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A Highly Sensitive Chemiluminometric Assay for Real-Time Detection of Biological Hydrogen Peroxide Formation

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Abstract

Hydrogen peroxide (H_2O_2) is a major reactive oxygen species (ROS) produced by various cellular sources, especially mitochondria. At high levels, H_2O_2 causes oxidative stress, leading to cell injury, whereas at low concentrations, this ROS acts as an important second messenger to participate in cellular redox signaling. Detection and measurement of the levels or rates of production of cellular H_2O_2 are instrumental in studying the biological effects of this major ROS. While a number of assays have been developed over the past decades for detecting and/or quantifying biological H_2O_2formation, none has been shown to be perfect. Perhaps there is no perfect assay for sensitively and accurately quantifying H_2O_2 as well as other ROS in cells, wherein numerous potential reactants are present to interfere with the reliable measurement of the specific ROS. In this context, each assay has its own advantages and intrinsic limitations. This article describes a highly sensitive assay for real-time detection of H_2O_2 formation in cultured cells and isolated mitochondria. This assay is based on the luminol/horseradish peroxidase-dependent chemiluminescence that is inhibitable by catalase. The article discusses the usefulness and shortcomings of this chemiluminometric assay in detecting biological H_2O_2 formation induced by beta-lapachone redox cycling with both cells and isolated mitochondria.

Keywords

Beta-Lapachone; Chemiluminescence; Hydrogen peroxide; Luminol; Oxidative stress; Reactive oxygen species; Redox cycling; Redox signaling

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1. OVERVIEW

Hydrogen peroxide (H_2O_2) is a non-radical reactive oxygen species (ROS). In contrast to superoxide anion radical, H_2O_2 is relatively stable and can readily cross cell membranes via aquaporin-facilitated transmembrane diffusion [1, 2]. Although high levels of H_2O_2 cause oxidative stress, leading to cell and tissue injury, at low concentrations and when its formation is tightly controlled, H_2O_2 acts as a second messenger, participating in cell signal transduction [3, 4]. Therefore, accurate and sensitive detection and measurement of H_2O_2 in biological systems are of critical importance for investigating the physiological and pathophysiological roles of this ROS. Over the past decades, a number of assays have been developed for the detection and/or quantification of biological H_2O_2 formation. These methods can be classified into the following four categories: (1) fluorescence-based assays (e.g., the *N*-acetyl-3,7-dihydroxyphenoxazine oxidation assay, also known as the Amplex Red assay, which is available as a kit from various commercial sources) [5]; (2) chemiluminescence (CL)-based assays (e.g., the luminol/horseradish peroxidase-dependent CL assay described in this article) [6, 7]; (3) electrode-based assays (e.g., the H_2O_2 electrode assay) [8]; and (4) the most recently developed redox protein-based assays [9, 10]. Although numerous assays are available, currently there is no well-established assay for sensitive and specific detection and/or measurement of intracellular H_2O_2 production. The method described in this article is primarily for the detection of extracellular H_2O_2. Therefore, there is a strong need for developing assays that allow sensitive and selective detection of intracellular H_2O_2. Nevertheless, when appropriately used in well-defined biological systems, many of the currently available assays, including the one described in this article, could be used to estimate the formation of intracellular H_2O_2.

2. METHOD PRINCIPLES

The assay described in this article involves the use of the CL technique. Hence, it is imperative to first introduce the basic chemical principles of CL and chemiluminometry in detecting biological ROS, including H_2O_2.

2.1. General Principles of CL and Chemiluminometry

CL is the emission of light with limited release of heat as a result of a chemical reaction. Given reactants A and B, with an excited intermediate E*, the light-emitting reaction can be written as follows: [A] + [B] → [E*] → [Products] + light. For example, if [A] is luminol and [B] is H_2O_2 in the presence of a suitable catalyst (e.g., myeloperoxidase or horseradish peroxidase), the following reactions occur: luminol + H_2O_2 → [3-APA*] → 3-APA + light, where 3-APA denotes 3-aminophthalate, and 3-APA* is the excited state. The decay of 3-APA* to a lower energy level causes photon emission (Figure 1). It should be noted that CL differs from fluorescence in that the electronically excited state is derived from the product of a chemical reaction rather than the more typical way of creating electronically excited states, namely, absorption of energy that occurs in the process leading to fluorescence.

By measuring the photon emission using a luminometer, the rate of H_2O_2 formation in a biological system can be determined. Because the above CL response is dependent on an exogenously added peroxidase, such as horseradish peroxidase (HRP), which does not cross

cell membranes, the luminol/HRP-dependent CL response is indicative of the level of H_2O_2 released into the extracellular milieu.

For the detection of a particular ROS, a CL probe (e.g., luminol) along with other required reagents (e.g., HRP) is incubated with the ROS-generating system (e.g., enzymes, cells, tissues), and the light emission resulting from the chemical reactions between the probe and the ROS is measured by a luminometer (e.g., the Berthold LB9505 Multi-Channel Biolumat or LB953 AutoLumat) or a liquid scintillation counter. CL imaging can also be applied to visualize ROS formation in situ in cultured cells or tissues [11, 12].

Similar to fluorescence-based methods, CL assays represent a highly sensitive way of detecting ROS in biological systems. However, many CL probes have limited specificity for different ROS, and this limitation also applies to many other ROS-detecting probes, such as fluorescence probes.

2.2. Assay Principle of the Luminol/HRP-Dependent CL

As noted earlier in Section 2.1, the CL probe luminol is oxidized by H_2O_2 in the presence of HRP to yield light emission. Hence, luminol-derived CL in the presence of exogenously added HRP can be used to sensitively and selectively detect extracellular H_2O_2 [6]. The kinetic mode of modern luminometers allows the real-time detection of the CL responses over time at various temperatures, which may yield important information on the rate of H_2O_2 flux in the extracellular milieu. Because H_2O_2 like water can readily diffuse across cell membranes via an aquaporin-dependent mechanism [1, 2], intracellularly generated H_2O_2 can be instantly released into the extracellular space. Hence, under well-defined conditions, by detecting extracellular H_2O_2 release, intracellular H_2O_2 formation can be estimated. This is particularly useful for studying chemical redox cycling inside cells to form H_2O_2 (Figure 2). It is noteworthy that in addition to H_2O_2, other oxidants might also be involved in the reactions leading to luminol/HRP-dependent CL. To determine the specificity of the luminol/HRP-dependent CL for detecting H_2O_2 formation, catalase can be added to the reaction mix to determine catalase-inhibitable luminol/HRP-dependent CL.

3. MATERIALS AND INSTRUMENTS

This article describes the catalase-inhibitable luminol/HRP-dependent CL assay for detecting H_2O_2 formation by two biological systems: cultured Lewis lung carcinoma (LLC) cells and intact mitochondria isolated from the LLC cells.

3.1. Major Materials

The major materials for the catalase-inhibitable luminol/HRP-dependent CL assay are listed alphabetically in Table 1.

3.2. Major Instruments

1. Luminometers: Berthold LB9505 Multi-Channel Biolumat or Berthold LB950 AutoLumat
2. Beckman Coulter DU-800 ultraviolet/visible (UV/Vis) spectrophotometer

3. Dounce tissue grinder
4. Water bath or dry bath
5. Cell culture equipment (CO_2 incubator, tissue culture hood, centrifuge, microscope, etc.)

4. PROTOCOLS AND STEPS

This section describes the detailed protocols and steps involved in the use of the catalase-inhibitable luminol/HRP-dependent CL assay to detect H_2O_2 generated from cultured LLC cells as well as isolated mitochondria in the presence of beta-lapachone, a potential anticancer compound that undergoes redox cycling to generate superoxide and H_2O_2 [13].

4.1. Cultured Cells

4.1.1. Assay Layout—Figure 3 illustrates the assay layout for determination of the catalase-inhibitable luminol/HRP-dependent CL resulting from H_2O_2 generated by redox cycling of beta-lapachone in LLC cells in CPBS at 37°C for 30 min. It should be noted that the incubation time can vary from a few seconds to a few hours depending on the experimental design.

4.1.2. Assay Description—The catalase-inhibitable luminol/HRP-dependent CL assay is used to detect extracellular H_2O_2 released from cultured LLCs. Briefly, LLC cells are cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells are fed every 2–3 days, and subcultured once they reach 80–90% confluence. For the CL measurement, LLC cells are harvested from the cultures and washed once in CPBS. As many as 1×10^6 cells are suspended in 1 ml CPBS in a CL tube containing 1 μM beta-lapachone, 10 μg/ml of HRP, and 10 μM luminol in the presence or absence of 500 units/ml of catalase, and the CL tube is immediately transferred to a Berthold LB9505 luminometer for recording the CL response at 37°C for 30 min. The results are expressed as the real-time photon emission curve (CL response) and the integrated CL response (the area under the curve, representing the total counts of photon emission over the 30 min of incubation time).

4.1.3. Preparation of Reagents

1. Luminol solution (2 mM in PBS): 7.96 mg luminol sodium (molecular mass = 199.14) dissolved in 2 ml PBS (aliquot into microfuge tubes and store at −20°C).
2. HRP solution (2 mg/ml in PBS): 4 mg HRP dissolved in 2 ml PBS (aliquot into microfuge tubes and store at −20°C).
3. Beta-Lapachone solution (1 mM in dimethyl sulfoxide): first prepare 10 mM beta-lapachone (molecular mass = 242.27) (add 2.06 ml dimethyl sulfoxide to a vial containing 5 mg beta-lapachone), and then dilute this 10 mM beta-lapachone solution in dimethyl sulfoxide to yield a 1 mM solution.

4. Catalase solution (100,000 units/ml in PBS): 50 mg catalase (2,300 units/mg) dissolved in 1.15 ml PBS (aliquot into microfuge tubes and store at −20°C).

4.1.4. Steps

1. Aliquot 1×10^6 cells into each of the 4 microfuge tubes followed by centrifugation to pellet the cells and discard of the medium. Keep the cell pellets on ice.
2. Add 5 μl of 2 mM luminol to each of the 4 CL tubes.
3. Add 5 μl HRP (2 mg/ml) to the CL tubes 1, 2, and 4, and 5 μl PBS to the CL tube 3.
4. Add 5 μl catalase (100,000 units/ml) to the CL tube 4, and 5 μl PBS to the CL tubes 1, 2, and 3.
5. Add 1 μl of 1 mM beta-lapachone to the CL tubes 2, 3, and 4, and 1 μl dimethyl sulfoxide (DMSO) to the CL tube 1.
6. Add 984 μl of 37°C-prewarmed air-saturated CPBS to the microfuge tube 1, as indicated in Step (1) above, that contains 1×10^6 cells to resuspend the cells. Transfer the entire cell suspension to the CL tube 1 immediately followed by mixing of the sample and recording of the CL response at 37°C for 30 min. Do the same for the remaining 3 samples. The background CL levels of the instrument are measured with the CL tubes containing all reactants/components but cells. The background CL levels are subtracted from the CL responses of the tubes containing cells so that the reported CL responses in the figures are due to cellular activity.

4.1.5. Calculations—The Berthold LB9505 multi-channel luminometer enables real-time measurement of the CL responses from each of the 4 samples (CL tubes) which are reported as the CL response curves (see Figure 3C) with the unit of the Y and X axis being counts of photon emission per minute (CPM) and minute, respectively. The luminometer automatically calculates the area under the curve for each of the CL response curves from the 4 samples. This is designated as the integrated CL (see Figure 3D) with the unit being total counts of photon emission over the 30 min of incubation time per 1×10^6 cells.

4.1.6. Other Considerations—The Berthold LB9505 multi-channel luminometer can measure up to 6 samples simultaneously, whereas the newer model of Berthold LB953 luminometer with auto-injectors can measure much more samples simultaneously under its kinetic mode. However, our laboratories have consistently noticed that the Berthold LB9505 luminometer is probably one of the most sensitive luminometers for detecting CL responses from biological systems, including tissues, intact cells, and isolated mitochondria, as well as other subcellular components. Both the Berthold LB9505 and LB953 luminometers are equipped with a temperature control unit, allowing the measurement of the CL responses at various temperatures including 37°C. Although this article describes the use of the Berthold LB9505 luminometer in detecting biological H_2O_2formation, the protocols can be easily adopted to detect biological H_2O_2 formation with other commercially available

luminometers if the detection sensitivity is not a major concern. As many of the commercially available luminometers are not equipped with the kinetic and temperature control units, to detect the time- and temperature-dependent CL responses, the sample CL tubes can be incubated at various temperatures in a water bath or dry bath and then transferred to the luminometer for the measurement of the CL responses at different time intervals to obtain a time-dependent response.

4.2. Isolated Mitochondria

4.2.1. Assay Layout—Figure 4 illustrates the assay layout for determination of the catalase-inhibitable luminol/HRP-dependent CL responses resulting from H_2O_2 generated by redox cycling of beta-lapachone in isolated mitochondria at 37°C for 30 min. It should be noted that the incubation time can vary from a few seconds to a few hours depending on the experimental requirement and the luminometer used.

4.2.2. Assay Description—The catalase-inhibitable luminol/HRP-dependent CL assay is used to detect H_2O_2 released from intact mitochondria isolated from LLC cells. Briefly, LLC cells are cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells are fed every 2–3 days, and subcultured once they reached 80–90% confluence. Mitochondria are isolated from the freshly harvested LLC cells according to the method described before [14]. Briefly, LLC cells (~3×10^7 cells) are harvested and washed once with PBS. The cell pellet is resuspended in 6 ml of sucrose buffer (250 mM sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% bovine serum albumin, pH 7.4) and homogenized in a Dounce tissue grinder (40 strokes with the tight pestle) on ice. The homogenate is centrifuged at 1,500 × *g* for 10 min at 4°C. The supernatant is collected and centrifuged at 10,000 × *g* for 10 min at 4°C. The resulting mitochondrial pellet is washed twice with the sucrose buffer and then resuspended in bovine serum albumin (BSA)-free sucrose buffer (250 mM sucrose, 10 mM Hepes, and 1 mM EGTA, pH 7.4). The mitochondrial protein is measured with Bio-Rad protein assay dye based on the method of Bradford [15] with BSA as the standard. The mitochondrial suspension is diluted to 1 mg/ml in the above BSA-free sucrose buffer and kept on ice for experiments within 3 hours. For the CL measurement, 5 μl of mitochondrial suspension (giving a final concentration of 0.05 mg mitochondrial protein/ml) is added to a CL tube containing the various reactants (250 μM succinate, 1 μM beta-lapachone, 10 μg/ml of HRP, and 10 μM luminol in the presence or absence of 500 units/ml of catalase) in the respiration buffer (in a final volume of 1 ml; 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 0.1% BSA, pH 7.4), and the CL tube is immediately transferred to the Berthold LB9505 luminometer for recording the CL responses at 37°C for 30 min. The results are expressed as the real-time photon emission (CL response curve) and the integrated CL response (the area under the curve, representing the total counts of photon emission over the 30 min of incubation time).

4.2.3. Preparation of Reagents

1. Luminol solution (2 mM in PBS): see Section 4.1.3 for preparation.

2. HRP solution (2 mg/ml in PBS): see Section 4.1.3 for preparation.
3. Beta-Lapachone solution (1 mM in dimethyl sulfoxide): see Section 4.1.3 for preparation.
4. Catalase solution (100,000 units/ml in PBS): see Section 4.1.3 for preparation.
5. Succinate solution (0.5 M in deionized water): 1.35 g sodium succinate dibasic hexahydrate (molecular mass = 270.14) dissolved in 10 ml deionized water (aliquot into microfuge tubes and store at −20°C).

4.2.4. Steps

1. Add 5 μl of 0.5 M succinate to each of the 4 CL tubes.
2. Add 5 μl of 2 mM luminol to each of the 4 CL tubes.
3. Add 5 μl of HRP (2 mg/ml) to the CL tubes 1, 2, and 4, and 5 μl PBS to the CL tube 3.
4. Add 5 μl catalase (100,000 units/ml) to the CL tube 4, and 5 μl PBS to the CL tubes 1, 2, and 3.
5. Add 1 μl of 1 mM beta-lapachone to the CL tubes 2, 3, and 4, and 1 μl dimethyl sulfoxide (DMSO) to the CL tube 1.
6. Add 974 μl of 37°C-prewarmed air-saturated mitochondrial respiration buffer to the CL tube 1 followed by the addition of 5 μl of the mitochondrial suspension (1 mg/ml). Upon mixing, the CL tube is transferred to the LB9505 luminometer for recording the CL response at 37°C for 30 min. Do the same for the remaining 3 samples. It is worth mentioning that the background CL levels of the instrument are measured with the CL tubes containing all reactants/components but mitochondria. The background CL levels are subtracted from the CL responses of the tubes containing mitochondria so that the reported data of CL responses in the figures are due to mitochondrial activity.

4.2.5. Calculations—The Berthold LB9505 multi-channel luminometer enables real-time measurement of the CL responses from each of the 4 samples (CL tubes) which are reported as the CL response curves (see Figure 4C) with the unit of the Y and X axis being CPM and minute, respectively. The luminometer automatically calculates the area under the curve for each of the CL response curves from the 4 samples. This is designated as the integrated CL (see Figure 4D) with the unit being total counts of photon emission over the 30 min of incubation time per 0.05 mg mitochondria.

4.2.6. Other Considerations—In addition to those mentioned in Section 4.1.6, the protocols described in this article can be easily adopted to study the effects of other mitochondrial substrates, such as pyruvate/malate, which provide NADH, thus feeding the electrons through the mitochondrial complex I. It is noteworthy that in addition to using this assay to study chemical redox cycling in mitochondria, the assay can also be employed to detect H_2O_2 formation by isolated mitochondria under various conditions. In this context, mitochondria can be isolated from cells or tissues under certain pathophysiological

conditions to determine the altered formation of mitochondrial H_2O_2. Notably, mitochondria are the major source of cellular H_2O_2under diverse physiological and pathophysiological conditions and mitochondria-derived H_2O_2 plays an important role in biology and medicine [16–18].

5. DISCUSSION OF ADVANTAGES AND LIMITATIONS

5.1. Advantages

As with other CL-based techniques, the biggest advantage of the luminol/HRP-dependent CL assay is its high sensitivity for detecting biological H_2O_2 formation. With the kinetic mode of the Berthold LB9505 (as well as the Berthold LB953) luminometer, the relative rate of formation of H_2O_2 in a biological system can be sensitively detected in a real-time manner. Examining the shapes of the CL response curves may also yield important insight into the dynamics of H_2O_2formation in a biological system under a particular physiological or pathophysiological condition.

5.2. Limitations

The luminol/HRP-dependent CL assay for detecting biological H_2O_2 formation is not without limitations. As noted earlier, this assay only detects the rate of H_2O_2 flux in the extracellular or extramitochondrial milieu. Because intracellularly formed H_2O_2 can readily penetrate the biomembranes to enter the extracellular/extramitochondrial milieu, detection of the rate of extracellular/extramitochondrial H_2O_2 flux can, however, be used to reflect the dynamics of H_2O_2 formation from the intracellular sources. Another shortcoming of the assay is related to its limited ability to directly quantify the absolute amounts of H_2O_2 formed in a biological system. To circumvent this limitation, a concurrently run experiment using known amounts of H_2O_2 may be considered to obtain a standard curve (i.e., a linear relationship between the integrated luminol/HRP-dependent CL and the amounts of exogenously supplemented H_2O_2). The exogenously supplemented H_2O_2 may be in the form of either a H_2O_2 solution of known concentration or preferentially a known rate of H_2O_2 formation by a standard enzymatic system, such as the glucose/glucose oxidase. Nevertheless, it should be borne in mind that due to the complexity of reactions involved in detecting H_2O_2 as well as other ROS in biological systems, the reliability of such a standard curve for quantifying biological H_2O_2 should be rigorously tested and validated.

6. CONCLUSION

As with many other assays and techniques for detecting biological H_2O_2 as well as other members of the ROS family, the luminol/HRP-dependent CL assay has its own advantages and limitations. The biggest advantage of this assay is its high sensitivity and real-time detection of H_2O_2 formation. On the other hand, a major limitation of the assay is its inability to directly detect intracellular H_2O_2 formation. In addition, the assay does not allow quantification of H_2O_2 in biological systems unless a concurrently run standard curve is employed. Nevertheless, as mentioned earlier, if appropriately used under controlled conditions along with the results being correctly interpreted, detection of H_2O_2 by the luminol/HRP-dependent CL assay can yield important information on the real-time

formation of this ROS in various biological systems under different physiological and pathophysiological conditions. In fact, detecting the changes in the relative rate of H_2O_2formation, rather than the absolute amounts of H_2O_2 formed, under different conditions, is frequently the goal of the experiments towards understanding the role of this important ROS in biology and medicine.

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ABBREVIATIONS

3-APA	3-aminophthalate
BSA	bovine serum albumin
CL	chemiluminescence
CPBS	complete phosphate-buffered saline
CPM	counts of photon emission per minute
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
PBS	phosphate buffered saline
ROS	reactive oxygen species

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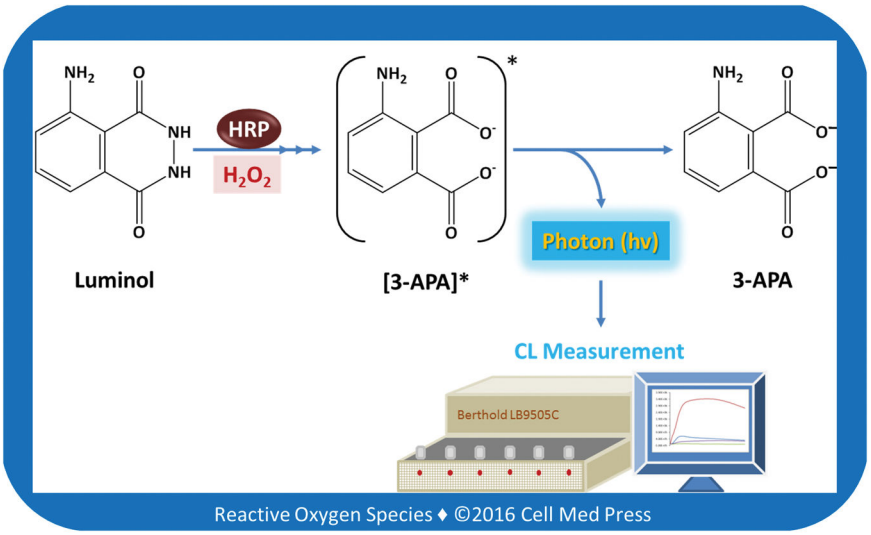

FIGURE 1. Schematic illustration of the major chemical reactions leading to luminol-dependent CL

As shown, in the presence of HRP, luminol reacts with hydrogen peroxide, leading to the eventual formation of the excited state of 3-aminophthalate (3-APA*), whose decay to a lower energy level results in photon emission, which is measured luminometrically as the chemiluminescence (CL) response.

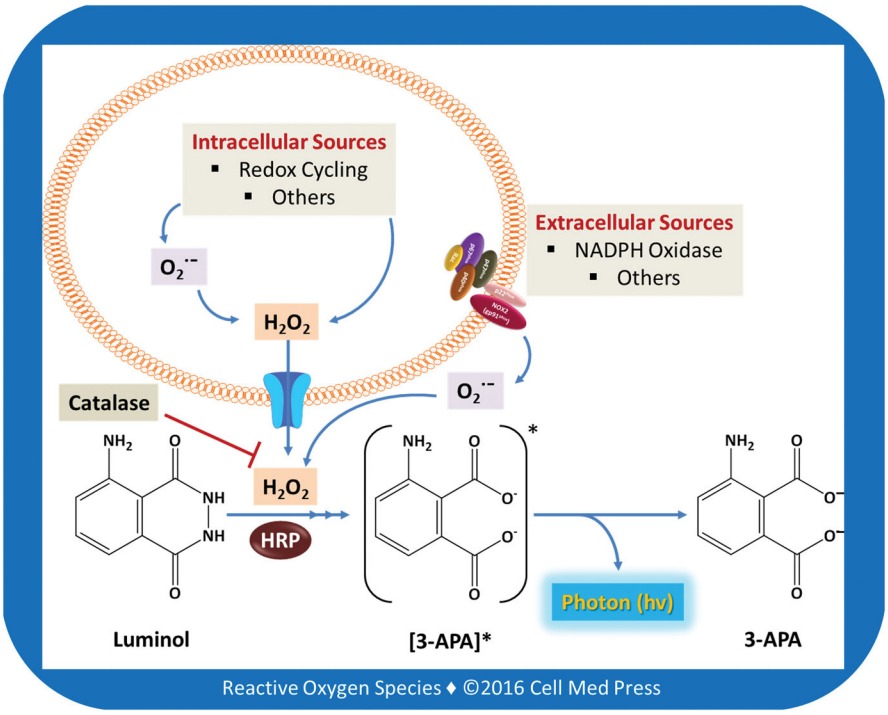

FIGURE 2. Detection of extracellular hydrogen peroxide by the luminol/HRP-dependent CL in cells

As depicted, H_2O_2 in the extracellular milieu may be either generated by membrane-associated enzymes such as NADPH oxidases or derived from intracellular sources (e.g., chemical redox cycling). In this context, intracellularly generated H_2O_2 can diffuse out of cells via an aquaporin-facilitated mechanism. Inclusion of exogenous catalase in the assay, i.e., detection of catalase-inhibitable luminol/HRP-dependent CL, makes it possible to specifically determine the flux of H_2O_2 in the extracellular milieu.

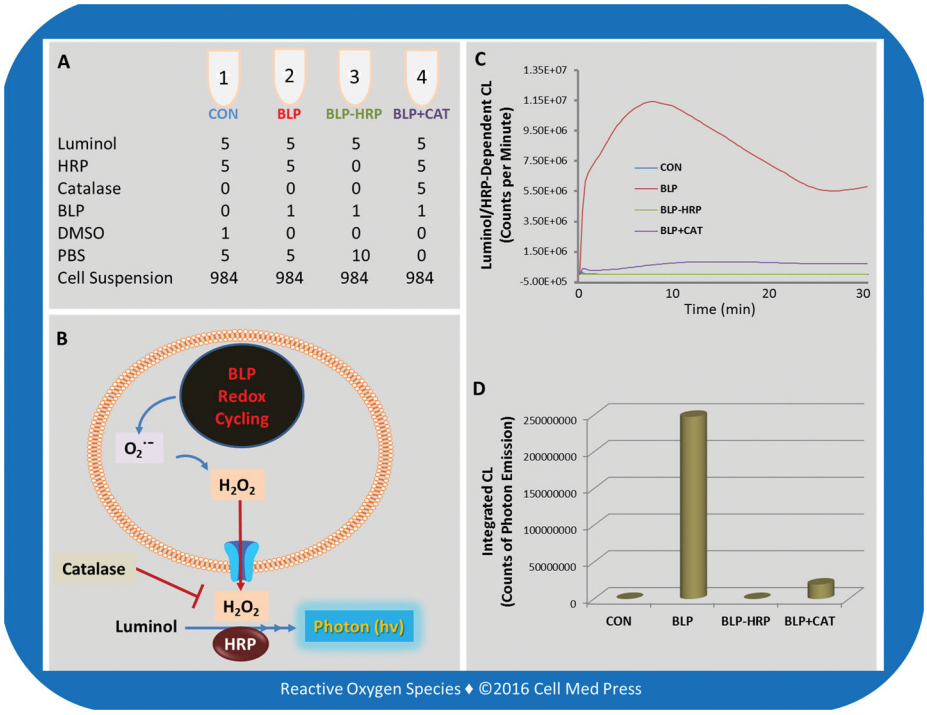

FIGURE 3. Assay layout for the catalase-inhibitable luminol/HRP-dependent CL responses in LLC cells treated with beta-lapachone

Redox cycling of beta-lapachone (BLP) in LLC cells generates superoxide which undergoes spontaneous or superoxide dismutase-catalyzed dismutation to form H_2O_2. The H_2O_2 formed can readily defuse out of cells and react with luminol in the presence of HRP, resulting in CL responses. Addition of catalase (CAT) inhibits the CL response by >92%, indicating that the assay under the present experimental conditions selectively detects extracellular H_2O_2. Notably, the CL response is completely abolished in the absence of added HRP, indicating that the CL response is dependent on the presence of HRP, thus confirming that the CL response occurs in the extracellular milieu. The unit in panel A is μl.

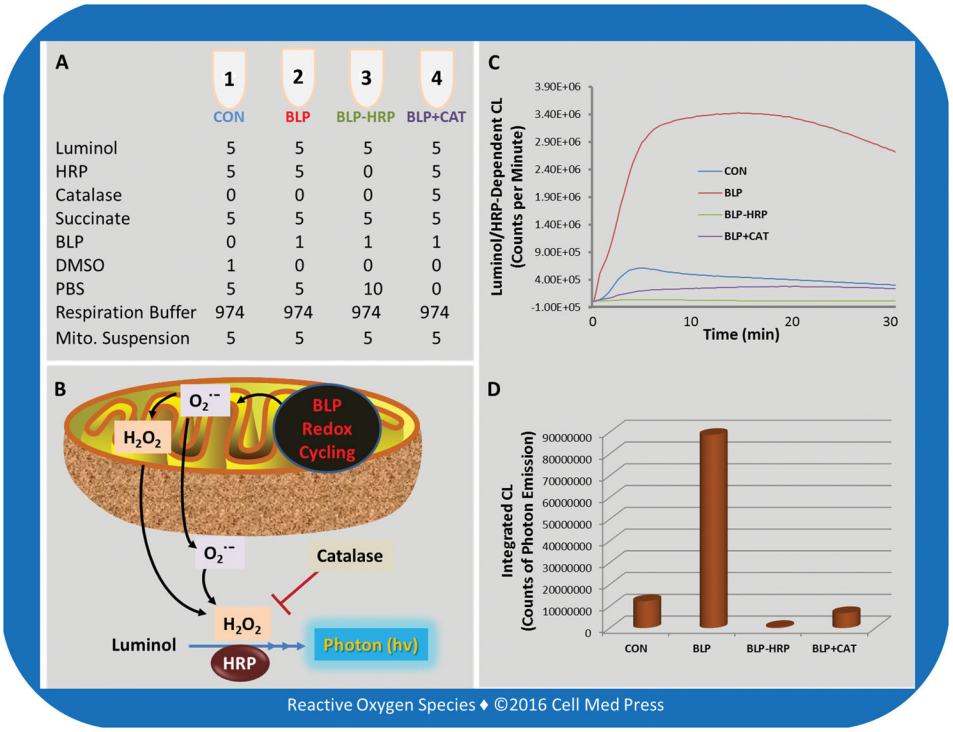

FIGURE 4. Assay layout for the catalase-inhibitable luminol/HRP-dependent CL responses in isolated mitochondria treated with beta-lapachone

Redox cycling of beta-lapachone (BLP) in succinate-driven mitochondria generates superoxide which undergoes spontaneous or superoxide dismutase-catalyzed dismutation to form H_2O_2. The H_2O_2formed can readily diffuse out of mitochondria and react with luminol in the presence of HRP, resulting in CL responses. Superoxide may also exit mitochondria and then undergo spontaneous dismutation, contributing to the extramitochondrial H_2O_2 formation. Addition of catalase (CAT) inhibits the CL response by >92%, indicating that the assay under the present experimental conditions selectively detects the formation of extramitochondrial H_2O_2. The CL response is completely abolished in the absence of added HRP, indicating that the CL response is dependent on the presence of HRP, confirming that the CL response occurs in the extramitochondrial milieu. The unit in panel A is μl. Mito. denotes mitochondrial.

Table 1

Major materials for the catalase-inhibitable luminol/HRP-dependent CL assay

Major Material	Vendor and Catalog Number	Note
Bio-Rad protein assay dye reagent concentrate	Bio-Rad; 5000006	For measurement of mitochondrial protein
Catalase	Sigma-Aldrich; C9322	Source: bovine liver; 2,300 units/mg
Complete phosphate-buffered saline (CPBS)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, 2.67 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, and 0.1% glucose, pH 7.4
Horseradish peroxidase (HRP)	Sigma-Aldrich; P8250	Type II; source: horseradish roots (*Amoracia rusticana*); molecular mass: ~44 kDa
Beta-Lapachone	Sigma-Aldrich; L2037	5 mg size
Lewis lung carcinoma (LLC) cells	ATCC; CRL-1642	LLC cells are derived from Lewis lung carcinoma in C57BL/6 mice.
Luminol (sodium salt)	Sigma-Aldrich; A4685	Luminol sodium salt is soluble in water (50 mg/ml), yielding a clear solution.
Mitochondrial isolation buffer	Investigator-prepared	Components: 250 mM sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% bovine serum albumin, pH 7.4
Mitochondrial respiration buffer	Investigator-prepared	Components: 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 0.1% bovine serum albumin, pH 7.4
Phosphate-buffered saline (PBS)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, and 2.67 mM KCl, pH 7.4
Succinate	Sigma-Aldrich; S2378	Sodium succinate dibasic hexahydrate