitor chelerythrine on the phosphorylation of a ~20 K Mr protein present in the mitochondrial fraction of rat retina. Brain Res 673:194–198.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with Folin phenol reagent. J Biol Chem 264:1450–1456.

Lubec G, Krapfenbauer K, Fountoulakis M. 2003. Proteomic in brain research: Potentials and limitation. Prog Neurobiol 69:193–211.

Matsumoto A, Okado A, Fujii T, Fujii J, Egashira M, Niikawa N, Taniguchi N. 1999. Cloning of the peroxiredoxin gene family in rats and characterization of the fourth member. FEBS Lett 443:246–250.

Mitsumoto A, Takanezawa Y, Okawa K, Iwamatsu A, Nakagawa Y. 2001. Variants of peroxiredoxins expression in response to hydroperoxide stress. Free Rad Biol Med 30:625–635.

Mitsumoto A, Takeuchi A, Katsuya A, Okawa K, Nakagawa Y. 2002. A subset of newly synthesized polypeptides in mitochondria from human endothelial cells exposed to hydroperoxide stress. Free Rad Biol Med 32:22–37.

Mizusawa H, Ishii T, Bannai S. 2000. Peroxiredoxin I (macrophage 23 kDa stress protein) is highly and widely expressed in the rat nervous system. Neurosci Lett 283:57–60.

Morigiwa K, Quan M, Murakami M, Yamashita M, Fukuda Y. 2000. P2 purinoceptor expression and functional changes of hypoxia-activated cultured rat retinal microglia. Neurosci Lett 282:153–156.

Mronga T, Stahnke T, Goldbaum O, Richter-Landberg C. 2004. Mitochondrial pathway is involved in hydrogen-peroxide-induced apoptotic cell death of oligodendrocytes. Glia 46:446–455.

Nakaso K, Kitayama M, Ishii T, Bannai S, Yanagawa T, Kimura K, Nakashima K, Ohma E, Yamada K. 1999. Effects of Kainate-mediated excitotxicity on the expression of rat counterparts of A170 and MSP23 stress proteins in the brain. Mol Brain Res 69:155–163.

Nakaso K, Kitayama M, Mizuta E, Fukuda H, Ishii T, Nakashima K, Yamada K. 2000. Co-induction of heme oxygenase-1 and peroxiredoxin 1 in astrocytes and microglia around hemorrhagic region in the rat brain. Neurosci Lett 293:49–52.

Netto ES, Chae HZ, Kang SW, Rhee SG, Stadman ER. 1996. Removal of hydrogen peroxide by thio-specific antioxidant enzyme (TSA) is involved with its antioxidant properties. TSA possesses thiol peroxidase activity. J Biol Chem 271:15315–15321.

Ohtsuka K, Suzuki T. 2000. Roles of molecular chaperones in the nervous system. Brain Res Bull 53:141–146.

Rhee SG, Kim KH, Chae HZ, Yim MB, Uchida K, Netto LE, Stadtman ER. 1994. Antioxidant defense mechanisms: A new thiol-specific antioxidant enzyme. Ann New York Acad Sci 738:86–92.

Ruffels J, Griffin M, Dickenson JM. 2004. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: Role of ERK1/2 in H_2O_2-induced cell death. Eur J Pharmacol 483:163–173.

Samali A, Cai J, Zhivotovsky B, Jones DP, Orrenius S. 1999. Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. EMBO J 18:2040–2048.

Sarafian TA, Verity MA, Vinters HV, Shih CCY, Shi L, Dong L, Shau HY. 1999. Differential expression of peroxiredoxin subtypes in human brain cell types. J Neurosci Res 56:206–212.

Schulze-Lohoff E, Hugo C, Rost S, Arnold S, Gruber A, Brune B, Sterzel RB. 1998. Extracellular ATP caused apoptosis and necroisis of cultured mesangial cells via $P2Z/P2X_7$ receptors. Am J Physiol 275:F962–F971.

Shan YX, Liu TJ, Su HF, Samsamshariat A, Mestril R, Wang PH. 2003. HSP10 and HSP60 modulate Bxl-2 family and mitochondria apoptosis signaling induced by doxorubicin in cardiac muscle cells. J Mol Cell Card 35:1135–1143.

Shau H, Golob SH. 1993. Modulation of natural killer-mediated lysis by red blood cells. Cell Immunol 116:60–72.

Shigemoto-Mogami Y, Koizumi S, Tsuda M, Ohsawa K, Kohsaka S, Inoue K. 2001. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. J Neurochem 78:1339–1349.

Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb Heidi Johnson JA. 2003. Coordinate regulation of glutathione biosynthesis and release of Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394–3406.

Sun SH, Lin L-B, Hung AC, Kuo J-S. 1999. ATP-stimulated Ca^{2+} influx and phospholipase D activities of a rat brain-derived type-2 astrocyte cell line, RBA-2, are mediated through $P2X_7$ receptors. J Neurochem 73:334–343.

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). Science 272:735–738.

Trembovler V, Abu-Raya S, Shohami E. 2003. Synergism between tumor necrosis factor-α and H_2O_2 enhances cell damage in rat PC12 cells. Neurosci Lett 353:115–118.

Vander Heiden MG, Chandel NS, Williamson EK, Schmacker PT, Thompson CB. 1997. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 91:559–562.

Wadia JS, Chalmers-Redman RME, Ju WJH, Carile GW, Philips JL, Fraser AD, Tatton WG. 1998. Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: Time course and modification by (–)-deprenyl. J Neurosci 18:932–947.

Wang CM, Chang YY, Kuo JS, Sun SH. 2002. Activation of $P2X_7$ receptors induced [^{3}H]GABA release from RBA-2 type-2 astrocyte cell line through a Cl^-/HCO_3^--dependent mechanism. Glia 37:8–18.

Wang CM, Chang YY, Sun SH. 2003. Activation of $P2X_7$ purinoceptor stimulated TGF-β1 mRNA expression via PKC/MAPK pathway in the RBA-2 type-2 astrocytes. Cell Signal 15:1129–1137.

Welch WJ. 1992. Mammalian stress response in cell physiology, structure/function of stress proteins and implications for medicine and disease. Physiol Rev 72:1063–1081.

Woo HA, Chae HZ, Hwang SC, Yang KS, Kang SW, Kim K, Rhee SG 2003. Reversing the inactivation of peroxiredoxins caused by cystein sulfinic acid formation. Science 300:653–656.

Wood ZA, Poole LB, Karplus PA. 2003a. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300:650–653.

Wood ZA, Schroder E, Harris JR, Poole LB. 2003b. Structure, mechanism and regulation of peroxiredoxins. Trends Biochem Sci 28:32–40.

Xanthoudakis S, Roy S, Rasper D, Hennessey T, Aubin Y, Cassady R, Tawa P, Ruel R, Rosen A, Nicholson DW. 1999. Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. EMBO J 18:2049–2056.

Yamamoto S, Seta KI, Morisco C, Vatner SF, Sadoshima J 2001. Chelerythrine rapidly induces apoptosis through generation of reactive oxygen species in cardiac myocytes. J Mol Cell Cardiol 33:1829–1848.