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In Vivo Bioluminescence Imaging of Nuclear Factor kappaB Activation: A Valuable Model for Studying Inflammatory and Oxidative Stress in Live Mice

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ABSTRACT | The nuclear factor kappaB (NF-κB) is a redox-sensitive transcription factor that plays a critical role in inflammation among other biological functions. This ROS Protocol article describes an in vivo bioluminescence imaging assay for assessing NF-κB activation using the commercially available transgenic mice carrying NF-κB response element-luciferase reporter gene (NF-κB-RE-Luc). Using the highly sensitive Berthold NightOwl LB981 in vivo bioluminescence imaging system, we are able to visualize the NF-κB activation in live mice under basal conditions, suggesting constitutive activation of NF-κB as a part of its fundamental biology. Treatment of mice with lipopolysaccharides (LPS) results in a drastic increase in bioluminescence, proving the validity of the model in assessing inflammatory stress. Treatment of mice with 3*H*-1,2-dithiole-3-thione (D3T), an activator of nuclear factor E-2 related factor 2 (Nrf2), led to a significant reduction in both basal and LPS-induced activation of NF-κB in the live mice, suggesting a value of this model in assessing drug efficacy in suppressing NF-κB activation and inflammatory stress. The protocols of this valuable model are detailed in this article along with a discussion of its potential use in studying disease conditions involving inflammatory and oxidative stress mechanisms and in assessing therapeutic modalities targeting the NF-κB signaling for disease intervention.

KEYWORDS | 3*H*-1,2-Dithiole-3-thione; In vivo bioluminescence imaging; Nuclear factor kappaB; Inflammatory stress; Oxidative stress

ABBREVIATIONS | D3T, 3*H*-1,2-dithiole-3-thione; LPS, lipopolysaccharides; NF-κB, nuclear factor kappaB; Nrf2, nuclear factor E-2 related factor 2; PBS, phosphate-buffered saline

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CONTENTS

1. OVERVIEW

In vivo imaging of biological activities in live animals has been becoming an important tool in biomedical research. In this context, in vivo bioluminescence imaging provides a unique approach to visualizing the biochemical alterations in tissues and organs of live animals while causing little or no harm, which is in contrast to radiological imaging that introduces significant damage to the subjects. Indeed, bioluminescence imaging has been widely used to study tumor growth and metastasis in diverse animal models [1–4].

Recently, a mouse model carrying a transgene containing 6 nuclear factor kappaB (NF-κB)-response elements from the CMVα (immediate early) promoter placed upstream of a basal SV40 promoter, and a modified firefly luciferase cDNA, namely, the NFκB-RE-luc mouse, has become available [5]. This mouse model on BALB/c background has been used to study the in vivo activation of NF-κB, a redox-sensitive transcription factor that plays important roles in regulating genes responsible for innate and adaptive immune responses as well as other biological processes, such as cell proliferation and survival, tumorigenesis, learning and memory, as well as addiction [6].

In this ROS Protocol article, we describe the detailed protocols for the in vivo imaging of NF-κB activation under basal conditions and following treatment with bacterial lipopolysaccharides. We also discuss the value of using this novel animal model in assessing inflammatory and oxidative stress conditions and their therapeutic intervention.

2. METHOD PRINCIPLES

The NFκB-RE-luc mouse was developed by Caliper Life Sciences (Hopkinton, MA, USA). The model was created by microinjecting a transgene containing 6 NFκB-responsive elements (RE) from the CMVα (immediate early) promoter placed upstream of a basal SV40 promoter, and a modified firefly luciferase cDNA (Promega pGL3). This transgene was microinjected into BALB/cJ zygotes. The resultant mice from founder line 31 were bred to BALB/cJ mice.

Taconic Biosciences (Hudson, NY, USA) received stock from Caliper Life Sciences in 2010, and the line was embryo transfer derived. In the production colony, the line is maintained by mating mice which are wild type to those which are hemizygous for the luciferase transgene. In response to diverse stimuli, including oxidants and lipopolysaccharides, NF-κB translocates to the nuclei, binding to the NF-κB response elements of the transgene, leading to the increased expression of luciferase, which gives rise to light emission upon reacting with D-luciferin given via injection (typically, via intraperitoneal injection) (**Figure 1**). As D-luciferin is absorbed rapidly and distributes throughout the body, tissues and organs with NF-κB activation will give out light that can be captured by a sensitive camera, thereby producing in vivo imaging of NF-κB activation. The intensity of the imaging correlates to the intensity of the light emission from different parts of the animal body, which in turn correlates to the intensity of the in situ NF-κB activation.

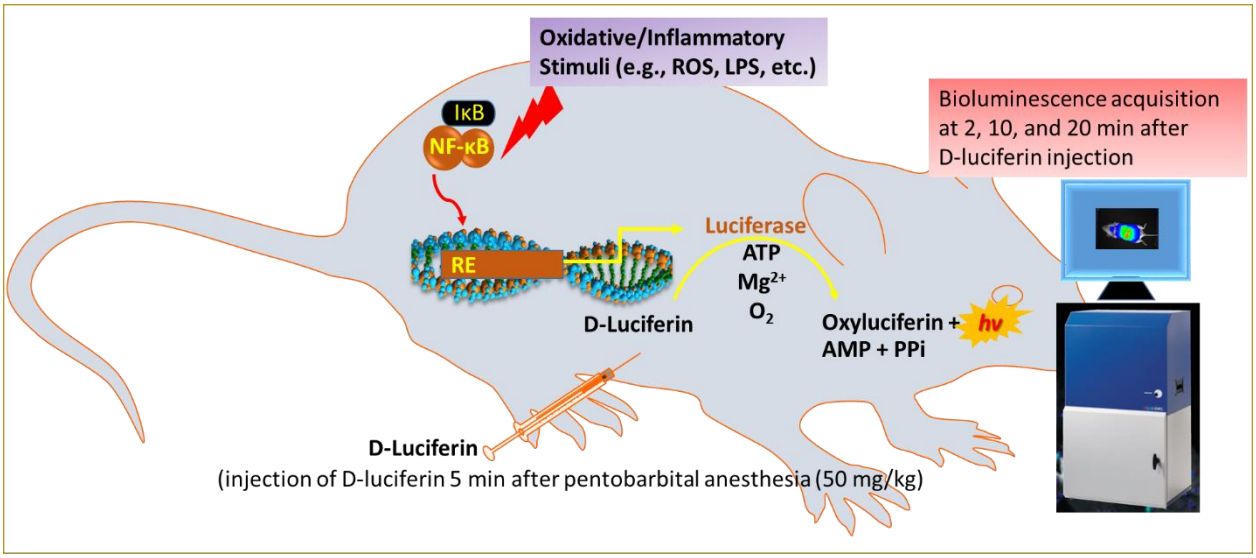

FIGURE 1. Schematic illustration of the assay principle and layout. The scheme depicts the in vivo bioluminescence imaging of NF-κB activation in live animals following inflammatory/oxidative stress using the NFκB-RE-luc mouse model.

3. MATERIALS AND INSTRUMENTS

3.1. Animals and Major Materials

(1) Animals: Male NFκB-RE-luc random transgenic mice on BALB/c background were obtained from Taconic Biosciences. Mice at the age of 7–8 weeks were used in the experiments. These mice were housed in an institutional animal research facility with a light period from 6 am to 6 pm. Purified AIN-93G chow (BioServ, NJ, USA) and water were available ad libitum. All mice were allowed to acclimate for at least one week prior to the experiments. The animal procedures were approved by the Institutional Animal Care and Use Committee in compliance with the pertinent U.S. Federal policy.

(2) D-Luciferin: D-Luciferin was obtained from PerkinElmer (Cat. No. 122799, Waltham, MA, USA). D-Luciferin was dissolved in Dulbecco's phosphate-buffered saline and stored at –90ºC.

(3) Dulbecco's phosphate-buffered saline (PBS): It was prepared by the investigators, and the components include 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, and 2.67 mM KCl, pH 7.4 in deionized water.

(4) 3*H*-1,2-Dithiole-3-thione (D3T): D3T was synthesized by SRI International (Menlo Park, CA, USA) with a reported purity of 99.0% (Lot No.13886JB-26), and this synthesis was sponsored by the United States National Institutes of Health/National Institute of Mental Health (NIH/NIMH, Bethesda, MD, USA).

(5) Lipopolysaccharides (LPS): LPS from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich (Cat. No. L2630, St. Louis, MO, USA) and stored at –90ºC.

(6) Pentobarbital: Pentobarbital (50 mg/ml) was manufactured by Abbott Laboratories (Chicago, IL, USA)

3.2. Major Instruments

(1) Berthold NightOwl LB981 bioluminescence imaging system (Wildbad, Germany).

4. PROTOCOL AND STEPS

4.1. Assay Layout

The assay layout is depicted in **Figure 1**.

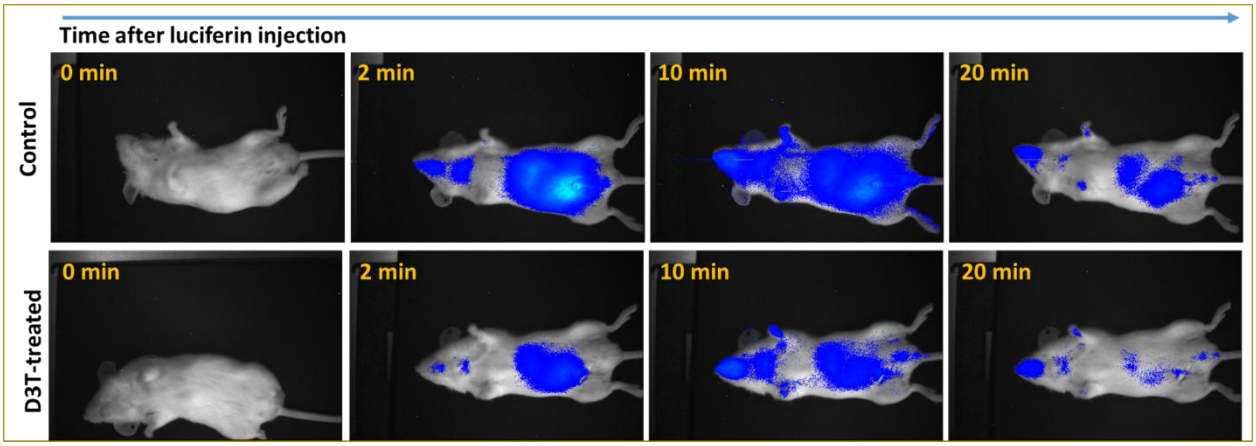

FIGURE 2. Representative in vivo bioluminescence imaging of constitutive NF-κB activation in NFκB-RE-luc mice and the effects of D3T treatment. The imaging was acquired at the indicated time points after injection of D-luciferin. D3T dosage was 0.3 mmol/kg body weight.

4.2. Assay Description

The in vivo imaging experiment is performed with a Berthold NightOwl LB981 imaging system (Wildbad, Germany) following intraperitoneal injection of D-luciferin (0.6 mg/30 g body weight) in a volume of 30 µl/30 g body weight (dissolved in PBS) to the anesthetized mouse. At different time points following D-luciferin injection, the images are acquired at room temperature for 2 min and analyzed using the WinLight32 software. The final images are created by overlaying the bioluminescence images on top of the photos of the respective animals. The 3D images are constructed using the WinLight32 software, and the rate of total light emission of the whole animal body area is calculated and expressed as counts (numbers of photons emitted) per second or counts per square mm of body surface.

4.3. Preparation of Reagents

(1) D-Luciferin solution (20 mg/ml in PBS): 20 mg D-luciferin dissolved in 1 ml cold PBS (aliquot into microfuge tubes and stored at −90ºC). Intraperitoneal injection of 30 µl of the above D-luciferin solution per 30 g body weight gives a final dosage of 0.6 mg/ 30 g body weight.
(2) D3T suspension (10 mg/ml vehicle): 50 mg D3T dispersed into 5 ml vehicle containing 25% glycerol and 1% cremophor. The molecular mass of D3T is 134.25. Oral gavage of 0.1 ml of the above D3T suspension per 25 g body weight gives to a final dosage of 0.3 mmol/kg body weight.
(3) LPS (2.5 mg/ml PBS): 10 mg LPS (one vial) dissolved in 4 ml cold PBS (aliquot into microfuge tubes and stored at −90ºC). Intraperitoneal injection of 0.1 ml of the above LPS solution per 30 g body weight gives a final dosage of 0.25 mg/30 g body weight.

4.4. Steps

(1) Anesthetize the mouse via intraperitoneal injection of pentobarbital (50 mg/ml, Abbott Laboratories, Chicago, IL, USA) at a dose of 50 mg /kg body weight. It typically takes 3–5 min for the animal to become completely anesthetized.
(2) Inject D-luciferin intraperitoneally at a dosage of 0.6 mg/30 g body weight and immediately transfer the animal to the Berthold LB981 imager and start acquisition at various time points following D-luciferin injection. Photographs are also taken at the corresponding time points.
(3) Overlay the image on top of the photograph to locate the body areas of light emission using the WinLight32 software and use the same software to calculate the rate of photo emission.

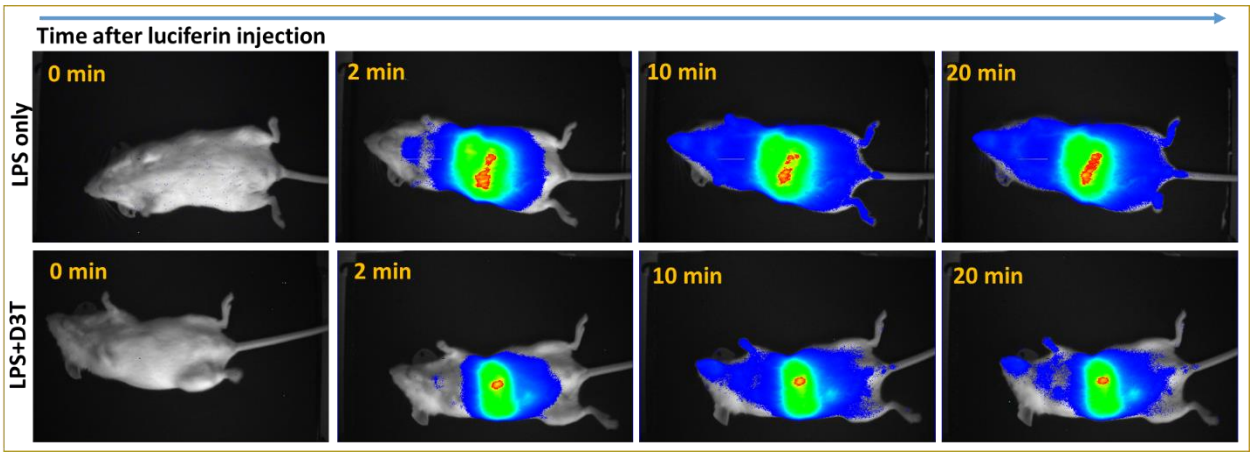

FIGURE 3. Representative in vivo bioluminescence imaging of LPS-induced NF-κB activation in NFκB-RE-luc mice and the effects of D3T treatment. The imaging was acquired at the indicated time points after injection of D-luciferin. D3T dosage was 0.3 mmol/kg body weight.

4.5. Calculations

The rate of photon emission is calculated using the WinLight32 software and expressed as counts per second and counts per square mm of whole body area. The data in the histograms represent mean ± SD of the values obtained at 2, 10, and 20 min following D-luciferin injection.

4.6. Other Considerations

Berthold NightOwl LB981 bioluminescence imaging system is highly sensitive and was able to pick up the light emission from live mice under basal conditions (**Figure 2**). The intensity of light emission was greatly increased upon bacterial LPS stimulation (**Figure 3**). The imaging intensity and shapes of NF-κB activation both under basal conditions and induced by LPS in mice treated with or without D3T (an antioxidative stress and anti-inflammation compound) showed dependence on the time points following D-luciferin injection. Light emission initially localized to the abdominal area immediately (2 min) after D-luciferin injection, and gradually spread to other areas, including the head and extremities. Notably, in LPS-treated animals, the imaging intensity and shapes became relatively stable between 2 and 20 min following D-luciferin injection, which would provide a wide time window for image acquisition and comparison between different animal groups (**Figure 3**). Measurement of the light emission as counts per square mm body surface or counts per second per whole body area at 2, 10, and 20 min after D-luciferase injection showed a moderate variation among the three time points (**Figure 4**). Although this article only presented in vivo imaging data on live mice, it is also possible to obtain imaging of individual organs and tissues immediately following collection from the live mice. Furthermore, cells and tissues can also be obtained for assessing the NF-κB activation via detecting luciferase activity using a luminometer. These studies would generate data on differential activation of NF-κB among different tissues and cells under various conditions.

5. DISCUSSION OF ADVANTAGES AND LIMITATIONS

The major advance of in vivo bioluminescence imaging is the real-time visualization of the underlying biochemical activities in different body areas of live mice without causing significant harm to the animals. As NF-κB plays critical roles in diverse biological processes, especially inflammatory responses [7], studying with the NFκB-RE-luc mice may yield important insights into the involvement of NF-κB signaling in disease processes and the development of

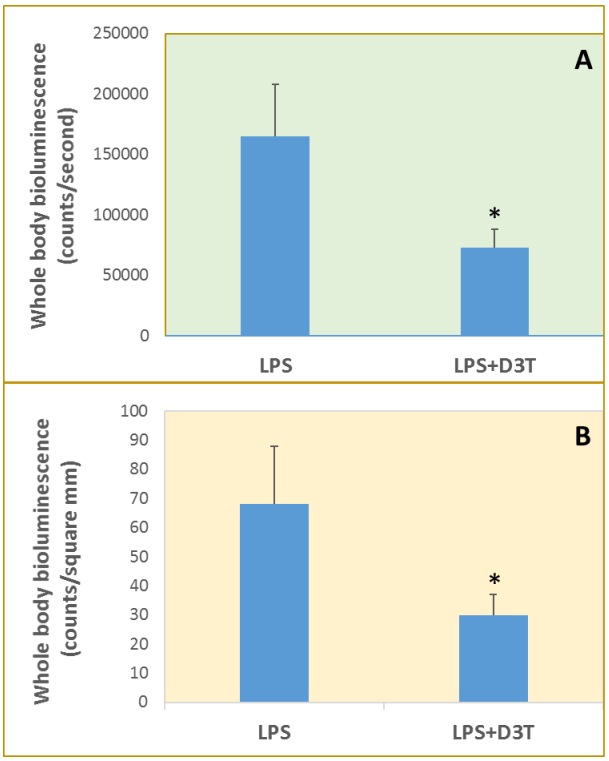

FIGURE 4. Integrated whole-body bioluminescence intensity derived from LPS-induced NF-κB activation in NFκB-RE-luc mice and the effects of D3T treatment. Refer to the legend of Figure 3 for experimental conditions. Data represent mean ± SD from the imaging intensity measurements at 2, 10, and 20 min after D-luciferin injection. *, $p < 0.05$ compared to the LPS alone group.

modalities for the intervention of conditions involving dysregulation of NF-κB signaling. In this context, as shown in **Figures 2–4**, administration of D3T, a potent activator of nuclear factor E2-related factor 2 (Nrf2) [8], led to a significant suppression of both basal and LPS-induced activation of NF-κB. Nrf2 activation has been reported to attenuate NF-κB activation via different pathways, including stimulation of phosphorylation of IkB-alpha, leading to reduced nuclear translocation of NF-κB [9]. Although D3T has been suggested to suppress NF-κB signaling in animals, our data for the first time demonstrated the ability of D3T to suppress systemic NF-κB activation in live animals, possibly via the activation of Nrf2 signaling. As NF-κB activation is involved in doxorubicin-induced cardiotoxicity as well as cancer cell resistance to doxorubicin [10–12], studies using the NFκB-RE-luc mouse model would provide critical insights into D3T efficacy in protecting against doxorubicin cardiotoxicity while enhancing its tumoricidal activity.

As with other bioluminescence models, the NFκB-RE-luc mouse model is not without limitations. The presence/load of the transgene may vary among different tissues and organs, and the basal light emission from the different body areas may not fully reflect the intensity of the NF-κB activation. The difference in distribution of D-luciferin to different tissues and organs may affect the eventual light emission. Furthermore, variations in the levels of tissue ATP and magnesium ions (Mg^{2+}) may also impact the light emission as the light-generating reaction is dependent on ATP and Mg^{2+}. These variations along with the light quenching properties of different tissues should be considered in the interpretation of the imaging data for comparisons either between the different body areas of the same animal or between different animals for the same body area.

6. CONCLUSION

Combination of the Berthold LB981 imaging system and NFκB-RE-luc mouse model appears to provide a unique approach to assessing NF-κB activation in live mice under both basal and inflammatory conditions and the effects of pharmacological intervention. As NF-κB signaling, inflammation, and oxidative stress are closely intertwined, this imaging modality may be used as a valuable tool to study the dynamics of inflammatory and oxidative stress and their therapeutic intervention in vivo.

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