ARTICLE
Journal of Cellular Biochemistry 114:1695–1703 (2013)

Journal of Cellular Biochemistry

The Mechanism Underlying the Effects of the Cell Surface ATP Synthase on the Regulation of Intracellular Acidification During Acidosis

Wen-juan Wang,[1] Xiao-xing Shi,[2] Yi-wen Liu,[1] Yi-qing He,[1] Ying-zhi Wang,[1] Cui-xia Yang,[1] and Feng Gao[1*]

[1]*Department of Laboratory Medicine, Shanghai Sixth People's Hospital of Shanghai, Shanghai JiaoTong University School of Medicine, Shanghai 200233, PR China*

[2]*Department of Laboratory Medicine, Shanghai Wujing General Hospital, PR China*

ABSTRACT

The F1F0 ATP synthase has recently become the focus of anti-cancer research. It was once thought that ATP synthases were located strictly on the inner mitochondrial membrane; however, in 1994, it was found that some ATP synthases localized to the cell surface. The cell surface ATP synthases are involved in angiogenesis, lipoprotein metabolism, innate immunity, hypertension, the regulation of food intake, and other processes. Inhibitors of this synthase have been reported to be cytotoxic and to induce intracellular acidification. However, the mechanisms by which these effects are mediated and the molecular pathways that are involved remain unclear. In this study, we aimed to determine whether the inhibition of cell proliferation and the induction of cell apoptosis that are induced by inhibitors of the cell surface ATP synthase are associated with intracellular acidification and to investigate the mechanism that underlines the effects of this inhibition, particularly in an acidic tumor environment. We demonstrated that intracellular acidification contributes to the cell proliferation inhibition that is mediated by cell surface ATP synthase inhibitors, but not to the induction of apoptosis. Intracellular acidification is only one of the mechanisms of ecto-ATP synthase-targeted antitumor drugs. We propose that intracellular acidification in combination with the inhibition of cell surface ATP generation induce cell apoptosis after cell surface ATP synthase blocked by its inhibitors. A better understanding of the mechanisms activated by ecto-ATP synthase-targeted cancer therapies may facilitate the development of potent anti-tumor therapies, which target this enzyme and do not exhibit clinical limitations. J. Cell. Biochem. 114: 1695–1703, 2013.

KEY WORDS: CELL SURFACE ATP SYNTHASE; TUMOR; INTRACELLULAR ACIDIFICATION; PROLIFERATION; APOPTOSIS

The treatment of cancer remains one of the most challenging questions in life science research. The cell surface F1F0 ATP synthase has been a recent target for cancer therapy [Mowery and Pizzo, 2008]. Since it was found to reside on the cell surface in 1994 [Shampo et al., 2011], the cell surface ATP synthase has been reported to participate in tumor immunity, tumor angiogenesis, and tumor cell survival in low extracellular pH environments [Vantourout et al., 2008; Pan et al., 2011].

ATP synthase is composed of a membrane F0 region and a catalytic F1 region. The F1 complex, which consists of the $\alpha 3\beta 3\gamma\delta\varepsilon$ subunits, catalyzes the synthesis of ATP from ADP (adenosine diphosphate) and inorganic phosphate [Wächter et al., 2011]. F0, which is a proton channel, pumps intracellular H^+ to the outer membrane in response to a concentration gradient [Usukura et al., 2012]. ATP synthase is bidirectional; thus, it can also hydrolyze ATP and pump H+ into the cell. Therefore, the cell surface ATP synthase

WenJuan Wang and Xiao-xing Shi contributed equally to this work.

Grant sponsor: The National High Technology Research and Development Program of China (863 Program); Grant number: 2008AA 02Z121; Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81071814, 81172027; Grant sponsor: Program of Shanghai Subject Chief Scientist; Grant number: 11XD1404000; Grant sponsor: Science and Technology Commission of the Shanghai Municipality (Key Technology Support Program); Grant number: 10411950500.

*Correspondence to: Feng Gao, Department of Laboratory Medicine, Shanghai Sixth People's Hospital of Shanghai, Shanghai JiaoTong University School of Medicine, 600 Yi-shan Road, Shanghai 200233, PR China.
E-mail: gao3507@126.com

Manuscript Received: 5 June 2012; Manuscript Accepted: 24 January 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 February 2013

DOI 10.1002/jcb.24511 • © 2013 Wiley Periodicals, Inc.

can perform both ATP synthesis and hydrolysis and regulate intracellular H+ concentration [Dong and Fillingame, 2010].

Ecto-ATP synthase on tumor cells may play an important role in cell survival in an environment with a low extracellular pH. ATP synthase inhibitors including antibodies, the natural inhibitor of ATP synthase and angiostatin, are able to induce tumor cell apoptosis under extracellular acidosis [Burwick et al., 2005; Chi and Pizzo, 2006; Chi et al., 2007; Zhang et al., 2008]. Ecto-ATP synthase is also involved in tumor angiogenesis. The blocking of ecto-ATP synthase on vascular endothelial cells by angiostatin or other inhibitors suppresses angiogenesis [Dass et al., 2007; Mowery and Pizzo, 2008; Zhang et al., 2008; Takahashi et al., 2010; Xiao-yan et al., 2011].

Although many studies have focused on the role of the cell surface ATP synthase, few reports have investigated the mechanism by which ecto-ATP synthase participates in the anti-tumor process. None of the previous studies has illustrated how the cell surface ATP synthase participates in signal transduction inside the cell. Thus, the molecular pathways downstream of the ecto-ATP synthase remain unknown. Because the intracellular pH is decreased and ATP generation is inhibited by treatment with ecto-ATP synthase inhibitors, we hypothesized that the ATP synthesis and H^+ regulation activities of this receptor might contribute to the intracellular signaling responses. It is known that a decreased intracellular pH inhibits cell proliferation and induces apoptosis [Zanke et al., 1998; Thangaraju et al., 1999; Wahl and Grant, 2000; Schelling and Jawdeh, 2008; Yang et al., 2008]. Thus, it appears that the downregulation of the intracellular pH contributes to the antitumor effects of ATP synthase-targeting drugs.

We used the Na^+/H^+ exchanger (NHE) inhibitor 5-ethylisopropyl amiloride (EIPA) to construct an intracellular acidification model that imitates the effect of ATP synthase inhibitors. The studies described in this manuscript were designed to determine whether intracellular acidification is associated with the inhibition of cell proliferation and the induction of cell apoptosis that are proliferation inhibition and cell apoptosis that are induced by surface ATP synthase inhibitors, specifically in an acidic tumor environment.

A high concentration of intracellular H^+ has been reported to indirectly increase the intracellular Ca^{2+} concentration [Tani and Neely, 1989; Harrison et al., 1992]. The excessive entry of calcium into a cell may damage the cell and even cause the cell to undergo apoptosis or necrosis via PKC translocation [Pongracz et al., 1996; Zhu et al., 1999]. PKC, which is downstream of nearly all of the membrane-associated signal transduction pathways, such as MAPK and Akt, mediates many types of biological functions [Ping et al., 1999; Saijo et al., 2002; Kim et al., 2008]. Moreover, intracellular acidification has been reported to induce cell apoptosis through the dephosphorylation of Akt [Schelling and Jawdeh, 2008]. Furthermore, the cell surface ATP synthase which is inhibited by angiostatin, has been found to mediate cell apoptosis through JNK [CHEN et al., 2008] and cytochrome *c* [Hanford et al., 2003]. Therefore, we hypothesized that the down regulation of intracellular pH might affect the PKC, MAPK, and Akt pathways. Thus, the activation of these signaling proteins was tested in this study to further investigate the molecular mechanism that underlines the induction of apoptosis in response to the inhibition of the cell surface ATP synthase.

ATP synthase is expressed on the surfaces of many types of cell, including normal and malignant cells. A549 cells, which are adenocarcinomic human alveolar basal epithelial cells, express high levels of ATP synthase on their cell surfaces [Chi and Pizzo, 2006]. Therefore, A549 cells were used as a positive control in our study. A better understanding of the mechanisms of ecto-ATP synthase will facilitate the development of more potent anti-tumor therapies that target on this enzyme, and do not have limited clinical applications.

MATERIALS AND METHODS

REAGENTS AND CELL CULTURE

Mc178-Ab, which is a monoclonal antibody against the cell surface F1F0 ATP synthase, was obtained from hybridoma cells as previously described and reconstituted in sterile PBS [Zhang et al., 2008]. The mouse isotype IgG and the SABC-Cy3 immunohistochemistry kit were purchased from Biostar (WuHan, China). Antibodies to phospho-PKC, phospho-Akt (Ser473), phospho-ERK1/2, phospho-JNK, and phospho-p38 were obtained from Cell Signaling Technology (Beverly, MA). Ethyl isopropyl amiloride (EIPA) was purchased from Sigma–Aldrich (Gillngham, UK). The acetomethoxy ester of 2′,7′-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein (BCECF-AM) and Fura2-AM in DMSO were purchased from Dojindo Co. (Kumamoto, Japan). The CellTiterGlo luminescence assay reagents were purchased from Promega (Madison, WI, USA), and the Annexin V-FITC and PI apoptosis kit was obtained from BD (Bender Medsystem, USA). All of the cell lines were purchased from the American Tissue Culture Collection (ATCC) and cultured in the recommended medium (Gibco, Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal calf serum (Gibco, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2.

DETECTION OF Ecto-ATP SYNTHASE EXPRESSION

A549, HUVEC, HepG2, L-02, 95-D, PC12, and CHO cells were cultured in fresh medium for 48 h and then digested with EDTA (0.1%). The cells were then blocked with 1% BSA for 30 min, incubated with an antibody to the β-subunit of ATP synthase for 60 min, and washed three times with PBS. The cells were then incubated with a FITC-conjugated goat anti-mouse antibody (Biostar, Wuhan, China) and washed. All of these steps were performed at 4°C. Propidium iodide (BD Biosciences, San Jose, CA) was added to in all of the samples to label the cells that had compromised membranes. The mean fluorescence intensity of FITC in 10,000 cells was quantified using a flow cytometer (Beckman–Coulter, Brea, USA), and those cells that expressed mitochondrial ATP synthase were excluded from the analysis. The expression of cell surface ATP synthase was confirmed by fluorescence.

CELL PROLIFERATION ASSAY

A549, 95-D, PC12, and CHO cells were cultured in 96-well plates for 24 h. The medium was then replaced with serum-free medium, and the cells were allowed to rest overnight to allow the cells to become quiescent. The cells were then incubated with fresh medium at pH 6.7 in a CO_2 atmosphere; during this incubation step, the cells were treated with 5% FCS, Mc178-Ab (200 μg/ml, 1:10 dilution), EIPA (25

or 45 μM), IgG (200 μg/ml, isotype control), epirubicin (5 μg/ml), PBS (1:10 dilution) or media alone for 24 and 48 h. The cell proliferation was measured through the MTT assay (Amresco, Sigma). After the medium was removed, 20 μl of 5 mg/ml MTT medium was added to each well. Fresh medium was then added to the wells to obtain a final volume of 100 μl/well. The cells were incubated for 4 h at 37°C in a tissue culture hood. After the media was removed, 150 μl of the MTT solvent (DMSO) was added. After the plates were shaken for 15 min, the absorbance at 570 nm was recorded using a microplate reader (Wellscan MK3, Labsystems Dragon).

ANALYSIS OF CELLULAR APOPTOSIS

The early apoptotic events were detected using AnnexinV-FITC and PI apoptosis staining kit as described by the manufacturer. Briefly, after 16 h of serum starvation, the A549 cells were separately treated with Mc178-Ab (200 μg/ml, 5% FCS), EIPA (25 or 45μM), IgG (200μg/ml, 5% FCS, isotype control) or medium alone (5% FCS) for 24 or 48 h. Treated cells were washed and digested with trypsin, resuspended at a concentration of 10^6 cells/ml, and incubated with binding buffer containing Annexin V-FITC (3 μM) and PI (5 μg/ml) for 10 min. The cells were then analyzed on a flow cytometer (Beckman–Coulter).

MEASUREMENT OF INTRACELLULAR pH

The intracellular pH was measured by luminescence spectrometry using BCECF/AM, as previously described with few modifications [Franck et al., 1996]. A549 cells were seeded at a density of 5,000 cells/well in a 96-well plate and incubated overnight. The cells were washed with HEPES buffer (20 mM HEPES, 153 mM NaCl, 5 mM KCl, and 5 mM glucose, pH 7.4) supplemented with 2 μg/ml BCECF-AM for 10 min at 37°C. After the cells were washed twice with HEPES buffer, the cells were treated with Mc178-Ab (200 μg/ml, 5% FCS), IgG (200 μg/ml, isotype control), oligomycin (10 μg/ml), EIPA (25 or 45 μM) or media alone (pH 6.7, negative control). To monitor the intracellular pH, the fluorescence of BCECF at 525 nm was recorded after excitation at 485 nm in a SpectraMax multifunctional microplate reader. A calibration curve for the intracellular pH was generated by incubating BCECF-loaded cells with media at different pH levels (HEPES buffer: 25 mM HEPES, 145 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.5 mM glucose; the pH levels were modified using 1 M NaOH).

DETERMINATION OF CELL SURFACE ATP GENERATION AND THE CONCENTRATION OF EXTRACELLULAR ATP

Prior to the assay, A549, 95-D, and CHO cells (10,000 per well) were incubated in 96-well plates overnight. The cells were treated with Mc178-Ab (200 μg/ml), oligomycin (5 ng/ml), IgG (200 μg/ml, isotype control), or media alone (negative control) for 60 min at 37°C in a 5% CO_2 incubator. The cells were then incubated with ADP (0.05 mM, Sigma, MO) for 30 s under normal or acidic conditions (the pH of the media was 7.2 or 6.7). The supernatants were harvested and used to determine the level of ATP generation. The supernatants, which were removed before the MTT medium was added in the cell proliferation assay, were also harvested. The extracellular ATP in the supernatants was measured using the CellTiterGlo luminescence assay (Promega, Madison, WI) as previously described [Chi and Pizzo, 2006]. The results were recorded with a SpectraMax multifunctional microplate reader (Bio-Tek, Winooski, USA).

MEASUREMENT OF INTRACELLULAR Ca^{2+}

The intracellular Ca^{2+} level was measured with a luminescence spectrometer using Fura2-AM, as previously described [Oakes et al., 1988]. A549 cells were incubated overnight at density of 10,000 cells/well in 96-well plate. The cells were washed with Hank's Balanced Salt Solution (HBSS, 8 g/L NaCl, 0.4 g/L KCl, 1 g/L glucose, 60 mg/L KH_2PO_4, 47.5 mg/L Na2HPO4, pH 7.2). Cells were loaded with 3 μM of Fura2-AM for 30 min at room temperature in HBSS. After twice washed with HBSS, cells were treated with Mc178-Ab (200 μg/ml, 5% FCS), EIPA (25/45 μM) and media only (pH 6.7) as a negative control. To monitor the intracellular Ca^{2+} levels, the fluorescence of Fura2 at 510 nm was recorded after excited at 340 nm by the SpectraMax multifunctional microplate reader.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

A549 cells were incubated with or without Mc178-Ab (200 μg/ml), EIPA (25/45 μM) for 30 min over microscope slides at 37°C. The cells were then fixed for 10 min with 4% paraformaldehyde at room temperature, permeabilized for 5 min with 0.1% Triton X-100 in PBS, blocked for 30 min with 1% BSA in PBS, and then stained with monoclonal anti PKC antibody for 18 h at 4°C. The cells were washed with PBS, and incubated with a secondary biotin-conjugated sheep anti-mouse IgG antibody for 45 min at room temperature. The cells were washed with PBS, and then incubated with cy3-conjugated avidin for 30 min at room temperature. The cells were subsequently washed and processed for confocal microscopy.

WESTERN BLOT ANALYSIS

Subconfluent A549 cells were plated in six-well plates and treated with serum-free media for 17 h. The cells were then stimulated by replacing the medium with new medium with or without Mc178-Ab (200 μg/ml, 5% FCS) or EIPA (45 μM) for 5, 20, or 30 min. The cells were washed with PBS and lysed with a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The cell lysates were resolved by SDS–PAGE (10% running, 5% stacking; Applied Biosystems) and transferred to PVDF membranes (0.45-mm pore size; Millipore, Bedford, MA). The membranes were incubated with the designated antibodies for 1 h at room temperature or overnight at 4°C. The immunodetection was performed using the Western-Light Chemiluminescent detection system according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Each experiment was repeated independently at least twice. The data from the independent experiments were pooled and analyzed using two-tailed Student's *t*-test. A difference was considered statistically significant if the *P*-value was less than 0.05.

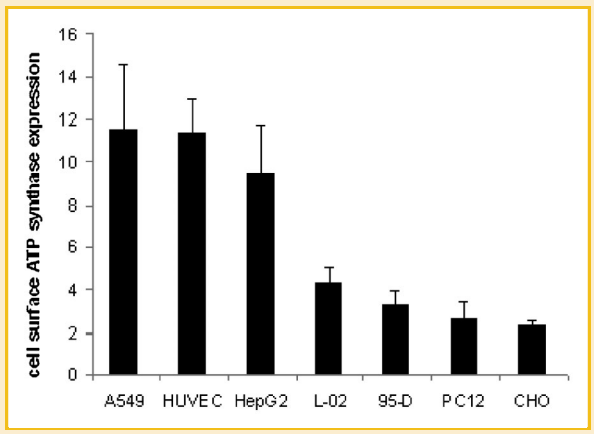

Fig. 1. ATP synthase localizes to the cell surface. The cell surface expression of ATP synthase was analyzed by flow cytometry. Ecto-ATP synthase was identified in all of the cell lines tested, which included both normal and malignant cells (A549, adenocarcinomic human alveolar basal epithelial cells; HUVEC, human umbilical vein cells; HepG2, liver hepatocellular cells; L-02, human normal liver cells; 95-D, human highly metastatic lung cancer cell line; PC-12, rat pheochromocytoma cell line; CHO, Chinese hamster ovary cells). Of the cell lines tested, the A549 cells and the CHO cells exhibited the highest and the lowest expression, respectively, of the cell surface ATP synthase. The level of expression was not associated with the degree of malignancy.

RESULTS

ATP SYNTHASE IS EXPRESSED ON THE CELL SURFACES

As reported by many studies, ATP synthase localizes not only to the inner surface of the mitochondrial but also to the extracellular surface in both benign and malignant cells [Moser et al., 1999, 2001]. The presence of ATP synthase was detected on the cell surface of all of the cell lines tested by flow cytometry. The human lung adenocarcinoma A549 cell line displayed the highest extracellular ATP synthase expression (Fig. 1), whereas the Chinese Hamster Ovary (CHO) cell line had the lowest expression. Therefore, these two cells lines were selected for further study to contrast the effects of the cell surface ATP synthase.

ATP SYNTHASE IS ABLE TO SYNTHESIZE ATP ON THE CELL SURFACE AND IS INHIBITED BY OLIGOMYCIN AND ANTI-ATP SYNTHASE ANTIBODY

If the cell surface ATP synthase is homologous to the mitochondrial ATP synthase, these enzymes may have the same catalysis ability. The most important function of ATP synthase on the inner mitochondrial membrane is the production of almost all of the ATP that is required by the cell. Therefore, we tested the activity of the cell surface ATP synthase by evaluating its ablity to synthesize ATP from ADP. In this experiment, the ATP generation was measured 20 s after the addition of an ADP solution to the cells. In the absence of ADP treatment, the ATP generation was negligible, which suggests that the majority of the ATP measured could be attributed to the synthase activity. We found that oligomycin and Mc178-Ab inhibited the ability of ATP synthase to synthesize ATP. Moreover, in A549 cells, Mc178-Ab showed markedly greater inhibition of ATP synthesis compared with oligomycin and an isotype control mouse IgG. Furthermore, Mc178-Ab and oligomycin were less effective at reducing ATP synthesis in CHO cells (Fig. 2).

EFFECTS OF CELL SURFACE ATP SYNTHASE INHIBITORS ON CELL PROLIFERATION AND APOPTOSIS

To evaluate the effects of ATP synthase inhibition on the cells, we conducted a proliferation assay using A549, 95-D, and CHO cells that were treated with the ATP synthase β-subunit antibody Mc178-

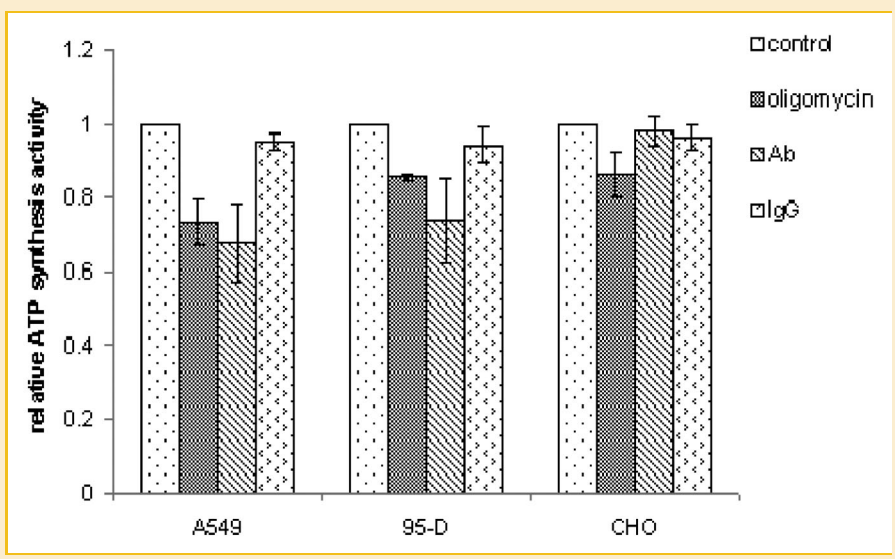

Fig. 2. ATP synthesis on the cell surface was inhibited by ATP synthase inhibitors. The generation of ATP on the surface of A549 cells was inhibited in the presence of 200 μg/ml anti-ATP synthase β subunit monoclonal antibody (Mc-178Ab) or 5 ng/ml oligomycin (positive control) compared with 200 μg/ml isotype mouse IgG (negative control). The inhibitory effects of the antibody treatment were most pronounced in the A549 cells, which had the highest cell surface ATP synthase expression among the cells tested.

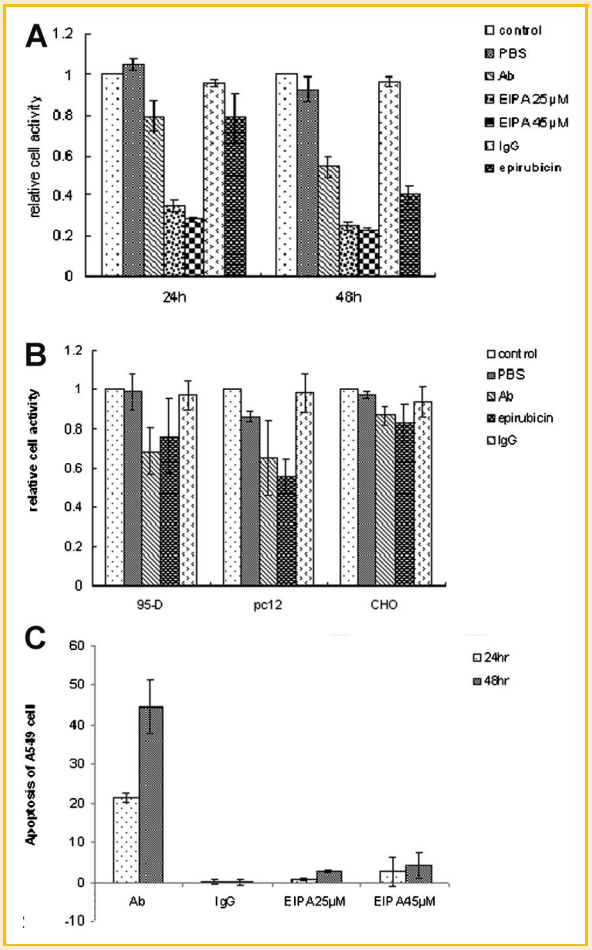

Fig. 3. ATP synthase inhibitors and EIPA inhibited cell proliferation and induced apoptosis. A: Cell proliferation was inhibited by Mc-178Ab and EIPA in A549 cells. The cell proliferation was measured using the MTT assay. A549 cells in the logarithmic growth phase were incubated with Mc178-Ab (200 μg/ml), epirubicin (5 μg/ml, positive control), IgG (200 μg/ml, isotype control), or EIPA (25 or 45 μM) for 24 and 48 h. B: Cell proliferation was inhibited by Mc-178Ab in 95-D, PC12 and CHO cells after 48 h. C: The apoptosis of A549 cells was induced by Mc-178Ab and EIPA. The apoptosis was determined by flow cytometry with annexin V-FITC and PI staining. Under acidic conditions, the apoptosis of A549 cells was significantly increased by treatment with 200 μg/ml Mc178-Ab compared with treatment with the isotype control IgG. A significantly lower degree of apoptosis was induced in the A549 cells by treatment with EIPA under the same conditions.

Ab. As shown in Figure 3, the cell proliferation was inhibited by the ATP synthase inhibitor, and this inhibition of the cell proliferation paralleled the expression of the cell surface ATP synthase. Upon exposure to Mc178-Ab, A549 cells, which exhibited the highest ecto-ATP synthase expression, showed a marked decrease in proliferation compared with the other cells tested. In contrast, CHO cells, which were found to have the lowest ecto-ATP synthase expression, exhibited the least proliferation inhibition in response to Mc178-Ab. Cell apoptosis assays were conducted to further explore this result. As shown in Figure 3c, a Mc178-Ab concentration of 200 μg/ml significantly induced apoptosis in A549 cells under acidic conditions. Compared with A549 cells, CHO cells treated with Mc178-Ab did not exhibit any obvious apoptosis (data not shown). The treatment with the isotype control mouse IgG did not induce apoptosis.

EXTRACELLULAR ATP WAS SLIGHTLY DECREASED BY ATP SYNTHASE INHIBITORS

Extracellular ATP has been reported to participate in cell proliferation and apoptosis depending on the concentration of ATP. The ATP generation by the cell surface ATP synthase was inhibited by the inhibitors treatments that were used in our study. The concentration of extracellular ATP was then tested. Resveratrol, an inhibitor of ATP synthase, was used as a positive control. The ATP concentrations in the supernatants of all the samples were low approximately 10 nM. The Extracellular ATP concentration was slightly decreased by treatment with the ATP synthase inhibitors, compared to pH6.7 control (Fig. 4A).

THE REGULATION OF INTRACELLULAR pH BY THE CELL SURFACE ATP SYNTHASE WAS DISRUPTED BY ATP SYNTHASE INHIBITORS

It is hypothesized that intracellular acidosis may contribute to cell proliferation inhibition and apoptosis. To investigate the mechanism through which ATP synthase inhibitors induced cell proliferation inhibition and apoptosis, we investigated whether the cell surface ATP synthase regulated intracellular pH. As shown in Figure 4A, the intracellular pH balance was disrupted by both oligomycin and Mc178-Ab. However, isotype IgG had no obvious effect on the intracellular pH. The intracellular pH of A549 cells was dropped by 0.5 pH units after a 2-h treatment with Mc178-Ab (Fig. 4B).

TREATMENT WITH THE H^+–Na^+ EXCHANGE INHIBITOR EIPA AS A MODEL OF LOW INTRACELLULAR pH

The above data, suggests that a decrease in the intracellular pH may contribute to the ATP synthase inhibitor-induced cell cytotoxicity. Thus, we constructed a model of low intracellular pH using EIPA. As shown in Figure 4B, the intracellular pH of A549 cells was down-regulated by treatment with EIPA (25 or 45 μM) to the levels achieved by treatment with Mc178-Ab. This down-regulation was rapid (observed at 2 h after treatment) and sustained.

A LOW INTRACELLULAR pH INDUCED THE INHIBITION OF A549 CELL PROLIFERATION BUT NOT APOPTOSIS

Cell proliferation and apoptosis assays were then performed to investigate the effects of a low intracellular pH. As shown in Figure 3, EIPA at a concentration of 25 or 45 μM induced the inhibition of cell proliferation, similarly to Mc178-Ab. However, although the EIPA treatment induced the same intracellular pH decrease that was induced by Mc178-Ab, EIPA did not induce any obvious cell apoptosis.

INTRACELLULAR Ca^{2+} WAS INCREASED BY LOW INTRACELLULAR pH

To determine whether the intracellular Ca^{2+} concentration in cells treated with cell surface ATP synthase inhibitors was disrupted, the intracellular Ca^{2+} concentration of A549 cells treated with Mc178-

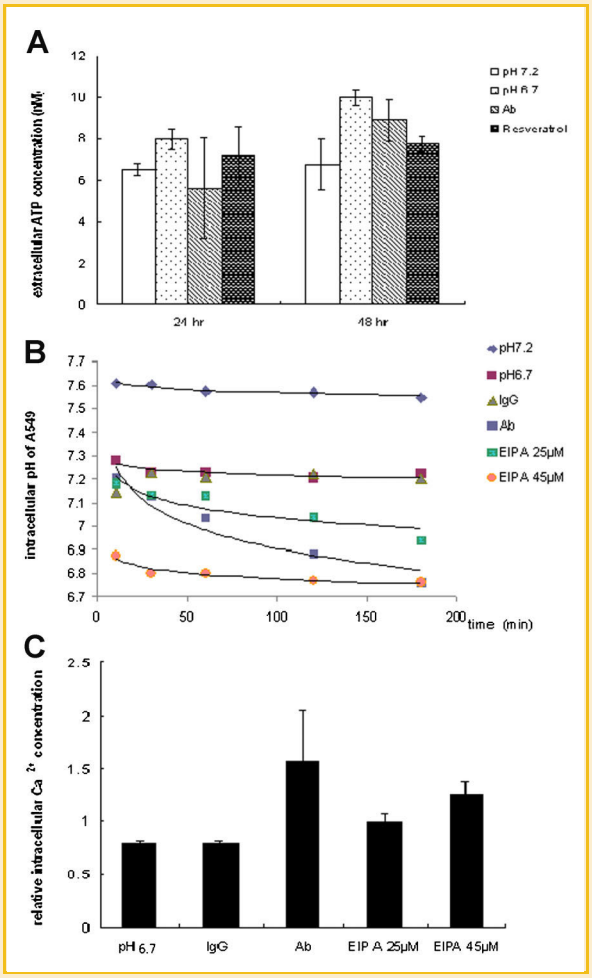

Fig. 4. The extracellular ATP concentration and intracellular pH and Ca^{2+} concentration as disrupted by an ATP synthase inhibitor and EIPA. A: Extracellular ATP in the supernatant of A549 cells that were treated with ATP synthase inhibitors for 24 or 48 h. Resveratrol, an inhibitor of ATP synthase, was used as a positive control. The concentrations of ATP in the supernatants of all of the samples were low (~10 nM). The extracellular ATP was slightly decreased by treatment with Mc178-Ab and resveratrol compared with the control at pH 6.7. B: The intracellular pH was measured through BCECF/AM luminescence spectrometry. A549 cells were incubated overnight at a cell density of 5,000 cells/well in a 96-well plate and then treated with Mc178-Ab (200 μg/ml, 5% FCS), IgG (200 μg/ml, isotype control), oligomycin (10 μg/ml, positive control), or EIPA (25 or 45 μM). The intracellular pH of the A549 cells was disrupted by both oligomycin and Mc178-Ab. The isotype IgG had no obvious effect on the intracellular pH. C: The concentration of intracellular Ca^{2+} was tested using the Fura2-AM fluorescent probe. The intracellular Ca^{2+} increased after treatment with the ATP synthase inhibitor or EIPA. The intracellular Ca^{2+} concentration of the A549 cells treated with ATP synthase inhibitor was higher than that in the cells treated with EIPA.

Ab and EIPA were tested. The concentration of intracellular Ca^{2+} was increased after treatment with the ATP synthase inhibitor or EIPA. Intracellular Ca^{2+} in A549 cells treated with ATP synthase was more than that the concentration measured in cells treated with EIPA.

THE PKC, MAPK, AND Akt SIGNALING ACTIVITIES WERE ALTERED BY Mc178-Ab AND EIPA TREATMENT

To further investigate the mechanism through which the intracellular pH decrease affects the cellular cytotoxicity, the molecular changes induced by Mc178-Ab and EIPA were characterized. A549 cells were treated and harvested at various time points. The cells that were treated with mouse IgG were used as isotype controls (data not shown). Both the ATP synthase inhibitor and EIPA induced the cell surface translocation of PKC. Compared with the control cells, the treatment with Mc178-Ab significantly increased the signal intensity of phosphorylated p38 and JNK and decreased the levels of phosphorylated ERK and Akt. The increase in the phosphorylation of p38 and JNK occurred rapidly (within 20 min after treatment). In comparison, EIPA significantly decreased the phosphorylated ERK and Akt levels, but did not induce p38 and JNK activation (Fig. 5).

DISCUSSION

Recent studies have highlighted that cell surface ATP synthases are expressed on normal and tumor cells and that these enzymes may be implicated in angiogenesis, lipoprotein metabolism, innate immunity, hypertension, the regulation of food intake, and other processes [Fu and Zhu, 2010; Terni et al., 2010; Pan et al., 2011; Vacirca et al., 2011]. However, the mechanism through which this synthase regulates the various physiological and pathophysiological programs, has not yet been elucidate. In this study, we demonstrate that the cell surface ATP synthases are expressed on the surfaces of cells in all lines tested, including normal and tumor cell lines. We also showed that an inhibitor to this catalytic synthase inhibits cell proliferation and induces cell apoptosis. This cytotoxicity was found to be associated with a decrease in the intracellular pH. Furthermore, the intracellular pH of A549 cells treated with EIPA, which is an inhibitor of the H^+/Na^+ exchanger, decreased to a level that was similar to that observed after Mc178-Ab treatment. Moreover, EIPA inhibited cell proliferation but did not induce apoptosis. Finally, the decrease in the intracellular pH induced the cell surface translocation of PKC. MAPK and Akt activation and/or inactivation were found to be associated with the cytotoxicity induced by the inhibition of the cell surface ATP synthase. However, only the downregulation of ERK and Akt contributed to the cell cytotoxicity that is induced by EIPA.

It was previously reported that angiostatin exhibited few side effects in a clinical trial [Kurup et al., 2006] and that the surface ATP synthase is the major receptor of angiostatin. Therefore, we hypothesized that the expression of the cell surface ATP synthase might be a feature of malignant cells and might be associated with cell malignancy. However, the cell surface ATP synthase was observed both in tumor cells, such as A549, HepG2, and 95-D cells, and in normal cells, such as HUVEC, L-02 and CHO cells. Moreover, the degree of expression of this synthase was not directly correlated with cell malignancy. Our results seem to conflict with the clinical trials of angiostatin. Angiostatin, which is an ATP synthase inhibitor, should bind, and thus be poisonous, to cells that express cell surface ATP synthase. However, angiostatin had few effects on

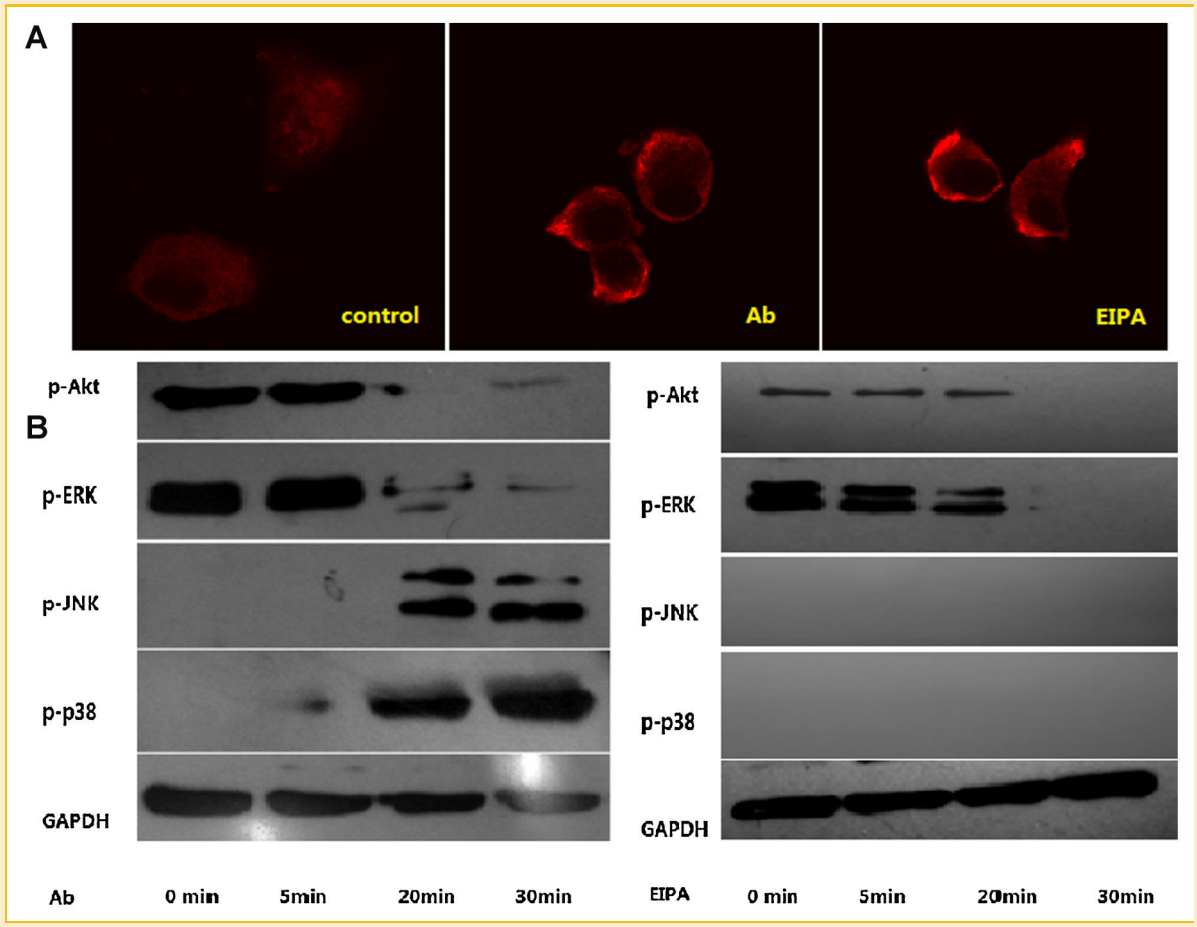

Fig. 5. The activities of the PKC, MAPKase, and Akt signaling proteins changed in response to Mc178-Ab and EIPA treatment. A: Representative immunofluorescence photomicrographs of control A549 cells (pH 6.7) show the presence of PKC in red. As shown, the treatment with the ATP synthase inhibitor induced the translocation of PKC to the membrane. B: Subconfluent A549 cells were treated with Mc178-Ab (200 μg/ml) or EIPA (45 μM) for 5, 20, or 30 min. The cells were then lysed, and the proteins were resolved by SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with the designated antibodies, and the immunodetection was performed using the Western-Light Chemiluminescent detection system (A, A549 cells treated with Mc-178Ab; B, A549 cells treated with EIPA).

normal cells; the only detected side effect was the development of new vascular endothelial cells in vivo. These data indicated that some other factors must affect the function of angiotensin in tumor cells. One of the differences between tumor and normal cells in vivo is their microenvironment. Normal cells are exposed to normal extracellular pH levels, whereas tumor cells may be exposed to acidosis, or hypoxia. This tumor environment, combined with ATP synthase inhibitors, may induce cell cytotoxicity.

The extracellular pH is very important for cell survival. Although the cell is able to self-regulate in response to a low or high external pH, the extracellular pH influences the cell metabolism and proliferation and can even induce apoptosis. Specifically, in an acidic environment, the cell must pump out an increased amount of H^+ to maintain the appropriate intracellular pH. The disruption of this balance induces cell apoptosis. Carbonic anhydrase inhibitors were recently proposed as antitumor agents because these proteins can significantly reduce cell proliferation and induce apoptosis by decreasing the intracellular pH [Laihia et al., 2010; Lelouvier and Puertollano, 2011]. Our study showed that the intracellular pH equilibrium was disrupted after treatment with ATP synthase inhibitors. This intracellular pH disruption was then investigated to determine whether it might contribute to the cytoxicity that was induced by the cell surface ATP synthase inhibitors.

EIPA, which is an inhibitor of the Na^+/H^+ antiporter, was used to construct a model of low intracellular pH. We demonstrated that intracellular pH was disrupted by EIPA in an acidic environment. Moreover, the decrease in intracellular pH that was induced by EIPA was similar to that induced by Mc178-Ab. Thus, the effect of the intracellular pH decrease was further investigated. Our data showed that the low intracellular pH induced by EIPA inhibited cell proliferation but did not promote apoptosis, in contrast to the effects induced by Mc178-Ab. These results suggest that the intracellular pH disruption may be associated with the inhibition of cell proliferation but not with apoptosis. Thus, some other mechanisms must contribute to the ATP synthase inhibitor induced-cytotoxicity.

In this study, we mostly focused on the downregulation of the intracellular pH that is induced by ATP synthase inhibitors. To

further investigate the role that the intracellular pH decrease plays in cell cytotoxicity, we evaluated the changes in the activity of several signaling proteins that occur in response to Mc178-Ab and EIPA treatment. A high concentration of intracellular H^+ will activate the Na^+/H^+ exchanger, which pumps Na^+ into the cell and H^+ out of the cell. A high cytoplasmic Na^+ concentration then activates the Na^+/Ca^{2+} exchanger, which pumps out intracellular Na^+ and pumps extracellular Ca^{2+} into the cell. Consequently, an increase in the intracellular Ca^{2+} concentration is indirectly elicited by a high concentration of intracellular H^+. An excessively elevated Ca^{2+} concentration triggers PKC translocation, which promotes cell apoptosis. As shown in our results, the intracellular Ca^{2+} concentration was elevated after treatment with the ATP synthase inhibitor; PKC was also translocated to the cell surface in response to this treatment.

The translocation of PKC has been reported to be downstream of nearly all of the membrane-associated signal transduction pathways, such as the MAPK and Akt pathways. The signaling proteins MAPKase and Akt are the focus of several recent tumor biology studies [Widenmaier et al., 2009; Hatzivassiliou et al., 2010; Kim and Choi, 2010; Li et al., 2010]. MAPKs, including ERK, JNK and p38 are closely associated with tumor cell proliferation, differentiation, motility, and death. The ERK signaling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, cytokines [Roberts and Der, 2007], and mediates cell proliferation. p38 MAPK is activated in response to a variety of cellular stresses and mediates cell apoptosis [Adachi et al., 2009; Hatzivassiliou et al., 2010]. JNK is potently activated by a variety of environmental stresses and mediates cell apoptosis [Lu et al., 2007]. Akt, also referred to as PKB, promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets [Cardone et al., 1998; Brunet et al., 1999].

We previously showed that the treatment with the ATP synthase inhibitor Mc178-Ab resulted in an increase in JNK and p38 phosphorylation and a decrease in ERK and Akt activities. To determine the effect of the Mc178-Ab-induced down-regulation of the intracellular pH on the activities of these cell signaling proteins, we examined these proteins in cells treated with EIPA (45 μM) by Western blot. EIPA-induced downregulation of the intracellular pH triggered the deactivation of ERK and Akt but did not increase the activation of p38 and JNK. These data are in agreement with previously reported results and show that the disruption of the intracellular pH inactivates ERK and Akt to inhibit cell proliferation in normal and tumor cells but does not affect cell apoptosis.

Another function of the cell surface ATP synthase is ATP generation and ATP hydrolysis, which depended on the electrochemical potential of protons across the cell membrane. An increasing number of studies have revealed that the extracellular ATP participated in a variety of physiological and pathological processes [Wu and Wu, 2008; Lee et al., 2011]. In particular, a high concentration of ATP was reported to induce cell apoptosis [Neary et al., 2003]. However, the concentration of extracellular ATP in our experiments did not reach this high pathological contraction. Cell surface ATP synthase has been found to localize to the caveolae [Moser et al., 2001]. Thus, the changes in the extracellular ATP concentration in the caveolar microenvironment cannot be ignored and may contribute to the mechanism which the ATP synthase inhibitor mediated cell cytotocity.

Interestingly, newborn vascular endothelial cells in vivo are exposed to a normal pH environment. These cells were reported to be sensitive to treatment with angiostatin in a clinical trial. We hypothesized that this finding may be the result of a high cell surface ATP synthase expression in the newborn vascular endothelial cells compared with other normal cells. The cell surface ATP synthase is necessary for cell survival under many conditions, not just in a low pH environment. This synthase is as important as the Na^+/H^+ antiporter in newborn vascular endothelial cells for the maintenance of the intracellular pH in a normal environment.

In conclusion, a disruption in the intracellular pH is only one of the mechanisms that underline the cytotoxicity induced by cell surface ATP synthase inhibitors during acidosis. Other factors, such as the extracellular ATP concentration in caveolae and hypoxia, may contribute to the mechanisms activated by cell surface ATP synthase-targeted cancer therapy.

REFERENCES

Adachi S, Shimizu M, Shirakami Y, Yamauchi J, Natsume H, Matsushima-Nishiwaki R, To S, Weinstein IB, Moriwaki H, Kozawa O. 2009. (−)-Epigallocatechin gallate downregulates EGF receptor via phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells. Carcinogenesis 30:1544–1552.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell (Cambridge, MA) 96:857–868.

Burwick NR, Wahl ML, Fang J, Zhong Z, Moser TL, Li B, Capaldi RA, Kenan DJ, Pizzo SV. 2005. An inhibitor of the F1 subunit of ATP synthase (IF1) modulates the activity of angiostatin on the endothelial cell surface. J Biol Chem 280:1740–1745.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282:1318–1321.

Chen YH, Huang YH, Wu HL, Wu MP, Chang WT, Kuo YZ, Lu KC, Wu LW. 2008. Angiostatin K1-3 induces E-selectin via AP1 and Ets1: A mediator for anti-angiogenic action of K1-3. J Thromb Haemost 6:1953–1961.

Chi SL, Pizzo SV. 2006. Angiostatin is directly cytotoxic to tumor cells at low extracellular pH: A mechanism dependent on cell surface-associated ATP synthase. Cancer Res 66:875–882.

Chi SL, Wahl ML, Mowery YM, Shan S, Mukhopadhyay S, Hilderbrand SC, Kenan DJ, Lipes BD, Johnson CE, Marusich MF, Capaldi RA, Dewhirst MW, Pizzo SV. 2007. Angiostatin-like activity of a monoclonal antibody to the catalytic subunit of F1F0 ATP synthase. Cancer Res 67:4716–4724.

Dass CR, Tran T, Choong PFM. 2007. Angiogenesis inhibitors and the need for anti-angiogenic therapeutics. J Dent Res 86:927–936.

Dong H, Fillingame RH. 2010. Chemical reactivities of cysteine substitutions in subunit a of ATP synthase define residues gating H+ transport from each side of the membrane. J Biol Chem 285:39811–39818.

Franck P, Petitipain N, Cherlet M, Dardennes M, Maachi F, Schutz B, Poisson L, Nabet P. 1996. Measurement of intracellular pH in cultured cells by flow cytometry with BCECF-AM. J Biotechnol 46:187–195.

Fu Y, Zhu Y. 2010. Ectopic ATP synthase in endothelial cells: A novel cardiovascular therapeutic target. Curr Pharm Des 16:4074–4079.

Hanford HA, Wong CA, Kassan H, Cundiff DL, Chandel N, Underwood S, Mitchell CA, Soff GA. 2003. Angiostatin4. 5-Mediated apoptosis of vascular endothelial cells. Cancer Res 63:4275–4280.

Harrison SM, Frampton JE, McCall E, Boyett MR, Orchard CH. 1992. Contraction and intracellular Ca^{2+}, Na^{+}, and H^{+} during acidosis in rat ventricular myocytes. Am J Physiol Cell Physiol 262:C348–C357.

Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJC, Stokoe D, Gloor SL, Vigers G. 2010. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464:431–435.

Kim EK, Choi EJ. 2010. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta (BBA)—Mol Basis Dis 1802:396–405.

Kim SW, Oleksyn DW, Rossi RM, Jordan CT, Sanz I, Chen L, Zhao J. 2008. Protein kinase C-associated kinase is required for NF-κB signaling and survival in diffuse large B-cell lymphoma cells. Blood 111:1644–1653.

Kurup A, Lin CW, Murry D, Dobrolecki L, Estes D, Yiannoutsos C, Mariano L, Sidor C, Hickey R, Hanna N. 2006. Recombinant human angiostatin (rhAngiostatin) in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer: A phase II study from Indiana University. Ann Oncol 17:97–103.

Laihia JK, Kallio JP, Taimen P, Kujari H, Kähäri VM, Leino L. 2010. Protodynamic intracellular acidification by cis-urocanic acid promotes apoptosis of melanoma cells in vitro and in vivo. J Invest Dermatol 130:2431–2439.

Lee JY, Karwatsky J, Ma L, Zha X. 2011. ABCA1 increases extracellular ATP to mediate cholesterol efflux to apoA-I. Am J Physiol Cell Physiol 301:C886–C894.

Lelouvier B, Puertollano R. 2011. Mucolipin-3 regulates luminal calcium, acidification, and membrane fusion in the endosomal pathway. J Biol Chem 286:9826–9832.

Li P, Jayarama S, Ganesh L, Mordi D, Carr R, Kanteti P, Hay N, Prabhakar BS. 2010. Akt-phosphorylated mitogen-activated kinase-activating death domain protein (MADD) inhibits TRAIL-induced apoptosis by blocking Fas-associated death domain (FADD) association with death receptor 4. J Biol Chem 285:22713–22722.

Lu GD, Shen HM, Chung MCM, Ong CN. 2007. Critical role of oxidative stress and sustained JNK activation in aloe-emodin-mediated apoptotic cell death in human hepatoma cells. Carcinogenesis 28:1937–1945.

Moser TL, Stack MS, Asplin I, Enghild JJ, Hojrup P, Everitt L, Hubchak S, Schnaper HW, Pizzo SV. 1999. Angiostatin binds ATP synthase on the surface of human endothelial cells. PNAS 96:2811–2816.

Moser TL, Kenan DJ, Ashley TA, Roy JA, Goodman MD, Misra UK, Cheek DJ, Pizzo SV. 2001. Endothelial cell surface F1-F0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin. PNAS 98:6656–6661.

Mowery Y, Pizzo S. 2008. Targeting cell surface F1F0 ATP synthase in cancer therapy. Cancer Biol Ther 7:1836–1838.

Neary JT, Kang Y, Willoughby KA, Ellis EF. 2003. Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP and P2 purinergic receptors. J Neurosci 23:2348–2356.

Oakes SG, Martin WJ, II, Lisek CA, Powis G. 1988. Incomplete hydrolysis of the calcium indicator precursor fura-2 pentaacetoxymethyl ester (fura-2 AM) by cells. Anal Biochem 169:159–166.

Pan J, Sun L, Tao Y, Zhou Z, Du X, Peng L, Feng X, Wang J, Li Y, Liu L, Wu S, Zhang Y, Hu S, Zhao W, Zhu X, Lou G, Ni J. 2011. ATP synthase ecto-α-subunit: A novel therapeutic target for breast cancer. J Transl Med 9:211.

Ping P, Zhang J, Huang S, Cao X, Tang XL, Li RCX, Zheng YT, Qiu Y, Clerk A, Sugden P. 1999. PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. Am J Physiol Heart Circ Physiol 277:H1771–H1785.

Pongracz J, Deacon EM, Johnson GD, Burnett D, Lord JM. 1996. Doppa induces cell death but not differentiation of U937 cells: Evidence for the involvement of PKC-βl in the regulation of apoptosis. Leukemia Res 20:319–326.

Roberts P, Der C. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26:3291–3310.

Saijo K, Mecklenbräuker I, Santana A, Leitger M, Schmedt C, Tarakhovsky A. 2002. Protein kinase C β controls nuclear factor κB activation in B cells through selective regulation of the IκB kinase α. J Exp Med 195:1647–1652.

Schelling JR, Jawdeh BGA. 2008. Regulation of cell survival by Na^{+}/H^{+} exchanger-1. Am J Physiol Renal Physiol 295:F625–F632.

Shampo M, Kyle R, Steensma D. 2011. Paul D. Boyer Nobel prize for work on ATP synthase. Mayo Clin Proc 86:e51.

Takahashi S, Shinya T, Sugiyama A. 2010. Angiostatin inhibition of vascular endothelial growth factor-stimulated nitric oxide production in endothelial cells. J Pharmacol Sci 112:432–437.

Tani M, Neely JR. 1989. Role of intracellular Na^{+} in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^{+}-Na^{+} and Na^{+}-Ca^{2+} exchange. Circ Res 65: 1045–1056.

Terni B, Boada J, Portero-Otin M, Pamplona R, Ferrer I. 2010. Mitochondrial ATP-synthase in the entorhinal cortex is a target of oxidative stress at stages I/II of Alzheimer's disease pathology. Brain Pathol 20:222–233.

Thangaraju M, Sharma K, Leber B, Andrews DW, Shen SH, Srikant CB. 1999. Regulation of acidification and apoptosis by SHP-1 and Bcl-2. J Biol Chem 274:29549–29557.

Usukura E, Suzuki T, Furuike S, Soga N, Saita E, Hisabori T, Kinosita K, Jr., Yoshida M. 2012. Torque generation and utilization in motor enzyme F0F1-ATP synthase. J Biol Chem 287:1884–1891.

Vacirca D, Barbati C, Scazzocchio B, Masella R, Rosano G, Malorni W, Ortona E. 2011. Anti-ATP synthase autoantibodies from patients with Alzheimer's disease reduce extracellular HDL level. J Alzheimer's Dis 26:441–445.

Vantourout P, Martinez LO, Fabre A, Collet X, Champagne E. 2008. Ecto-F1F0-ATPase and MHC-class I close association on cell membranes. Mol Immunol 45:485–492.

Wächter A, Bi Y, Dunn SD, Cain BD, Sielaff H, Wintermann F, Engelbrecht S, Junge W. 2011. Two rotary motors in F-ATP synthase are elastically coupled by a flexible rotor and a stiff stator stalk. Proc Natl Acad Sci 108:3924–3929.

Wahl M, Grant D. 2000. Effects of microenvironmental extracellular pH and extracellular matrix proteins on angiostatin's activity and on intracellular pH. Gen Pharmacol 35:277–285.

Widenmaier SB, Ao Z, Kim SJ, Warnock G, McIntosh CHS. 2009. Suppression of p38 MAPK and JNK via Akt-mediated inhibition of apoptosis signal-regulating kinase 1 constitutes a core component of the β-cell pro-survival effects of glucose-dependent insulinotropic polypeptide. J Biol Chem 284:30372–30382.

Wu SJ, Wu JY. 2008. Extracellular ATP-induced NO production and its dependence on membrane Ca2+ flux in Salvia miltiorrhiza hairy roots. J Exp Bot 59:4007–4016.

Xiao-yan Chi, Yan-jie Wang, Yue-li Hou. 2011. Effects of angiostatin on growth and apotosis of vascular endothelial cells in vitro. Lab Sci 2:040.

Yang L, Mei Y, Xie Q, Han X, Zhang F, Gu L, Zhang Y, Chen Y, Li G, Gao Z. 2008. Acidification induces Bax translocation to the mitochondria and promotes ultraviolet light-induced apoptosis. Cell Mol Biol Lett 13:119–129.

Zanke BW, Lee C, Arab S, Tannock IF. 1998. Death of tumor cells after intracellular acidification is dependent on stress-activated protein kinases (SAPK/JNK) pathway activation and cannot be inhibited by Bcl-2 expression or interleukin 1β-converting enzyme inhibition. Cancer Res 58:2801–2808.

Zhang X, Gao F, Yu L, Peng Y, Liu H, Liu J, Yin M, Ni J. 2008. Dual functions of a monoclonal antibody against cell surface F1F0 ATP synthase on both HUVEC and tumor cells. Acta Pharmacol Sin 29:942–950.

Zhu DM, Fang WH, Narla RK, Uckun FM. 1999. A requirement for protein kinase C inhibition for calcium-triggered apoptosis in acute lymphoblastic leukemia cells. Clin Cancer Res 5:355–360.