O_2 and Ca^{2+} Fluxes as Indicators of Apoptosis Induced by Rose Bengal–Mediated Photodynamic Therapy in Human Oral Squamous Carcinoma Cells

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Abstract

Objective: Photodynamic therapy (PDT) triggers various cellular responses and induces cell death via necrosis and/or apoptosis. This study evaluated the feasibility of using O_2 and Ca^{2+} fluxes as indicators of apoptosis induced by rose bengal (RB)-mediated PDT in human oral squamous carcinoma cells (Cal27 cells). **Methods:** Intracellular reactive oxygen species (ROS) generation was assessed by the dichloro-dihydro-fluorescein diacetate (DCFH-DA) method. Real-time O_2 and Ca^{2+} flux measurements were performed using the noninvasive micro-test technique (NMT). Apoptosis of the PDT-treated cells was confirmed by 4'6-diamidino-2-phenylindole-dilactate staining. The activation of apoptosis-related molecules was examined using Western blot. We assayed the effects of the fluctuation of O_2 and Ca^{2+} flux in response to PDT and the apoptotic mechanism, by which ROS, O_2 , and Ca^{2+} synergistically may trigger apoptosis in PDT-treated cells. **Results:** Real-time O_2 and Ca^{2+} flux measurements revealed that these indicators were involved in the timely regulation of apoptosis in the PDT-treated cells and were activated 2 h after PDT treatment. RB-mediated PDT significantly elicited the generation of ROS by approximately threefold, which was critical for PDT-induced apoptosis. Cytochrome c and cleaved caspase-3, caspase-9 and poly ADP ribose polymerase (PARP) were overexpressed, and the data provided evidence that 2 h was considered to be the key observation time in RB-mediated PDT-induced apoptosis in Cal27 cells. **Conclusions:** Our collective results indicated that the effects of O_2 and Ca^{2+} fluxes may act as a real-time biomonitoring system of apoptosis in the RB-PDT-treated cells. Also, RB-mediated PDT can be a potential and effective therapeutic modality in oral squamous cell carcinoma.

Introduction

ORAL SQUAMOUS CELL CARCINOMA (OSCC) is a serious public health problem worldwide, with 389,000 new cases per year; its high mortality rate of 50% has remained unchanged for decades.^{1–3} Despite the significant advances in clinical applications, such as chemotherapy, radiotherapy, and surgery, many severe side effects and toxicity appear.⁴ Therefore, further exploration of therapeutic strategies for OSCC is necessary.

Photodynamic therapy (PDT) is a minimally invasive and potent therapy approved for the treatment of cancer and non-oncological disorders.^{5,6} The oral cavity is an easily accessible area for PDT activation; therefore, PDT could be developed for the diagnosis and treatment of oral diseases.^{7–9} PDT is based on a requirement for the simultaneous presence of three components: photosensitizer (PS), molecular

oxygen, and visible light. Ideally, the PS is taken up and accumulates preferentially in the targeted cells.¹⁰ PS for PDT generally include dyes [e.g., rose bengal (RB) and methylene blue], drugs (e.g., tetracyclines and chlorpromazine), and endogenous porphyrins.¹¹ RB is an anionic water-soluble xanthene dye that has been used as an ophthalmic diagnostic modality to assess damage of the cornea and conjunctiva. Our preliminary study has shown that RB staining may be a valuable diagnostic test for the detection of oral precancerous and malignant lesions.^{12–14} RB is applied as a photodynamic sensitizer during PDT for effective and safe delivery in subcellular loci and target tissue; it is also used in PDT for the clinical treatment of uterine cervix carcinoma and metastatic melanoma.¹⁵ PSs can be highly specific or slightly broad in diverse organelles, including the endoplasmic reticulum (ER), mitochondria, Golgi apparatus, lysosomes, and plasma membrane.¹⁶ PS activation during

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light exposure elicits photochemical reactions, which produce lethal toxic agents, such as singlet O₂ and other reactive oxygen species (ROS), leading to cell apoptosis/death and tissue destruction.^{17–19} The effects of PDT depend upon the simultaneous presence of PSs, light irradiation, and molecular O₂. Molecular O₂ is essential for promoting the production of highly toxic ROS, which damage cellular constituents and lead to cell death. Cell death by PDT requires the interaction of excited PSs with molecular O₂, and then causes the photochemical formation of ROS, such as singlet O₂ and hydroxyl radicals, which can cause severe damage to target cells.^{20,21} In addition, the interplay between Ca²⁺ and ROS delicately controls apoptosis in PDT-treated cells. Clearly, Ca²⁺ is crucial in apoptotic signalling, and the PDT-induced increase in Ca²⁺ is regulated by ROS generation. However, the regulatory mechanism underlying the mutual interaction of Ca²⁺ and ROS with apoptosis remains unclear.²²

A number of studies have explained the three forms of programmed cell death (autophagic cell death, necrosis, and apoptosis). However, little is known about the dynamic and continuous intracellular processes after PDT. The present study aims to demonstrate the various cellular responses and regulatory mechanisms of PDT-induced apoptosis in human oral squamous carcinoma cells. It also aims to investigate whether or not O₂ and Ca²⁺ fluxes can be used as indicators of apoptosis induced by RB-mediated PDT. O₂ and Ca²⁺ fluxes around PDT-treated cells were measured using the noninvasive micro-test technique (NMT). The system established in this study is applicable to assay the interaction of O₂ and Ca²⁺ fluxes with apoptosis.

Materials and Methods

Cell cultures

Oral squamous carcinoma Cal27 cells obtained from the American Type Culture Collection (ATCC) were maintained at 37°C in a humidified atmosphere (90%) containing 5% CO₂. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). These cells were used in further experimental procedures after confirming the absence of any contamination.

RB treatment and PDT

Cells were incubated with 10 μM RB (Sigma-Aldrich, Louis, MO) in Dulbecco's modified Eagle's medium for 60 min in the dark, and then washed twice with phosphate-buffered saline (PBS). Prior to irradiation, the culture medium was replaced with PBS (0.2 mol/L, pH 7.4) without phenol red to avoid undesired photosensitising effects. Irradiation was performed by placing the dishes under a homemade light emitting diode at 530 ± 15 nm (the total light dose was 1.6 J/cm² with a uniform light fluence rate of 20 mW/cm², the total output power was 1000 mW, and the beam diameter was ~0.5 cm) for 90 sec. The cells were rinsed twice with PBS, transferred to growth medium, and then allowed to recover for different times.

Measurement of intracellular ROS generation

Intracellular ROS generation was assessed in arbitrary units by the dichloro-dihydro-fluorescein diacetate (DCFH-

DA) method. The fluorogenic substrate DCFH-DA (Invitrogen, Carlsbad, CA) was oxidized to highly fluorescent DCF by ROS and used to monitor intracellular ROS dose generation. Treated and control cells were incubated in a medium containing 20 μM DCFH-DA at 37°C in the dark for 30 min. The cells were harvested at various times, washed with PBS, and then resuspended in 1 mL of PBS. The resultant intensity of fluorescence was measured in the FL-2 channel of a FACScan flow cytometer and analysed immediately using CELLQuest software (Becton Dickinson, USA).

Net O₂ flux measurement

The net O₂ flux of the cultured Cal27 cells was measured using the NMT technique (BIO-IM Series; YoungerUSA, LLC, Amherst, MA) with ASET 2.0 (Sciencewares Inc., Falmouth, MA), and iFluxes 1.0 (YoungerUSA, LLC, Amherst, MA) software.^{23,24} Pt/Ir polarographic O₂ microelectrodes (tip diameter: 2–4 μm, YG-O2-ME03, YoungerUSA, LLC) were used to detect dissolved O₂ at a polarisation voltage of –750 mV as described earlier.^{24,25} The O₂ microelectrode vibrated in 30 μm excursion at a frequency of 0.3–0.5 Hz. A MI-402 type of electrode (Microelectrodes, Inc., New Hampshire) was used as the reference electrode to complete the circuit. The O₂ microelectrode was positioned vertically 400 μm above the cell for 3–5 min before the background signal (Blank) was recorded. Then, the same microelectrode was moved back and positioned 5 μm from the cell membrane to record O₂ fluxes for 8–10 min throughout the experiments. At least 10 cells per treatment ($n \geq 10$) were used for O₂ flux measurements. All experiments were repeated 8–10 times to ensure the validity of the physiological trends. Flux experiments were conducted in the YoungerUSA (Xuyue Beijing) NMT Service Center. The Net O₂ flux was calculated according to Fick's first law of diffusion: $J = -D_0 (dc/dx)$, where J is the O₂ flux (unit: pmol/cm²/sec), D_0 is the O₂ diffusion coefficient, dx is the distance of the microelectrode vibrated repeatedly from one point to another perpendicular to the sample surfaces, and dc is the O₂ concentration difference between these two points. JCal 1.0 is an Excel spread sheet (<http://youngerusa.com/jcal> or <http://ifluxes.com/jcal>) that was used to calculate the net O₂ flux.

Net Ca²⁺ flux measurement

The net Ca²⁺ flux was also obtained in the YoungerUSA (Xuyue Beijing) NMT Research Service Center with NMT (BIO-IM Series; YoungerUSA, LLC, Amherst, MA) running ASET 2.0 (Sciencewares Inc., Falmouth, MA) and iFluxes 1.0 (YoungerUSA, LLC, Amherst, MA) software (17). ASET 2.0 and iFluxes 1.0 can integrate and coordinate differential voltage signal collection, motion control, and image capture simultaneously. Ca²⁺-selective microelectrodes were freshly fabricated prior to each NMT test. Silanized glass microelectrodes (inner Φ4 ± 1 μm, YG-IS-ME02; YoungerUSA, LLC) were initially backfilled with solution (100 mM CaCl₂) to ~1 cm in length and then front-filled with ~25 μm liquid ion exchange (LIX) (Ca²⁺: YG-LIX-Ca01, YoungerUSA, LLC).

YG-LIX-Ca01 was constructed and optimized for NMT experiments following the procedures from cat#21048

(Sigma-Aldrich, Louis, MO). Before and after Ca^{2+} flux measurement, the microelectrode was calibrated with the aforementioned culture medium with different concentrations of Ca^{2+} (0.1 and 0.01 mM). Only electrodes with Nernstian slope > 25 mV/decade for Ca^{2+} were used in this study. The Ca^{2+} ion flux was calculated based on Fick's first law of diffusion: $J = -D_0 (dc/dx)$, where J is the ion flux (unit: $\text{pmol}/\text{cm}^2/\text{s}$), D_0 is the Ca^{2+} diffusion coefficient, dx is the distance of the microelectrode moved from one point to another perpendicular to the surfaces of root hair at a frequency of ~ 0.3 Hz, and dc is the Ca^{2+} concentration differences between the two points. The microelectrodes were positioned $1\text{--}2\ \mu\text{m}$ away from the samples by a computer-controlled NMT system.

The net Ca^{2+} flux was calculated by using JCal 1.0 (a free MS Excel spreadsheet, youngerusa.com/jcal or ifluxes.com/jcal).

Cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.²⁶ Cal27 cells (1×10^4) were seeded into a 96 well culture plate and treated with $10\ \mu\text{M}$ RB for 60 min in the dark. This process was performed in the presence or absence of $10\ \text{mM}$ *N*-acetyl cysteine (NAC) (Sigma-Aldrich, Louis, MO). The antioxidant NAC was used as a ROS scavenger. The cells were irradiated at a 530 ± 15 nm light emission diode for 90 sec, and then washed completely with PBS to remove the red color of RB. The medium was changed to a growth medium containing 10% FBS and then incubated for different times. The cells were labelled with MTT solution ($5\ \text{mg}/\text{mL}$) for 4 h, and the resulting formazan was solubilized with dimethyl sulfoxide ($100\ \mu\text{M}$). The absorbance of the prepared solution was determined in a 96 well microplate by a microplate reader (Titertek Multiskan MCC) using a 540 nm filter.

Apoptosis assay

Apoptotic cells were determined by simultaneous staining of cells with propidium iodide (PI) and annexin V (annexin V-FITC). Briefly, treatment was conducted according to the experimental setup, and the cells were washed twice with cold PBS and resuspended in binding buffer (HEPES supplemented with $2.5\ \text{mM}$ CaCl). The cells were incubated with annexin V-FITC ($0.5\ \text{mL}$) on ice for 20 min in the dark and then washed once in ice-cold HEPES buffer. The stained cells were analyzed by a FACScan flow cytometer. The data were expressed as percentages of cells (per 100,000 events read) as derived from the dot plots generated by CellQuest software (Becton Dickinson, USA).

Western blot analysis

According to the experimental protocol, cells were washed with PBS and harvested using mammalian protein extraction reagent (Pierce, Rockford, IL) containing 0.1% protease inhibitor (Roche, Indianapolis, IN) at various time points. The cells were then centrifuged at $14,000g$ for 15 min, and the supernatant was collected. Cytosol protein (cytochrome c) was extracted, and protein concentrations were determined using a bicinchoninic acid protein assay

reagent kit (Pierce, Rockford, IL). Samples containing equal amounts of protein were loaded onto 12% sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE) gels and transferred onto polyvinylidene fluoride membranes (NEN Life Science, Boston, MA). The membranes were blocked for 1 h in Tris-buffered saline with 0.05% Tween-20 (TBST) containing 5% nonfat dry milk and incubated with the following antibodies: cytochrome c; cleaved caspase 9, caspase 3, and poly ADP ribose polymerase (PARP) (Cell Signaling, Danvers, MA); and actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturers' instructions at 4°C overnight. The membranes were washed three times in TBST before adding rabbit immunoglobulin G (IgG)-horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and washed again three times. Immunoreactive bands were visualised with a Western Lightning ECL kit (Amersham Biosciences) and exposed to X-ray films (Kodak, Rochester, NY). Equal loading of samples was confirmed by probing the membranes with HMGB1 antibody (Cell Signaling).

Statistical analysis

Differences between means were evaluated by ANOVA and Student's *t* test. Statistical analysis was performed using SPSS for Windows version 17.0. Statistical significance was set at $p < 0.05$.

Results

RB-mediated PDT stimulated ROS generation

We examined the possible mechanisms involved after the induction of apoptosis by RB-mediated PDT. To date, the proposed mechanisms of PDT-induced apoptosis mainly focus on the generation of intracellular ROS. PDT can trigger ROS generation by exciting PSs. Therefore, we monitored the intracellular ROS generation at different times after PDT by measuring the conversion of non-fluorescent DCFH-DA to fluorescent DCF by using flow cytometry. As shown in Fig. 1A, the intracellular ROS production was immediately and significantly increased after irradiation in Cal27 cells. The ROS level was threefold higher in the PDT-treated cells than in the control and RB alone-treated cells. The ROS levels in the PDT group displayed a continuous twofold increase up to 24 h after PDT treatment and remained higher than that in the other groups. We administered the special ROS scavenger NAC to further investigate the function of ROS in RB-PDT-induced damage in Cal27 cells. The addition of NAC ($10\ \text{mM}$) in the PDT group attenuated fluorescence. The corresponding decrease of the mean fluorescence intensity indicated that NAC scavenged the generation of ROS, and cell destruction was inhibited.

Inhibition of cell viability after RB-mediated PDT

The viability of Cal27 cells after RB-mediated PDT was assessed with the MTT assay. To examine the effects of PDT on Cal27 cell proliferation, cells were treated with $10\ \mu\text{M}$ RB for 1 h and then irradiated for 90 sec. Results showed that PDT inhibited the growth and viability of the cells (Fig. 1B). The data of optical density curve obtained

FIG. 1. Reactive oxygen species (ROS) levels and viability of photodynamic therapy (PDT)-treated Cal27 cells. The values are reported as percentage of optical density (OD). The OD of the untreated cells at 0 min time point is considered to be 100%. **(A)** Intracellular ROS generation was measured in untreated cells, rose bengal (RB)-treated cells, and PDT-treated cells or in combination with *N*-acetyl cysteine (NAC)-treated (PDT+NAC-treated) cells by flow cytometry at the indicated time points after treatment. **(B)** Viability was assessed by MTT assay. All PDT treatment values were significantly different ($p < 0.01$) compared with the other three groups. Data represent mean $\pm$ SD of three experiments.

through the MTT assay also suggested that PDT reduced the viability of Cal27 cells. However, no significant differences were detected between the control and RB-treated groups. The PDT-induced inhibition of cell viability was repaired by the administration of 10 mM NAC. These results indicated that PDT can trigger cell death of Cal27 cells immediately after PDT treatment, but a small number of surviving cells presented the same growing pattern continuing up to 72 h later.

ROS generation changes both net O₂ and Ca²⁺ fluxes

Cal27 cells were measured in culture using NMT to evaluate the effect of ROS generation on both net O₂ and Ca²⁺ fluxes. NMT is a noninvasive and continuous measurement method that uses microelectrodes; its time series data are contiguous and show a continuous trend. RB-mediated PDT directly triggered similar fluctuating changes in both net O₂ and Ca²⁺ fluxes (Fig. 2). The data indicated that the net O₂ influx was stable at the initial stage, immediately decreased at 1 h after PDT in Cal27 cells, and then peaked at 2 h. By contrast, the net O₂ influx of the PDT-treated cells gradually increased from 2 to 24 h after recovery. The PDT-treated cells showed higher uptake than

the control or cells treated with RB alone at 6 h after recovery. The addition of 10 mM NAC in the cells also inhibited the effect of PDT, and barely restrained the net O₂ influx.

The variation rate of the net Ca²⁺ efflux in the PDT-treated cells was significantly higher than that in the control or cells treated with RB alone. This rate peaked at 2 h after recovery. The net Ca²⁺ efflux fluctuated and converted into influx, and then reverted into efflux from 3 to 24 h after recovery. Similar to the previous experiment, a special ROS scavenger (10 mM NAC) was administered, and the cells were repaired after PDT, without significant changes in the net Ca²⁺ efflux.

Given the similar variation trend, we speculated that ROS generation can simultaneously influence the net O₂ influx and net Ca²⁺ flux in the PDT-treated cells at 2 h after irritation. Our results indicated that the net O₂ and Ca²⁺ fluxes could serve as early indicators of apoptosis after PDT.

Apoptosis induction in Cal27 cells after RB-mediated PDT

This study evaluated whether or not the growth inhibitory effects of RB-mediated PDT were associated with apoptosis

FIG. 2. (A) Oxygen influx time course for Cal27 cells in culture dish after treatment. (B) Ca²⁺ flux time course for a single Cal27 cell in culture dish after treatment. (C) Photomicrograph of noninvasive micro-test technique; Scale bar: 50 μm. Ca²⁺ selective microelectrodes positioned next to a single Cal27 cell in a culture dish (left). The Pt/Ir polarographic oxygen microelectrodes positioned next to Cal27 cells in a culture dish (right). The experiment was repeated in five different culture dishes.

induction. The PDT-induced apoptosis was determined by annexin V-FITC. The results of flow cytometry showed that PDT significantly increased the number of annexin V-positive apoptotic cells in the control cells and in those treated with RB alone. As shown in Fig. 3A, the apoptotic effect of PDT was time dependent. The percentage of apoptotic cells gradually increased to 30.57% at 24 h after RB-PDT. In addition, the PDT-induced apoptosis was effectively blocked by pretreatment with 10 mM NAC. This result demonstrated that PDT triggered apoptosis in Cal27 cells by ROS generation.

The expression levels of apoptosis-related proteins were measured by Western blot to analyze the molecular mechanisms that regulate apoptosis. The activation of the PDT-induced apoptotic pathway was characterized by examining the release of cytochrome c and the cleavage of caspase-3, caspase-9, and PARP at 24 h after recovery. As shown in Fig. 3B and C, RB-PDT triggered the rapid activation of the

FIG. 3. (A) Apoptosis in Cal27 cells was evaluated using annexin V-FITC by the flow cytometric method. Photodynamic therapy (PDT) treatment value was significantly different ($p < 0.01$) with respect to untreated or rose bengal (RB)-treated or PDT + *N*-acetyl cysteine (NAC)-treated cells. Experiments were conducted in triplicate. (B and C) The expression levels of cytochrome c, cleaved caspase-3, cleaved caspase-9, poly ADP ribose polymerase (PARP), and actin were detected in PDT cells by Western blot analysis. The representative bands from three independent experiments are shown.

intrinsic pathway after irradiation, whereas no noticeable activation was observed in the untreated cells. The cytosolic release of cytochrome c was detected at the beginning of the treatment. The expression level increased from 2 to 4 h after recovery and then started to weaken afterwards. Caspase-9 cleavage occurred at 2 h of recovery after PDT and then peaked at 6 h. Similarly, the onset of caspase-3 cleavage occurred at 2 h. At 6 h after PDT, the level of cleaved caspase-9 decreased, but the level of cleaved caspase-3 was consistently high up to 24 h post-PDT. PDT also increased the level of cleaved PARP. The cleavage of PARP started at 2 h and then peaked at 12 h after PDT. PARP is a known endogenous caspase substrate with important functions in apoptosis.

These results showed that RB-mediated PDT initially triggered the release of cytochrome c and then upregulated caspase-3 and caspase-9 at 2 h after recovery. Simultaneously, the molecular expression of cleaved PARP was observed. Therefore, we proposed that PDT stimulated the apoptosis signalling pathway and induced apoptosis in Cal27 cells, accompanied by quick changes in the net O₂ and Ca²⁺ fluxes at 2 h after recovery.

Discussion

PDT is an efficient and safe auxiliary treatment method of cancer and non-oncological disorders. This study used RB-mediated PDT, a process that involves RB, light irradiation, and molecular O₂, to inhibit the growth and proliferation of oral squamous carcinoma cells. The inhibitory and apoptotic effects of RB-mediated PDT were evaluated in Cal27 cells. The results initially showed that the net O₂ and Ca²⁺ fluxes could serve as early indicators of apoptosis after PDT treatment.

RB is a promising photosensitizing drug because it produces long-term cytotoxic effects by inducing ROS generation through a photodynamic reaction.²² Photoactive RB molecules were first restored by intracellular esterases in the ER and the Golgi apparatus, which were found to be the primary sites of photodamage, and then distributed dynamically throughout the cytoplasm. In addition, evidence has also been provided for the extensive alteration of other organelles such as the mitochondria and the cytoskeleton, and microscopical and biochemical parameters demonstrated that apoptotic cell death does occur.²⁷ In the present study, the cytotoxic and apoptotic effects of RB-mediated PDT were attributed to ROS generation and initially examined in Cal27 cells. RB-mediated PDT could inhibit cell proliferation time-dependently and the differences were growing significant compared with other groups. No cytotoxic effect was observed in the cells treated with RB alone, and NAC pretreatment alleviated the cytotoxic effects on cell viability.

After photon absorption, the PS transforms from its ground singlet state to an excited singlet state, which can either form free radicals or radical ions by hydrogen atom extraction or electron transfer to biological substrates, solvent molecules, or O₂.^{20,28} These radicals can interact with ground-state molecular O₂ to produce ROS. In some PDT paradigms, the rapid increase in intracellular Ca²⁺ after photosensitization has been associated with PDT-induced apoptosis in photosensitized mouse lymphoma cells.²⁹ Intracellular Ca²⁺ overload with consequent mitochondrial Ca²⁺ uptake, increase in cellular pro-oxidant state, and generation of free fatty acids are known factors that favor permeability transition pore (PTP) opening. The apoptotic signals induced by PDT are commonly associated with various photoactive molecules, and likely synergize each other's cytotoxic effects.³⁰

The generation of ROS in response to PDT and the mechanism by which ROS, O₂, and Ca²⁺ synergistically trigger apoptosis in PDT-treated cells have yet to be elucidated. To the best of our knowledge, this study is the first to report that the net O₂ and Ca²⁺ fluxes could serve as indicators of PDT-induced apoptosis using NMT. We provided evidence that RB-mediated PDT triggered apoptosis in Cal27 cells through ROS generation. The PDT-treated cells

had threefold higher ROS level than the control cells (Fig. 1A). The high level of ROS production is the main cause of PDT-induced apoptosis. As free radicals, ROS can react with most biological macromolecules. Therefore, ROS can not only change cell structure but also cause oxidative stress. ROS generation damages Ca²⁺ homeostasis and mitochondrial functions, as well as stimulating subsequent Ca²⁺ release by mitochondrial apoptosis. Concomitant net O₂ influx in cells, which is a key factor in regulating PTP opening and cytochrome c release at the mitochondria and ER sites, may alter Ca²⁺ fluxes in favor of mitochondrial membrane potential (MMP) induction.³¹

Chan indicated that the rise in [Ca²⁺]_i induced by RB-mediated PDT is primarily attributed to calcium release from intracellular stores, such as those found in the ER, mitochondria, nucleus, and/or calcium-binding proteins.²² Also, the present study described that the net O₂ and Ca²⁺ fluxes induced by ROS generation at the early stage were quickly mediated after RB-PDT-treatment in Cal27 cells. The prominent feature was that both net O₂ and Ca²⁺ fluxes in Cal27 cells peaked at 2 h of recovery after being PDT treated. This 2 h sensitive time point also appeared in the apoptosis test chart and Western blot analysis (Fig. 3B). Moreover, these biochemical events were effectively eliminated upon NAC pretreatment (Fig. 3A). This result confirmed that PDT altered the net O₂ and Ca²⁺ fluxes regulated by ROS generation.

The data on the net O₂ and Ca²⁺ fluxes, which were monitored during live cell culture, were timely, continuous, and dynamic. As shown in Fig. 2B, the 2 h point after RB-PDT indicated that the decrease in the net O₂ flux at the early stage was caused by intracellular ROS generation. By contrast, the increase in the net O₂ flux at 2 h after recovery showed quick O₂ uptake in Cal27 cells. O₂ is essential for promoting the production of highly toxic ROS, resulting in serious apoptosis. Similarly, the net Ca²⁺ efflux increased at the early period, reversed to influx, and then peaked at 2 h after recovery. These results indicated that the increase of intracellular Ca²⁺ induced the net Ca²⁺ efflux at the early stage and then triggered apoptosis. The occurrence of apoptosis increased the amount of Ca²⁺ flowing into the cell. These biochemical events, including net O₂ influx and Ca²⁺ flux, were consistent with the occurrence of apoptosis. Therefore, the net O₂ and Ca²⁺ fluxes could serve as early indicators of apoptosis after PDT treatment, and 2 h was considered to be the key observation time.

Although PDT induced cell death in diverse types of cancer cell lines, the molecular mechanisms involved in each death event may be different because of the induction of PSs and cancer cell lines.^{32,33} Apoptosis is responsible for PDT-mediated tumor inhibition in several cancer cell lines.^{21,34,35} Recent data have indicated that apoptotic caspases act in extrinsic and intrinsic pathways. Initiator caspases-8/-10 and -9 directly activate the effector procaspases-3 and -7.³⁶ PDT is involved in the intrinsic or mitochondrial apoptotic pathway.³⁷⁻³⁹

In the present study, annexin V-FITC apoptosis detection revealed that the percentage of apoptotic cells gradually increased up to 30.57%. This phenomenon was accompanied by rapid ROS generation. Quick ROS accumulation provoked mitochondrial damage and activated the intrinsic apoptotic pathway. To define the onset of apoptotic

pathways, our data tracked the cytosolic release of cytochrome c immediately after irradiation. The cytosolic release triggered the gradual expression of cleaved caspase-9, caspase-3, and PARP at 2 h after recovery (Fig. 3B). Also, these data provided evidence that 2 h was considered to be the key observation time in RB-mediated PDT-induced apoptosis in Cal27 cells. Some studies have shown that collapse or depolarization of mitochondria is one of the first measurable events during apoptosis, and that the release of cytochrome c into the cytosol activates the intrinsic apoptotic pathway to induce cell death.⁴⁰ These data demonstrated that RB-mediated PDT triggered the generation of intracellular ROS. This event was followed by the release of cytochrome c from the mitochondria, the cleavage of caspase activation, and other processes that lead to apoptosis in Cal27 cells.

Conclusions

In conclusion, the extent of photodamage and apoptosis after RB-mediated PDT is multifactorial. Establishing a real-time monitoring system will be a powerful method to elucidate the signal transduction network, which includes the net O₂ and Ca²⁺ fluxes and ROS in photosensitized cells. ROS function as upstream regulators of net O₂ and Ca²⁺ fluxes and activate cytochrome c, caspase-9, caspase-3, and PARP during PDT-induced apoptosis in OSCC cells.

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Author Disclosure Statement

No competing financial interests exist.

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