ATP Promotes Cell Survival Via Regulation of Cytosolic [Ca\textsuperscript{2+}] and Bcl-2/Bax Ratio in Lung Cancer Cells

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**Running Title:** ATP increases Bcl-2/Bax ratio by inducing sustained Ca\textsuperscript{2+} influx

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Abstract

Adenosine triphosphate (ATP) is a ubiquitous extracellular messenger elevated in the tumor microenvironment. ATP regulates cell functions by acting on purinergic receptors (P2X and P2Y) and activating a series of intracellular signaling pathways. We examined ATP-induced Ca\(^{2+}\) signaling and its effects on anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins in normal human airway epithelial cells and lung cancer cells. Lung cancer cells exhibited two phases (transient and plateau phases) of increase in cytosolic [Ca\(^{2+}\)] (\([\text{Ca}^{2+}]_{\text{cyt}}\)) caused by ATP, while only the transient phase was observed in normal cells. Removal of extracellular Ca\(^{2+}\) eliminated the plateau phase increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) in lung cancer cells, indicating that the plateau phase of \([\text{Ca}^{2+}]_{\text{cyt}}\) increase is due to Ca\(^{2+}\) influx. The distribution of P2X (P2X1-7) and P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) receptors was different between lung cancer cells and normal cells. Pro-apoptotic P2X7 was nearly undetectable in lung cancer cells, which may explain why lung cancer cells showed decreased cytotoxicity when treated with high concentration of ATP. The Bcl-2/Bax ratio was increased in lung cancer cells following treatment with ATP; however, the anti-apoptotic protein Bcl-2 demonstrated more sensitivity to ATP than pro-apoptotic protein Bax. Decreasing extracellular Ca\(^{2+}\) or chelating intracellular Ca\(^{2+}\) with BAPTA-AM significantly inhibited ATP-induced increase in Bcl-2/Bax ratio, indicating that a rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) through Ca\(^{2+}\) influx is the critical mediator for ATP-mediated increase in Bcl-2/Bax ratio. Therefore, despite high ATP levels in the tumor microenvironment, which would induce cell apoptosis in normal cells, the decreased P2X7 and elevated Bcl-2/Bax ratio in lung cancer cells may enable tumor cells to survive. Increasing the Bcl-2/Bax ratio by exposure to high extracellular ATP may, therefore, be an important selective pressure promoting transformation and cancer progression.

Keywords: Purinergic receptor; calcium signaling; Bcl-2; Bax; cell apoptosis; cell proliferation
Introduction

There is growing interest in the role of ATP in the development of cancer. ATP, well known as an intracellular molecular energy source, also functions as an extracellular messenger (2). ATP receptors are purinergic receptors (P2 receptors) and include the ligand-gated ion channel family of P2 receptors (P2X1-7) as well as the G protein-coupled receptor family of P2 receptors (P2Y1-2, P2Y4, P2Y6 and P2Y11-14) (3, 31). Activation of P2X receptors, which are nonselective cation channels formed by three homomeric or heteromeric P2X subunits, directly results in Na⁺ and Ca²⁺ influx through the cell plasma membrane, leading to membrane depolarization, which in turn activates voltage-gated Na⁺ and Ca²⁺ channels and causes the firing of action potentials. Activation of P2Y receptors, which are G protein-coupled receptors (GPCR), increases cytosolic free Ca²⁺ concentration ([Ca²⁺]_{cyt}) by inducing Ca²⁺ release from intracellular stores (e.g., sarcoplasmic or endoplasmic reticulum) and Ca²⁺ influx through store-operated (SOC) and/or receptor-operated (ROC) Ca²⁺ channels. P2X and P2Y signaling is not only responsible for inducing action potentials in excitable cells (e.g., neurons), but also have been implicated in cell proliferation, differentiation and apoptosis in non-excitable cells (e.g., epithelial cells) (5). In a lung cancer cell line, A549, extracellular ATP, UTP and UDP have been shown to stimulate proliferation that is dependent on the P2Y₂ and P2Y₆ receptors (44).

Extracellular or intercellular ATP concentration is reported to be very low (1-5 µM) in normal healthy tissues; however, it is significantly increased (to >100 µM) in the tumor microenvironment (26). The effect of increased extracellular ATP on cancer cell function is, however, dependent of the expression of different P2 receptors and changes in cytosolic [Ca²⁺]_{cyt} due to activation of the different P2 receptors. Recent observations imply that differential expression of P2X and P2Y receptors may determine the kinetically-distinct patterns of ATP-
mediated increases in \([\mathrm{Ca}^{2+}]_{\text{cyt}}\); the different patterns of \(\mathrm{Ca}^{2+}\) signaling may lead to differential effects on cancer cells (e.g., proliferation vs. apoptosis) (34). This underscores the necessity of further characterization of P2 receptors in tumor cells, which could lead to novel therapeutic strategies that target purinergic signaling in the treatment of cancer.

Intracellular \(\mathrm{Ca}^{2+}\) plays a critical role in the regulation of processes relevant to tumorigenesis, including cell proliferation, migration and apoptosis (1, 22, 34). The role of \(\mathrm{Ca}^{2+}\) channels and \(\mathrm{Ca}^{2+}\) pumps in tumorigenesis is achieved through altered global or local changes in \([\mathrm{Ca}^{2+}]_{\text{cyt}}\). The remodeling of \(\mathrm{Ca}^{2+}\) signals in cancer cells is due potentially to aberrant expression of different \(\mathrm{Ca}^{2+}\) channels, \(\mathrm{Ca}^{2+}\) pumps and \(\mathrm{Ca}^{2+}\) transporters (22, 30). For example, it has been shown that P2Y2 and P2Y4 receptor expression in A549 cells promotes an increase in \([\mathrm{Ca}^{2+}]_{\text{cyt}}\) and induces \(\mathrm{Ca}^{2+}\)-dependent release of ATP and UTP as part of a positive feedback loop (4).

The Bcl-2 protein family consists of both anti-apoptotic and pro-apoptotic members, which are traditionally considered to reside in or translocate to mitochondria and function as apoptotic regulators by modulating mitochondrial membrane permeability. The ratio between anti- and pro-apoptotic Bcl-2 proteins determines whether cells survive or die (13). The Bcl-2 families of proteins have also been detected in other subcellular compartments including the endoplasmic reticulum, nuclear membrane, plasma membrane and within the cytosol. Furthermore, the Bcl-2 family of proteins have been implicated in regulating \([\mathrm{Ca}^{2+}]\) homeostasis in these cellular compartments (15, 28, 41), while changes in \([\mathrm{Ca}^{2+}]_{\text{cyt}}\) also exert effect on the expression of the anti- or pro-apoptotic Bcl-2 proteins. The Bcl-2 family of proteins also functions to relay \(\mathrm{Ca}^{2+}\) signals to regulate cell apoptosis and proliferation. We hypothesize that there is a feedback loop in which \(\mathrm{Ca}^{2+}\) signaling regulates the expression of Bcl-2 family members resulting in an increase of the Bcl-2/Bax ratio.
In this report, we show that extracellular ATP induces only a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal lung epithelial cells; however, ATP induces a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ followed by a plateau phase (or a slowly-declining phase) increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ in lung cancer cells (H23 and A549) that is dependent on extracellular $\text{Ca}^{2+}$. Furthermore, extracellular treatment with ATP increases the ratio of the anti-apoptotic protein, Bcl-2, to the pro-apoptotic protein, Bax (Bcl-2/Bax ratio) in lung cancer cells, but not in normal lung epithelial cells. We also demonstrate that chelation of intracellular $\text{Ca}^{2+}$ (with BAPTA-AM) or removal of extracellular $\text{Ca}^{2+}$ inhibits the ATP-induced increase of Bcl-2/Bax ratio in lung cancer cells. These data imply that extracellular ATP, by selectively inducing a sustained or long-lasting increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in lung cancer cells (but not in normal lung epithelial cells), promotes cell survival and enhances lung tumor growth through increase of Bcl-2/Bax ratio. Inhibiting ATP-induced $\text{Ca}^{2+}$ influx in lung cancer cells may lead to novel therapeutic approaches for lung cancer.
Materials and Methods

Chemicals and Drugs. Adenosine 5-triphosphate disodium salt hydrate (ATP), Uridine 5-triphosphate trisodium salt hydrate (UTP), α,β-Methylene adenosine 5-triphosphate lithium salt (α,β−meATP), β,γ-Methyleneadenosine 5-triphosphate disodium salt (D-β,γ- meATP) and TNP-ATP hydrate were prepared as concentrated stock solutions in distilled water. U-73122 hydrate, BAPTA-AM and cyclopiazonic acid (CPA) were prepared as concentrated stock solutions in dimethyl sulfoxide (DMSO). All stock solutions (in water, DMSO) were aliquoted and kept frozen at -20ºC until use. On the day of experiments, aliquots of the stock solutions were diluted in HEPES-buffered bath solution to the final concentrations for each drug. The pH values of all solutions were measured after addition of the drugs and adjusted to 7.4. All drugs were from Sigma Chemical, unless otherwise indicated.

Cell Culture. Human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell line BEAS-2B (American Type Culture Collection, Manassas, VA) were cultured in DMEM medium (Corning, Cellgro, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were routinely cultured in 20% O2 and 5% CO2 at 37 °C.

Measurement of [Ca2+]cyt. Human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell line were grown at 50%-60% confluence on 25-mm diameter circular glass cover slips. Cells were loaded for 60 minutes in room temperature (23°C) with 4 μM fura-2 acetoxymethyl ester (fura-2/AM; Invitrogen/Molecular Probes, Eugene, OR) in HEPES-buffered solution. Cover slips with cells were placed into the recording chamber of an
inverted fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (S Plan Fluor 20×/0.45 ELWD; Nikon), an EM-CC camera (Evolve; Photometrics, Tucson, AZ) and the NIS Elements 3.2 software (Nikon), and superfused with HEPES-buffered solution for 30 min to wash out residual extracellular fura-2/AM and allow sufficient time for intracellular esterase to convert fura-2/AM to fura-2. With excitation at 340 and 380 nm, the emitted fluorescence was acquired at 520 nm by a fluorescent objective lens and an EM-CCD camera using NIS Elements 3.2 software. Intracellular Ca\(^{2+}\) concentration is expressed as 340/380 fluorescence ratio within an area of interest in a cell recorded every 2 seconds. The HEPES-buffered solution contained (in mM) 137 NaCl, 5.9 KCl, 1.8 CaCl\(_2\), 1.2 MgCl\(_2\), 14 glucose, and 10 HEPES (pH was adjusted to 7.4 with 10 N NaOH). The Ca\(^{2+}\)-free solution was prepared by replacing 1.8 mM CaCl\(_2\) with equimolar MgCl\(_2\) and adding 1 mM EGTA to chelate residual Ca\(^{2+}\). Measurement of [Ca\(^{2+}\)]\(_{cyt}\) was carried out at room temperature (23°C). For each experimental replicate we quantitated the fluorescence ratios for at least 20 cells.

**Western Blotting.** Total protein was isolated from human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell lines that were lysed in 1× RIPA buffer (BioRad, Hercules, CA) at 4°C. Protein was loaded on a 15% acrylamide gel, transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA), and immunoblotted with anti-Bcl-2 and (SAB4500003; 1:1000; Sigma-Aldrich) and anti-Bax monoclonal antibody (B3428; 1:4000; Sigma-Aldrich). Signals were detected using a Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA). The protein levels were normalized to β-actin (sc-9104, 1:1000; Santa Cruz Biotechnology) and expressed in arbitrary units.
**RT-PCR and Real-Time RT-PCR.** Total RNA was isolated from human non-small cell lung cancer cell line A549 and H23 and human airway epithelial cell line by the TRIzol method. The extracted RNA was quantified by spectrophotometry at 260 nm. The synthesis and polymerase chain reaction were carried out using a Platinum PCR SuperMix (Invitrogen, Grand Island, NY) with 3-step cycling. The relative expression levels of P2X and P2Y receptors were normalized against the amount of GAPDH mRNA in the same RNA extract. Quantitative real-time RT-PCR was performed on a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad) using an iTaq™ Universal SYBR Green Supermix (Bio-Rad) following the instructions of the manufacturer. All samples were run in triplicate, and GAPDH was used as an internal control. The expression level of P2YR mRNA or P2XR mRNA was normalized to GAPDH and was expressed as a ratio relative to GAPDH.

**Cell Viability Assay.** Viability of human non-small cell lung cancer cell line A549 and H23 and normal human airway epithelial cell line after treatments with DMEM with 10% FBS, 100μM ATP in DMEM with10% FBS, and 1mM ATP DMEM with10% FBS, was determined by the Trypan blue dye exclusion assay. Control and treated cells were incubated with Trypan blue solution for 3-5 minutes and then counted on Countess (Invitrogen, Grand Island, NY). Cell numbers per milliliter cell suspension were determined in each preparation and the percentage of cells that excluded Trypan blue stain was indicated as a measure of cell viability, which is expressed in percentage (%).

**Statistical Analysis.** Pooled data are shown as the mean ± S.E. The statistical significance between two groups was determined by Student’s t-test. The statistical significance among
groups was determined by Scheffé’s test after one-way analysis of variance. Significant
difference is expressed in the figures or figure legends as *$P<0.05$ or #$P<0.05$. 


Results

Extracellular ATP induces a prolonged $[\text{Ca}^{2+}]_{\text{cyt}}$ response in lung cancer cells but not in normal lung epithelial cells

Extracellular ATP increases $[\text{Ca}^{2+}]_{\text{cyt}}$ through activation of different P2 receptors. To determine if extracellular application of ATP induces different patterns of intracellular $\text{Ca}^{2+}$ signaling in normal and lung cancer cells, we treated cells with 100 $\mu$M of ATP and measured $[\text{Ca}^{2+}]_{\text{cyt}}$ using the $\text{Ca}^{2+}$ indicator fura-2. We compared a normal lung airway epithelial cell line (BEAS-2B) to two lung cancer cell lines (H23 and A549) that reflect the heterogeneity of human lung cancer (see Table 1 for details). Two different patterns ATP-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ were observed in normal and lung cancer cells: a transient increase or slowly-declining increase (Fig. 1A-C). ATP induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal cells with a mean duration of 120 seconds (Fig. 1A-C). In lung cancer cells, ATP induced the same rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, however, the $[\text{Ca}^{2+}]_{\text{cyt}}$ rise that followed transient increase was sustained for a longer period of time (a plateau increase) in H23 (mean duration of 660 seconds) and A549 cells (mean duration of 700 seconds). While 95% and 99% of H23 and A549 lung cancer cells responded to ATP, only 30% of normal cells responded (Fig. 1D). Of the responding cells, we observed three patterns: a) Pattern 1, a transient increase followed by a plateau increase; b) Pattern 2, a transient increase followed by a slowly-declining increase; and c) Pattern 3, only a transient increase (Fig. 1B). Pattern 3 represents the highest proportion of normal responding cells (78%) (Fig. 1E). In contrast, Pattern 1 represents the highest proportion of responding lung cancer cells (H23, 70%; A549, 55%) (Fig. 1E). The $[\text{Ca}^{2+}]_{\text{cyt}}$ increase during the plateau phase was higher in A549 cells (mean of 0.06 (ratio 340/380)) as compared to H23 cells (mean of 0.18 (ratio of 340/380)). The results demonstrate that lung cancer cells respond to extracellular ATP with a prolonged increase...
in \([\text{Ca}^{2+}]_{\text{cyt}}\). Since the ATP-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) is mainly caused by activating different P2 receptors, the ATP-induced plateau phase of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in lung cancer cells (but not in normal cells) suggests that ATP receptors (P2X or P2Y receptors) may be expressed differently in lung cancer cells versus normal cells.

In the next set of experiments, we performed RT-PCR and real-time RT-PCR to examine and compare the relative expression levels of P2X receptors (P2X2, P2X3, P2X4, P2X5, P2X6, P2X7) and P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) in normal and lung cancer cells. We observed a statistically significant (\(P<0.05\)) increase in expression of several of the P2X receptors and the P2Y receptors in lung cancer cells as compared to normal cells (P2X3: H23=2.05-fold, A549=3.02-fold; P2X4: H23=4.05-fold, A549=4.08-fold; P2X5: H23=11.00-fold, A549=3.45-fold. P2Y1: H23=4.55-fold, A549=2.37-fold; P2Y2: H23=3.44-fold, A549=14.53-fold; P2Y4: H23=7.12-fold, A549=4.18-fold; P2Y6: H23=12.64-fold, A549=24.84-fold) (Fig. 1F-I). Our findings suggest that the prolonged increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ATP in lung cancer cells may be due to upregulated expression of P2 receptors.

**Lung cancer cells are more sensitive to extracellular ATP than normal cells**

To determine if the pattern of ATP-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) is concentration-dependent, we treated normal (BEAS-2B) and lung cancer cells (H23 and A549) with different concentrations of ATP. In lung cancer cells, ATP induced an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) starting at 1 \(\mu\text{M}\); however, ATP induced an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) that was detectable at a higher concentration of 10 \(\mu\text{M}\) in normal cells (Fig. 2A). Both H23 and A549 cells that respond to ATP have a plateau phase of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) when treated with higher concentrations of ATP (100 \(\mu\text{M}\)). While H23 cells also respond to lower concentrations of ATP (1 \(\mu\text{M}\) and 10 \(\mu\text{M}\)) with a plateau phase
of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, A549 cells respond to the lower concentrations of ATP with $\text{Ca}^{2+}$ oscillations. To determine the total amount of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ over time, we calculated the area under the curve (AUC). Lung cancer cells exhibited a dose-dependent increase in the AUC starting at 10 $\mu$M when compared to normal cells ($P<0.05$) (Fig. 2B). We observed that the transient phase of increase (induced by ATP starting at 1 $\mu$M) and plateau phase of increase (induced by ATP starting at 10 $\mu$M) in $[\text{Ca}^{2+}]_{\text{cyt}}$ were significantly higher in H23 and A549 cells than in normal cells (Fig. 2C-D). Furthermore, we observed that in all cell types the amplitude of the transient increase (Fig. 2C) and the amplitude of the plateau increase (Fig. 2D) maximized at the ATP concentration of $10^{-6}$ to $10^{-5}$ M. Further increasing ATP concentration from $10^{-5}$ M to $10^{-3}$ M did not further increase the amplitude of both transient and plateau increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 2C-D). These observations suggest that lung cancer cells are more sensitive to extracellular ATP, and that the downstream intracellular $\text{Ca}^{2+}$ signaling may be of higher intensity with a more prolonged duration in lung cancer cells than in normal cells.

Ca$^{2+}$ influx is required for ATP-induced plateau phase of increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ in lung cancer cells

It is known that ATP can increase $[\text{Ca}^{2+}]_{\text{cyt}}$ by inducing $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores and/or $\text{Ca}^{2+}$ influx from extracellular source via the GPCR P2Y receptors. To determine if the observed ATP-induced plateau phase of increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ is dependent on extracellular $\text{Ca}^{2+}$, we treated cells with ATP in the absence of extracellular $\text{Ca}^{2+}$. In the absence of extracellular $\text{Ca}^{2+}$, only 5% of the normal cells (BEAS-2B) responded to ATP with a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 3A-C). In H23 cells, ATP induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in 95% of the cells, however, the plateau phase increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ was no longer observed (Fig. 3A-C).
In A549 cells, ATP induced either transient increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) or \(\text{Ca}^{2+}\) oscillations in 97% of the cells and, similar to H23 cells, the plateau phase of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) was no longer observed (Fig. 3A-C). Extracellular ATP induced transient increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in both H23 and A549 cells superfused either with \(\text{Ca}^{2+}\)-containing (99% of H23 and A549 cells) solution or with \(\text{Ca}^{2+}\)-free solution (95% of H23 and A549 cells) (Fig. 3C). These data suggest that normal cells require extracellular \(\text{Ca}^{2+}\) for ATP to induce a transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\). In contrast, cancer cells do not require extracellular \(\text{Ca}^{2+}\) for ATP to induce a transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\). Our results also suggest that \(\text{Ca}^{2+}\) influx from extracellular source is responsible for the ATP-induced plateau phase of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) observed in lung cancer cells.

Next, we wanted to investigate the potential mechanism that mediates \(\text{Ca}^{2+}\) influx in lung cancer cells (H23 an A549). Upon activation of P2Y receptors, ATP may mobilize \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) stores by IP3 and activation of the IP3 receptor (a \(\text{Ca}^{2+}\) release channel) on the sarcoplasmic (SR) or endoplasmic (ER) reticulum membrane. To determine if depletion or partial depletion of intracellular \(\text{Ca}^{2+}\) stores (SR) by ATP could induce store-operated \(\text{Ca}^{2+}\) entry (SOCE), we first treated cells with ATP in \(\text{Ca}^{2+}\)-free solution, and observed a transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) (due apparently to \(\text{Ca}^{2+}\) leak from the intracellular stores) in both normal and lung cancer cells. We then superfused the cells with 1.8 mM \(\text{Ca}^{2+}\)-containing solution and observed that restoration of extracellular \(\text{Ca}^{2+}\) induced a second increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) which was due apparently to \(\text{Ca}^{2+}\) influx through store-operated \(\text{Ca}^{2+}\) channels (Fig. 4A). In both normal and lung cancer cells, the second increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ATP-mediated SOCE showed no statistically significant differences [0.08±0.010 (340/380 ratio) for normal cells; 0.08±0.003 for H23 cells; and 0.11±0.01 for A549 cells] (Fig. 4B)(19, 44). Next, we treated cells with cyclopiazonic acid (CPA), a reversible inhibitor of the SR/ER \(\text{Ca}^{2+}\)-pump (SERCA), passively
depletes Ca$^{2+}$ from the SR and induces SOCE. We observed that the second increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to extracellular Ca$^{2+}$ restoration-mediated SOCE was significantly lower in H23 cells (0.10±0.004) than in normal cells (0.45 ± 0.022, $P<0.05$); however, we did not observe a significant difference in A549 cells (0.40±0.010) compared with normal cells (Fig. 4C, G). When ATP was then introduced to the cells in the presence of extracellular Ca$^{2+}$, we observed that the second increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was significantly higher than that in the presence of extracellular Ca$^{2+}$ without ATP in both normal and lung cancer cells (0.51±0.010 for normal cell; 0.13±0.003 for H23 cells; 0.47±0.008 for A549 cells; $P<0.05$ vs. the value in the absence of ATP) (Fig. 4D, F and G).

To determine if P2X receptors are involved, we pretreated cells with TNP-ATP (P2X receptor inhibitor). TNP-ATP treatment significantly attenuated the amplitude of the second increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by extracellular Ca$^{2+}$ restoration with ATP in both normal and lung cancer cells (0.46±0.012 for normal cell; 0.01±0.003 for H23 cells; 0.44±0.0047 for A549 cell; $P<0.05$ vs. the value in the absence of TNP-ATP) (Fig. 4E, F and G). Taken together, these data suggest that ATP-induced Ca$^{2+}$ influx is at least partially mediated through store-operated Ca$^{2+}$ channels (SOCC) and P2X receptors.

**P2X and P2Y receptors are both required for ATP-induced increases in [Ca$^{2+}]_{\text{cyt}}$ in lung cancer cells**

Activation of P2X and P2Y receptors is known to mediate changes in intracellular Ca$^{2+}$ through different mechanisms. For example, activation of P2X receptors, nonselective cation channels, directly mediate Ca$^{2+}$ influx through the receptors, while activation of P2Y receptors, GPCRs, mediates Ca$^{2+}$ influx through receptor-operated and store-operated Ca$^{2+}$ channels and/or...
Ca\(^{2+}\) mobilization from the SR/ER as a result of G protein-mediated increases in and diacylglycerol (DAG) and inositol trisphosphate (IP\(_3\)). To determine if P2X receptors are responsible for the ATP-induced plateau phase of [Ca\(^{2+}\)]\(_{cyt}\) increase in lung cancer cells (H23 and A549), we treated cells with the P2X receptor inhibitors PPADS and TNP-ATP (14, 16). We observed that both PPADS and TNP-ATP decreased the ATP-induced transient phase of [Ca\(^{2+}\)]\(_{cyt}\) increase in normal cells and the ATP-induced transient and plateau phases of [Ca\(^{2+}\)]\(_{cyt}\) increases in lung cancer cells (Fig. 5A). To determine if P2X receptor activation can directly increase [Ca\(^{2+}\)]\(_{cyt}\), by Ca\(^{2+}\) influx through the receptors, we treated cells with P2X receptor activators βγ-meATP and αβ-meATP (11, 23). We observed that αβ-meATP induced small increase in [Ca\(^{2+}\)]\(_{cyt}\) in both normal and lung cancer cells; while, βγ-meATP only induced a small increase in [Ca\(^{2+}\)]\(_{cyt}\) in A549 cells but not in normal cells and H23 cells (Fig. 5B).

It is known that P2Y receptors are G protein-coupled receptors that can activate phospholipase C (PLC) to increase [Ca\(^{2+}\)]\(_{cyt}\) via increasing inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG). U73122, a PLC inhibitor, attenuated ATP-induced transient phase of [Ca\(^{2+}\)]\(_{cyt}\) increase in both normal and lung cancer cells. Only a small increase in [Ca\(^{2+}\)]\(_{cyt}\) was induced by ATP in H23 and A549 cells, but not in normal cells, after U73122 treatment (Fig. 5C) (42).

We next examined if the P2Y receptor activator UTP can increase [Ca\(^{2+}\)]\(_{cyt}\) (35). We observed that only transient or oscillatory increases of [Ca\(^{2+}\)]\(_{cyt}\) (without the plateau phase) were observed in normal and lung cancer cells in response to UTP (Fig. 5C). These observations suggest that both P2X and P2Y receptors are involved in the ATP-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) in normal and lung cancer cells, of which P2X receptors are involved in Ca\(^{2+}\) influx from extracellular source and P2Y receptors are involved in Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores.
The specific subtypes of P2X receptors or P2Y receptors involved cannot be discriminated due to the non-specificity of the inhibitors and activators. Future studies are needed to identify the specific subunits of P2X and P2Y receptors involved in ATP-mediated increases in \([\text{Ca}^{2+}]_{\text{cyt}}\).

**Lung cancer cells have a decreased cytotoxic response to extracellular ATP compared to normal cells**

RT-PCR data have previously shown that the expression level of P2X7 mRNA was very low in lung cancer cells (H23 and A549) as compared to normal cells (BEAS-2B) (20, 21, 39). The P2X7 receptor is known to be involved in non-Bcl-2/Bax mediated cell apoptosis (29, 44).

The loss of P2X7 in lung cancer cells is surprising, given that numerous studies have reported that ATP can exert cytotoxic effect on several tumor cell types (6, 12, 33, 43). Therefore, we investigated if extracellular ATP has a differential effect on cell growth processes (including cell apoptosis and cell proliferation) in normal cells versus lung cancer cells.

There was no effect of 100 μM ATP on cell viability in both normal and lung cancer cells in comparison to their control during ATP stimulation at 24, 48, and 72 hour, respectively (Fig. 6A). However, a decrease in cell viability was observed when normal cells were treated with 1 mM ATP for 48 hours (75.33% ±9.23 vs. control of 89.25% ±3.74, \(P<0.05\)) and 72 hours (73.16% ±5.23 vs. control of 90.23% ±3.61, \(P<0.05\)). Decreased cell viability was also observed in H23 lung cancer cells at 72 hour (65.00% ±6.61 vs. control of 81.00% ±3.88, \(P<0.05\)) with 1 mM ATP, while no change of cell viability was observed in A549 lung cancer cells (Fig. 6A).

We next examined the ability of ATP to affect cell proliferation. Cell proliferation was inhibited in normal cells treated with 100 μM ATP for at 72 hours \((4.25\times10^6 ± 0.45 \text{ vs. control of } 7.22\times10^6 ± 2.00, P<0.05)\) and with 1 mM ATP for 48 hours \((3.06\times10^6 ± 0.81 \text{ vs. control of }\)
5.69×10^6 ± 0.89, \( P<0.05 \)) and 72 hours (3.06×10^6 ± 1.03 vs. control of 7.22×10^6 ± 2.00, \( P<0.05 \)) (Fig. 6B). There was also a small decrease in cell proliferation in lung cancer cells at 72 hour when cells were treated with 100 \( \mu \)M ATP (1.03×10^6 ± 0.59 vs. control of 1.74×10^6 ± 0.50 for H23 cells; 4.08×10^6 ± 0.88 vs. control of 5.82×10^6 ± 0.97 for A549 cells, \( P<0.05 \)). However, no change in cell proliferation was observed in lung cancer cells when treated with 1mM ATP (Fig. 6B).

These data indicate that relatively low concentration of ATP (micromole) negatively modulates cell proliferation but not cell viability in lung cancer cells. Higher concentration of ATP (in the millimolar range), however, do not affect cell proliferation in lung cancer cells, but induce decreased cell viability in H23 cells. In normal cells, in which P2X7 receptors are expressed at a higher level, we observed consistent effects of ATP on cell viability and cell proliferation. Future studies are needed to determine if the absence of P2X7 uncouples cell viability and proliferation in lung cancer cells.

**ATP increases the Bcl-2/Bax ratio in lung cancer cells**

Since the lung cancer cells (H23 and A549) express low levels of the P2X7 receptor, we considered whether ATP could modulate cell apoptosis by other means. Anti-apoptotic Bcl-2 is well known for its classic role in blocking cytochrome \( c \) release from mitochondria and thereby inhibiting apoptosis; its expression is upregulated in many tumor cells (9, 10). Bax, a member of the Bcl-2 family of proteins, has a pro-apoptotic role that opposes Bcl-2; as such, high Bcl-2/Bax ratio represents an anti-apoptotic cell state. Therefore we examined the expression levels of both Bcl-2 and Bax proteins to determine if extracellular ATP affects Bcl-2/Bax ratio in normal and lung cancer cells.
The cells were first treated with vehicle (water) for 0, 2, 4, 8, 16, 24 hours; Bcl-2 and Bax proteins expression levels, as well as Bcl-2/Bax ratio, showed no change in both normal and lung cancer cells (Fig. 7A, D and E). We observed that Bcl-2 protein expression level was higher in basal condition (0 hour) in lung cancer cells than in normal cells, while the Bax protein expression level showed no difference between normal and lung cancer cells (Fig. A, D). We next treated cells with ATP (100 µM and 1 mM) for 0, 2, 4, 8, 16, 24 hours. In normal cells, both Bcl-2 and Bax proteins expression levels, as well as Bcl-2/Bax ratio, were not changed following treatment with ATP (100 µM and 1 mM) (Fig. 7B, C, F, G, H and I). In lung cancer cells (H23 and A549), Bcl-2 protein expression was upregulated following treatment with ATP (100 µM and 1 mM) from 2 hours to 24 hours, while Bax protein level was negligibly changed or slightly (but not significantly) increased (Fig. 7B, C, F, H). The Bcl-2/Bax ratio was significantly increased following treatment of lung cancer cells (H23 and A549) with ATP (100 µM and 1 mM) (Fig. 7G, I). We observed that Bcl-2, rather than Bax, was more sensitive to ATP in lung cancer cells H23 and A549. In normal cells, because of the low expression level of Bcl-2 protein, the Bcl-2/Bax ratio was much lower and less affected by ATP than in lung cancer cells. However, consistent with the decreased viability observed in normal cells, Bax protein expression was upregulated in these cells following treatment with ATP. These data suggest that an ATP-induced increase of the Bcl-2/Bax ratio in lung cancer cells may be the mechanism by which lung cancer cells escape from ATP-induced cytotoxic effects.

**Intracellular Ca^{2+} is required for extracellular ATP to increase Bcl-2/Bax ratio in lung cancer cells**
To understand the potential mechanism by which ATP increases Bcl-2/Bax ratio, we focused on ATP-induced [Ca$^{2+}$]$_{cyt}$ increase, which is one of the major downstream messengers of P2 receptor (P2X and P2Y) activation. Cells were treated with BAPTA-AM, a membrane-permeable Ca$^{2+}$ chelator, for 30 min before treatment with ATP (100 μM and 1mM) to determine if intracellular Ca$^{2+}$ is required for regulation of Bcl-2 and Bax proteins expression (37). BAPTA-AM pre-treatment blocked the ATP-induced increase of [Ca$^{2+}$]$_{cyt}$ in both normal and lung cancer cells (H23 and A549) (Fig. 8A, B). We also treated cells with ATP (100 μM and 1mM) for 0, 2, 4, 8, 16, 24 hours, respectively, after BAPTA-AM treatment to determine if intracellular Ca$^{2+}$ is required for ATP-induced increase in Bcl-2/Bax ratio. In both normal and lung cancer cells, chelation of intracellular Ca$^{2+}$ with BAPTA-AM preclude the ATP-induced changes in Bcl-2 and Bax protein expression and Bcl-2/Bax ratio (Fig. 8C-H). These data suggest that an increase of intracellular Ca$^{2+}$ is required for extracellular ATP to increase Bcl-2/Bax ratio in lung cancer cells.

**Ca$^{2+}$ influx is required for ATP-induced increase of Bcl-2/Bax ratio in lung cancer cells**

Because ATP induces two phases of increase in [Ca$^{2+}$]$_{cyt}$ in lung cancer cells (H23 and A549), the transient phase (due to Ca$^{2+}$ release) and the plateau phase (due to Ca$^{2+}$ influx), we investigated which phase is responsible for the ATP-induced increase of Bcl-2/Bax ratio. To determine the role of Ca$^{2+}$ influx, we decreased the extracellular Ca$^{2+}$ concentration in the media to 80 nM to inhibit Ca$^{2+}$ influx while avoiding cell death. Under these conditions, we observed that ATP (100 μM and 1mM) induced only transient or oscillatory increases in [Ca$^{2+}$]$_{cyt}$ without a plateau phase in both normal and lung cancer cells (Fig. 9A, B). We also treated cells with ATP (100 μM and 1mM) for 0, 2, 4, 8, 16, 24 hours in 80 nM Ca$^{2+}$ media and then measured
expression levels of Bcl-2 and Bax proteins. In both normal and lung cancer cells, Bcl-2 and Bax proteins expression levels, as well as Bcl-2/Bax ratio, showed no change during ATP treatment (100 μM and 1 mM) in the extracellular media containing low (80 nM) Ca²⁺ concentration (Fig. 9C-H). These data suggest that Ca²⁺ influx and the resulting plateau phase of increase, or sustained increase, in [Ca²⁺]_{cyt} are required for the enhancement of Bcl-2/Bax ratio in lung cancer cells.

To further verify that Ca²⁺ influx is responsible for the ATP-induced increase of Bcl-2/Bax ratio in lung cancer cells, we performed experiments to maintain the ATP-induced plateau phase of increase (Ca²⁺ influx) in [Ca²⁺]_{cyt} but to eliminate the transient phase of increase (Ca²⁺ release) in [Ca²⁺]_{cyt}. To accomplish this, we treated cells with U73122, an inhibitor of phospholipase C that is downstream of the P2Y receptors, before and during ATP (100 μM and 1mM) treatment. Indeed, we observed that pre-treatment of cells with U73122 not only eliminated ATP-induced transient increase in [Ca²⁺]_{cyt} as expected, but also inhibited Ca²⁺ influx (plateau phase) in both normal and lung cancer cells (Fig. 10A, B). When we treated both normal and cancer cells with ATP (100 μM and 1 mM) following U73122 treatment for 0, 2, 4, 8, 16, 24 hours, we did not see any changes in the Bcl-2/Bax ratio (Fig. 10C-H). Accordingly, blockade of P2Y receptors that signal through the PLC/IP3/DAG signaling cascade to induce Ca²⁺ release and influx, we were able to eliminate both the transient and plateau phases of increase in [Ca²⁺]_{cyt}. These data suggest that the P2X receptors (nonselective cation channels) are not the major channels for ATP-induced Ca²⁺ influx in lung cancer cells. Furthermore, these data also suggest that the Ca²⁺-dependent regulation of Bcl-2/Bax ratios is not mediated by Ca²⁺ influx through P2X receptors. However, U73122 has been shown to inhibit phospholipase A2 (PLA2) and 5-lipoxygenase as well. To our knowledge, these enzymes are not involved in Bcl-
2/Bax protein regulation. Further studies are needed to focus on investigating the channels that mediate ATP-induced Ca\(^{2+}\) influx required for the plateau phase in lung cancer cells.
Discussion

In addition to functioning as a molecular energy source, ATP is also an important extracellular messenger and agonist. In this study, we have shown that ATP induces different Ca\(^{2+}\) signaling patterns in normal lung airway epithelial cells verses lung cancer cells. Purinergic receptors P2Xs and P2Ys are ATP receptors that are widely expressed on multiple cell types, including tumor cells. Our study showed that P2Xs and P2Ys exhibit different expression distribution in normal and lung cancer cells. Our data show that P2X\(_3\), P2X\(_4\) and P2X\(_5\) receptors and P2Y\(_1\), P2Y\(_2\), P2Y\(_4\) and P2Y\(_6\) receptors are expressed more highly in lung cancer cells than in normal cells. Indeed, P2Xs and P2Ys are known to respond to ATP to regulate tumorigenic behaviors including cell proliferation and apoptosis.

The Ca\(^{2+}\) signal is also known to be involved in many cellular processes that promote tumorigenesis (1, 22). Ca\(^{2+}\) signaling components that determine Ca\(^{2+}\) signaling homeostasis change their expression or function in the occurrence and development of disease (30). The remodeling of Ca\(^{2+}\) signaling that occurs in cancer cells is achieved through differential expression of specific Ca\(^{2+}\) pumps and channels (30). Although alternative Ca\(^{2+}\) signaling is not necessary for cancer initiation, the consequence of onset may contribute to tumor progression. Our study showed that ATP induces markedly Ca\(^{2+}\) influx in lung cancer cells but not in normal cells. The most probable candidate channels for Ca\(^{2+}\) influx are P2Xs, because P2Xs per se are Ca\(^{2+}\)-permeable cation channels (31). As discussed earlier, H23 and A549 lung cancer cells had significantly higher levels of P2X\(_3\), P2X\(_4\), and P2X\(_5\) expression compared to normal cells. Studies have shown that P2X receptors desensitize ATP fast and mediate short-time inward current (36), which may underlie the limited Ca\(^{2+}\) influx induced by the activation of P2X receptors shown in P2X inhibitor and agonists data.
Store-operated Ca\textsuperscript{2+} entry (SOCE) through GPCR-activated store-operated Ca\textsuperscript{2+} channels (SOC) become the next most plausible over-functioning Ca\textsuperscript{2+} influx pathway in consideration of over-expressed P2Y receptors (GPCRs) in lung cancer cells. P2Ys function as G protein-coupled receptors and can cause Ca\textsuperscript{2+} release from the intracellular Ca\textsuperscript{2+} stores to increase cytosolic [Ca\textsuperscript{2+}] upon ATP activation (3, 31, 44). ATP (by active depletion of Ca\textsuperscript{2+} from the intracellular stores) and CPA (by passive depletion of Ca\textsuperscript{2+} from the intracellular stores) both induced SOCE, but ATP/CPA-induced SOCE in cancer cells was not enhanced, but rather reduced, in H23 lung cancer cells. This result was surprising because aggressive cancer cells would be expected to show stronger Ca\textsuperscript{2+} signaling intensity in consideration of sustained proliferation and migration. Although it has been demonstrated in proliferative pulmonary artery smooth muscle cells that enhanced SOCE promotes cell proliferation and growth, its effect on tumor growth is less clear (38, 46). Several studies have shown that, in cancer, decreased SERCA2 expression leads to low ER Ca\textsuperscript{2+} capacity, which then lowers the risk of mitochondrial Ca\textsuperscript{2+} overload that triggers cell apoptosis (27, 40). In addition, overexpressed anti-apoptotic protein Bcl-2 was shown to reduce ER Ca\textsuperscript{2+} content in part by interaction with IP3 receptors (25). However, other studies have shown that overexpressed Bcl-2 protein conserved SR/ER Ca\textsuperscript{2+} content by upregulating SERCA2 (7). These conflicting results suggest that the relationship between Bcl-2 protein and ER Ca\textsuperscript{2+} content depends on cell context. It is of great interest that our results show both H23 and A549 lung cancer cells had significantly higher levels of Bcl-2 protein expression as compared to normal epithelial lung cells. Thus, the low SOCE in cancer cells may result from reduced ER Ca\textsuperscript{2+} capacity, which is due to overexpressed Bcl-2 protein. Therefore, it is possible that low SOCE is important to survival if it normally couples to reduced ER Ca\textsuperscript{2+} capacity to regulate mitochondrial Ca\textsuperscript{2+} loading. Both SOCE and P2X-mediated Ca\textsuperscript{2+} influx participate in
the Ca\textsuperscript{2+} influx induced by ATP, however, they are not critically significant to the different kinetic Ca\textsuperscript{2+} signal in lung cancer cells as compared to normal cells.

Cell viability result that showed lung cancer cells exhibit resistance to the cytotoxicity of high concentration of ATP in comparison to normal cells is consistent with published data that showed low (nearly undetectable) expression levels of P2X\textsubscript{7}-R in lung cancer cells (20, 21, 39). P2X\textsubscript{7} is considered to act as a pro-apoptotic factor through increasing cytomembrane permeability activated by a high concentration of ATP (9, 29). However, significantly decreased cell viability was observed in H23 lung cancer cells at late (72 hours) culture but not in A549 lung cancer cells. These two lung cancer cell lines chosen in this study reflect the heterogeneity of the human lung cancer progression pathway, involving, for example, the expression of P53, Myc, Ras. This allows us to speculate that, despite both being lung carcinoma, each has its own particular response to environmental stimuli. We also showed in both normal and lung cancer cells that ATP affected cell proliferation. It is interesting to note that a lower concentration of ATP reduced cell proliferation in both H23 and A549 lung cancer cells, while reduced cell proliferation was not observed with a higher concentration of ATP. These findings are inconsistent with cell viability results that indicated more cell death in a high concentration of ATP while the cells remained healthy in low concentration. These results suggest that ATP induced cell apoptosis and cell proliferation are through separate pathways, and each sensitizes to a specific concentration range of ATP.

It is worth mentioning here that A549 lung cancer cells have been reported to secrete extracellular matrix components that have ATP binding sites. In our study, A549 lung cancer cells start to respond to ATP at 1 μM, which is the same as H23 lung cancer cells. A strong increase in intracellular Ca\textsuperscript{2+} increase was observed in A549 lung cancer cells after ATP
stimulation, and P2X and P2Y receptor inhibitors weakened ATP-induced intracellular Ca\(^{2+}\) increase in A549 lung cancer cells. These observations suggest that although extracellular matrix components secreted by A549 lung cancer cells might bind ATP, the binding ability is not sufficient to eliminate ATP action on P2 receptors. Therefore, the lack of effect of ATP on cell viability of A549 lung cancer cells is not due to competing binding of ATP to extracellular matrix components.

There are data suggesting that very high intracellular Ca\(^{2+}\) level can promote cell death through necrosis, whereas sustained and lower intracellular Ca\(^{2+}\) increases induced by milder insults promote cell death through apoptosis (8, 24). In this study, our data showed that ATP induces slow and long term phase of \([\text{Ca}^{2+}]_{\text{cyt}}\) increase in lung cancer cells, but that ATP at both high and relatively lower levels rarely affected cell viability, which indicates cell apoptosis. This leads us to propose that ATP may enhance the ability of anti-apoptosis of lung cancer cells in some way to desensitize the cytotoxicity of Ca\(^{2+}\) overload. Thus, we focused on the Bcl-2/Bax ratio, the change of which determines cell fate. Our data showed that ATP promotes anti-apoptosis by increasing Bcl-2/Bax ratio in lung cancer cells. It is of great interest that cancer cells respond to extracellular ATP by increasing survival capacities via multiple anti-death pathways that include low P2X\(_7\) expression, high Bcl-2 expression and an increased Bcl-2/Bax ratio.

To provide some insight on the mechanism of ATP-modulated Bcl-2/Bax ratio in lung cancer cells, we abolished ATP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase by using BAPTA-AM and eliminated Ca\(^{2+}\) influx through treating cells in low extracellular Ca\(^{2+}\) media, and both ways lead to unchanged Bcl-2/Bax ratio in lung cancer cells. Additionally, U73122 was applied to abolish Ca\(^{2+}\) mobilizing from intracellular Ca\(^{2+}\) stores rather than extracellular Ca\(^{2+}\) influx. However,
calcium image data showed that U73122 also reduced Ca\textsuperscript{2+} influx significantly. Cells treated with U73122 showed no change in the ratio of Bcl-2/Bax after ATP treatment. PLC inhibition leads to attenuation or blockade of two other Ca\textsuperscript{2+} influx pathways, store-operated Ca\textsuperscript{2+} entry through SOC and receptor-operated Ca\textsuperscript{2+} entry through ROC even when P2Y receptors are activated by ATP. The U73122 data indicate that, although the P2X family is one of the mechanisms that mediated ATP-induced Ca\textsuperscript{2+} influx, the P2X contribution to ATP-induced total Ca\textsuperscript{2+} influx is limited or, by itself, is insufficient to affect Bcl-2 expression and the Bcl-2/Bax ratio. These results suggest that a sustained increase in cytosolic Ca\textsuperscript{2+} due to Ca\textsuperscript{2+} influx from the extracellular environment is the critical mediator between extracellular ATP and an enhanced Bcl-2/Bax ratio in lung cancer cells. Thus, determining Ca\textsuperscript{2+} channels and transporters leading to sustained [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in lung cancer cells would provide an important therapeutic target eliminating ATP-mediated increase in Bcl-2/Bax ratio and, therefore, attenuating the ability of anti-apoptosis of lung cancer cells. In this study, we did not determine possible pathways that lead to enhanced Ca\textsuperscript{2+} influx in lung cancer cells as compared to normal cells. Ca\textsuperscript{2+} influx through P2X channels or SOCE showed no difference between cancer cells and normal cells. Other channels like VDCC and ROCC or Ca\textsuperscript{2+} extrusion and sequestration mechanisms such as the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase in the plasma membrane or SERCA on the membrane of SR or ER might be the reasons, which would be of great interest in a future study.

This study has highlighted the role of ATP in the extracellular microenvironment with regard to tumor cell growth \textit{in vitro}. Enhancing the anti-apoptotic ability of ATP may be an important selective pressure promoting oncogene transformation and cancer progression. We have shown for the first time that ATP increases the Bcl-2/Bax ratio through a sustained increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in lung cancer cells. Additionally, we have demonstrated that Ca\textsuperscript{2+} from the
extracellular environment mediates this ATP-induced increase in Bcl-2/Bax ratio. This study has several implications for lung cancer biology. It suggests that tumor cells may adapt to high ATP in the tumor microenvironment through low P2X7 receptor expression to escape apoptosis and also turn ATP into a stimulus to inhibit apoptosis. This conversion of harm to benefit is important for the aggressive behavior of tumors. Despite the exogenous application of ATP having anti-neoplastic action on tumors, as implicated by several studies, because of its cytotoxicity on low-tolerance normal cells, this side effect weakens its therapeutic value. Thus, understanding this ATP-induced anti-apoptotic pathway observed in lung cancer cells would be of therapeutic value to facilitate the development of cancer specific targeted therapies.
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Reference


**Figure legends**

**Figure 1.** ATP-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and P2X and P2Y purinergic receptor expression in normal airway epithelial cell line and lung cancer cell lines. (A) Representative images showing different patterns of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in normal (upper panels) and lung cancer cells (middle and lower panels) before, during and after application of 100 μM ATP. (B) Representative traces showing different patterns of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in normal (upper panels) and lung cancer cells (middle and lower panels) before, during and after application of 100 μM ATP. (C) Representative traces from B were overlaid to show the differences of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in normal and lung cancer cells in each pattern. (D) Summarized data showing the proportion of activated cells in each of normal and lung cancer cells (n=4-14). (E) Summarized data showing the proportion of each pattern of changes of $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells (n=4-14 separated experiments). (F) RT-PCR data showing the expression level of P2X$_2$, P2X$_3$, P2X$_4$, P2X$_5$, P2X$_6$ and P2X$_7$ purinergic receptors in normal and lung cancer cells (n=5). (G) RT-PCR data showing the expression level of P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$ and P2Y$_{11}$ purinergic receptors in normal and lung cancer cells (n=4). (H) Summarized data (mean ± SE) from real-time RT-PCR showing the expression level of P2X$_2$, P2X$_3$, P2X$_4$, P2X$_5$, P2X$_6$ and P2X$_7$ purinergic receptors in normal and lung cancer cells. (n=3, *$P<0.05$ vs. Normal). (I) Summarized data (mean ± SE) from real-time PCR showing the expression level of P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$ and P2Y$_{11}$ purinergic receptors in normal and lung cancer cells. (n=3, *$P<0.05$ vs. Normal).

**Figure 2.** Dose response curves of area under the curve (AUC), transient and plateau amplitude of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells. (A) Representative
traces showing changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) before, during and after application of 1 nM, 100 nm, 1 μM, 10 μM, 100 μM and 1 mM in normal (upper panel) and lung cancer cells (middle and lower panel). (B) Summarized data (mean ± SE) showing the dose response curve of area under the curve (AUC) of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ATP in normal and lung cancer cells. (n=3 *P<0.05 vs. Normal). (C) Summarized data (mean ± SE) showing dose response curve of transient amplitude of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ATP in normal and lung cancer cells. (n=3, *P<0.05 vs. Normal). (D) Summarized data (mean ± SE) showing dose response curve of plateau amplitude of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ATP in normal and lung cancer cells. (n=3, *P<0.05 vs. Normal).

**Figure 3.** ATP-induced changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in the absence of extracellular Ca\(^{2+}\) in normal and lung cancer cells. (A) Representative traces showing changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in normal and lung cancer cells before, during and after application of 100 μM ATP when depriving extracellular Ca\(^{2+}\) in individual cells. (B) Representative traces showing changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in normal and lung cancer cells before, during and after application of 100 μM ATP when depriving extracellular Ca\(^{2+}\) in average. (C) Summarized data showing the proportion of activated cells in 1.8 mM and 0 mM extracellular Ca\(^{2+}\) in normal and lung cancer cells, respectively (n=3-14).

**Figure 4.** ATP-induced store operated Ca\(^{2+}\) entry (SOCE) and extracellular Ca\(^{2+}\) influx in normal and lung cancer cells. (A) Representative traces showing changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in normal and lung cancer cells treated with 100 μM ATP in the absent of extracellular Ca\(^{2+}\) and subsequent \([\text{Ca}^{2+}]_{\text{cyt}}\) changes during and after application of 1.8 mM extracellular Ca\(^{2+}\). (B) Summarized data (mean ± SE) showing the amplitude of increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by 1.8mM extracellular Ca\(^{2+}\) after
application of 100 μM ATP in the absence of extracellular Ca^{2+}. n=5, *P<0.05 vs. Normal. (C) Representative traces showing changes in [Ca^{2+}]_{cyt} in normal and lung cancer cells before, during and after application of 10 μM cyclopiazonic acid (CPA, an inhibitor of SERCA) and subsequent [Ca^{2+}]_{cyt} changes during and after applying 1.8 mM extracellular Ca^{2+}. (D) Representative traces showing changes in [Ca^{2+}]_{cyt} in normal and lung cancer cells before, during and after application of 10 μM CPA and subsequent [Ca^{2+}]_{cyt} changes during and after applying 100 μM ATP in 1.8 mM extracellular Ca^{2+}. (E) Representative traces showing changes in [Ca^{2+}]_{cyt} in normal and lung cancer cells before, during and after application of 10 μM CPA and subsequent [Ca^{2+}]_{cyt} changes during and after applying 100 μM ATP in 1.8 mM extracellular Ca^{2+} during treated by 1 μM TNP-ATP (P2XR blocker). (F) Overlaid curves of panel C, D and E. (G) Summarized data (mean ± SE) showing the amplitude of second peak of [Ca^{2+}]_{cyt} increases in normal and lung cancer cells induced by 1.8 mM extracellular Ca^{2+} or 1.8 mM extracellular Ca^{2+} plus 100 μM ATP after the application of 10 μM CPA, and amplitude of second peak of [Ca^{2+}]_{cyt} increases in normal and lung cancer cells induced by 1.8 mM extracellular Ca^{2+} plus 100 μM ATP after the application of 10 μM CPA during TNP-ATP treatment. (n=3-10, *P<0.05 vs. Control, #P<0.05 vs. Normal).

Figure 5. Effect of P2X and P2Y inhibitors on ATP-induced changes of [Ca^{2+}]_{cyt} and intracellular Ca^{2+} changes induced by P2X and P2Y activators. (A) Representative traces showing changes in [Ca^{2+}]_{cyt} before, during and after application of 100 μM ATP in normal and lung cancer cells (n=4-14) (left panel); Representative traces showing changes in [Ca^{2+}]_{cyt} before, during and after application of 100 μM ATP in the present of 10 μM PPADS in normal and lung
cancer cells (n=4-5) (middle panel); Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during and after application of 100 μM ATP in the present of 100 nM TNP-ATP in normal and lung cancer cells (n=3) (right panel). (B) Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during and after application of 100 μM βm-ATP in normal and lung cancer cells (n=3) (left panel); Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during and after application of 100 μM αβ−meATP in normal and lung cancer cells (n=3) (right panel). (C) Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during and after application of 100 μM ATP in the presence of 10 μM U73122 in normal and lung cancer cells (n=3) (left panel); Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during and after application of 100 μM UTP (n=3) (right panel).

**Figure 6.** Effect of ATP on cell viability and cell proliferation in normal and lung cancer cells. (A) Summarized data (mean ± SE) showing cell viability of normal (left panel) and lung cancer cells (middle and right panels) treated with ATP (0 μM, 100 μM, 1 mM), (n=4 separated experiments, *$P<0.05$ vs. Control). (B) Summarized data (mean ± SE) showing cell proliferation of normal (left panel) and lung cancer cells (middle and right panels) treated with ATP (0 μM, 100 μM or 1 mM), (n=4 separated experiments, *$P<0.05$ vs. Control).

**Figure 7.** Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells. (A) Western blot analysis of Bcl-2 and Bax expression in normal (upper panel) and lung cancer cells (middle and lower panels) treated with vehicle (water) for 0, 2, 4, 8, 16, and 24 hours. β-actin is used as a control. (B) Western blot analysis of Bcl-2 and Bax expression in
normal (upper panel) and lung cancer cells (middle and lower panels) treated with 100 μM ATP for 0, 2, 4, 8, 16, and 24 hours. β-actin is used as a control. (C) Western blot analysis of Bcl-2 and Bax expression in normal (upper panel) and lung cancer cells (middle and lower panels) treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours. β-actin is used as a control. (D) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with vehicle for 0, 2, 4, 8, 16 and 24 hours. (n=3). (E) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with vehicle for 0, 2, 4, 8, 16 and 24 hours. (n=8, *P<0.05 vs. Normal). (F) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours. (G) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours. (n=8, *P<0.05 vs. Normal). (H) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours. (I) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours. (n=7, *P<0.05 vs. Normal).

Figure 8. Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells after application of BAPTA-AM. (A) Representative traces showing changes in [Ca^{2+}]_{cyt} before, during and after application of 100 μM ATP after treatment with 25 μM BAPTA-AM in normal and lung cancer cells. (B) Representative traces showing changes in [Ca^{2+}]_{cyt} before, during and after application of 1 mM ATP after treatment with 25 μM BAPTA-AM in normal and lung cancer cells. (C) Western blot analysis of Bcl-2 and Bax expression in
normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. β-actin is used as a control. (D) Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. β-actin is used as a control. (E) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. (F) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. (n=5, *P<0.05 vs. Normal). (G) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. (H) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after application of 25 μM BAPTA-AM. (n=5, *P<0.05 vs. Normal).

Figure 9. Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells in low extracellular Ca^{2+} culture conditions. (A) Representative traces showing changes in [Ca^{2+}]_{cyt} in normal and lung cancer cells before, during and after application of 100 μM ATP in media containing 80 nM extracellular Ca^{2+}. (B) Representative traces showing changes in [Ca^{2+}]_{cyt} in normal and lung cancer cells before, during and after application of 1 mM ATP in media containing 80 nM extracellular Ca^{2+}. (C) Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 100 μM ATP in media containing 80 nM
extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. β-actin is used as a control. (D) Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 1 mM ATP in media containing 80nM extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. β-actin is used as a control. (E) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 μM ATP in media containing 80 nM extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. (F) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer treated with 100 μM ATP in media containing 80 nM extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. (n=3, *\(P<0.05\) vs. Normal). (G) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 1 mM ATP in media containing 80nM extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. (H) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 1 mM ATP in media containing 80 nM extracellular Ca\(^{2+}\) for 0, 2, 4, 8, 16 and 24 hours. (n=3, *\(P<0.05\) vs. Normal).

**Figure 10.** Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells with treatment of U73122. (A) Representative traces showing changes in \([Ca^{2+}]_{cyst}\) in normal and lung cancer cells before, during and after application of 100 μM ATP after treatment with 10 μM U73122. (B) Representative traces showing changes in \([Ca^{2+}]_{cyst}\) in normal and lung cancer cells before and after application of 1 mM ATP after treatment with 10 μM U73122. (C) Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122. β-actin is used as a control. (D) Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122.
β-actin is used as a control. (E) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer treated 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122. (F) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer treated 100 μM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122. (n=3, *P<0.05 vs. Normal). (G) Summarized data (mean ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122. (H) Summarized data (mean ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer treated with 1 mM ATP for 0, 2, 4, 8, 16 and 24 hours after treatment with 10 μM U73122. (n=3, *P<0.05 vs. Normal).
Table 1 Characteristics of the human lung cancer cell lines

<table>
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<th>Cell line</th>
<th>Cell type</th>
<th>Disease</th>
<th>myc* expression</th>
<th>src* expression</th>
<th>ras* expression</th>
<th>Keratin* expression</th>
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<td>BEAS-2B</td>
<td>Epithelial</td>
<td>Normal</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>H23</td>
<td>Epithelial</td>
<td>Adenocarcinoma; Non-small cell lung cancer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>A549</td>
<td>Epithelial</td>
<td>Carcinoma</td>
<td>-</td>
<td>-</td>
<td>+ (47)</td>
<td>+ (17, 32)</td>
</tr>
</tbody>
</table>

*gene/#protein
Figure 1
Transient Rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Ratio)

Plateau Rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Ratio)

Rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Area Under Curve)

Figure 2
Figure 3
**Figure 4**

(A) Normal, H23, A549

(B) Rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Ratio)

(C) Normal, H23, A549

(D) ATP, CPA, ATP + TNP-ATP

(E) ATP, CPA, TNP-ATP

(F) CPA only, CPA + ATP, CPA + ATP + TNP-ATP

(G) Rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Ratio)
Figure 5
Figure 6
Figure 7

[Graphs showing protein levels and Bcl-2/Bax ratios under different conditions of ATP concentration over time.]

- **A** Control (Vehicle)
- **B** ATP (100 μM)
- **C** ATP (1 mM)
- **D** Normal
- **E** ATP (0 M)
- **F** Normal
- **G** ATP (100 μM)
- **H** Normal
- **I** ATP (1 mM)

*Time in ATP (h)*

*Protein Level (Arbitrary Unit)*

*Bcl-2/Bax Ratio*
**Figure 8**

**A**

Normal
Control BAPTA-AM H23
Control BAPTA-AM A549
Control BAPTA-AM

**B**

Normal
Control BAPTA-AM H23
Control BAPTA-AM A549
Control BAPTA-AM

**C**

ATP (100 μM)

H23
A549

**D**

ATP (1 mM)

H23
A549

**E**

Normal

H23

A549

**F**

Normal H23 A549

**G**

Normal

H23

A549

**H**

Normal H23 A549