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Massachusetts Institute of Technology

Low Level Laser Therapy activates NF-kB via Generation of Reactive Oxygen Species in Mouse Embryonic Fibroblasts

Aaron Chih-Hao Chen[1,2,] [*], Praveen R Arany[1,3,] [*], Ying-Ying Huang,[1,4] Elizabeth M Tomkinson[1,5], Taimur Saleem[1,6], Fiona E Yull[7], Timothy S Blackwell[7], Michael R. Hamblin[1,4,8,][†]

[1] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston MA
[2] Boston University School of Medicine, Graduate Medical Sciences, Boston MA
[3] Harvard School of Dental Medicine, Boston MA
[4] Department of Dermatology, Harvard Medical School
[5] Smith College, Northampton MA
[6] Aga Khan Medical School, Karachi, Pakistan
[7] Department of Medicine and Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN
[8] Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA.
[*] equal contributions
[†] corresponding author: BAR414, 40 Blossom Street, Boston, MA. 02114; hamblin@helix.mgh.harvard.edu

ABSTRACT

Despite over forty years of investigation on low-level light therapy (LLLT), the fundamental mechanisms underlying photobiomodulation remain unclear. In this study, we isolated murine embryonic fibroblasts (MEF) from transgenic NF-kB luciferase reporter mice and studied their response to 810-nm laser radiation. Significant activation of NF-kB was observed for fluences higher than 0.003 J/cm^2. NF-kB activation by laser was detectable at 1-hour time point. Moreover, we demonstrated that laser phosphorylated both IKK α/β and NF-kB 15 minutes after irradiation, which implied that laser activates NF-kB via phosphorylation of IKK α/β. Suspecting mitochondria as the source of NF-kB activation signaling pathway, we demonstrated that laser increased both intracellular reactive oxygen species (ROS) by fluorescence microscopy with dichlorodihydrofluorescein and ATP synthesis by luciferase assay. Mitochondrial inhibitors, such as antimycin A, rotenone and paraquat increased ROS and NF-kB activation but had no effect on ATP. The ROS quenchers N-acetyl-L-cysteine and ascorbic acid abrogated laser-induced NF-kB and ROS but not ATP. These results suggested that ROS might play an important role in the signaling pathway of laser induced NF-kB activation. However, the western blot showed that antimycin A, a mitochondrial inhibitor, did not activate NF-kB via serine phosphorylation of IKK α/β as the laser did. On the other hand, LLLT, unlike mitochondrial inhibitors, induced increased cellular ATP levels, which indicates that light also upregulates mitochondrial respiration. ATP upregulation reached a maximum at 0.3 J/cm^2 or higher. We conclude that LLLT not only enhances mitochondrial respiration, but also activates the redox-sensitive transcription factor NF-kB by generating ROS as signaling molecules.

Keywords: photobiomudulation, low level laser therapy, reactive oxygen species, NF-kB transcription factor, luciferase reporter mice

1 INTRODUCTION

Low level light (or laser) therapy (LLLT) has been used for more than forty years to promote healing, reduce pain and inflammation, and prevent tissue death [1, 2]. Despite many investigations on the subject, the therapy remains controversial largely due to uncertainties about the fundamental molecular and cellular mechanisms responsible for transducing signals from the photons that are incident on the cells to the biological effects that take place in the illuminated tissues.

It has been reasonably well established that mitochondria are a principal intracellular target of red and near-infra-red light [3]. Cytochrome c oxidase (unit IV of the mitochondrial respiratory chain) is a chromophore that absorbs light as far into the infra-red as 1000-nm [4]. There have been reports of increased cytochrome c oxidase activity after

LLLT [5] and many reports of increased ATP synthesis after light delivery to isolated mitochondria [6]. Additional evidence of the role of cytochrome c oxidase as a chromophore in LLLT has been provided by action spectra studies from Karu's laboratory in Russia [7] and from Eells and Wong-Riley in Wisconsin [8]. Many genes have their transcription upregulated (or down regulated) after illumination of cells with various wavelengths and fluences of light. For instance, illumination of human fibroblasts with 628-nm light emitting diode led to altered expression of 111 genes (68 up, 43 down) classified according to ten different functions [9].

Nuclear factor kappa B (NF-kB) is a transcription factor regulating multiple gene expression [10], and has been shown to govern various cellular functions, including inflammatory and stress-induced responses and survival [11]. NF-kB activation is governed by negative feedback by IkB, an inhibitor protein that binds to NF-kB, but can undergo ubiquitination and proteasomal degradation [12] thus freeing NF-kB to translocate to the nucleus and initiate transcription [13]. Understanding the activation mechanisms that govern NF-kB may be important in studying tissue repair or even cancer progression. NF-kB is a redox-sensitive transcription factor [14], that has been proposed to be the sensor for oxidative stress [15]. Reactive oxygen species (ROS) can both activate NF-kB [16], and have been shown to be involved in NF-kB activation by other stimuli such as tumor necrosis factor alpha (TNFα), phorbol ester, and interleukin (IL)-1 [17]. Several laboratories have observed the formation of ROS in cells in vitro after LLLT [18-21], and it has been proposed that ROS are involved in the signaling pathways initiated after photons are absorbed by the mitochondria in cells [22].

In the present report, we describe the effect of light from an 810-nm laser on mouse embryonic fibroblasts (MEF) isolated from a transgenic NF-kB luciferase reporter (HLL) mouse [23]. These mice have been genetically engineered so that luciferase expression is driven by the NF-kB-dependent portion of the human immunodeficiency virus-1 long terminal repeat. They have been used to carry out molecular imaging using a bioluminescence camera of inflammation after various stimuli such as TNFα, lipopolysaccharide and IL-1 [24]. We reasoned that these cells would be ideal to test the hypothesis that LLLT activated NF-kB. Depending on how the assay was set up, luciferase-luciferin could also be used to measure intracellular ATP levels after LLLT.

2 MATERIALS AND METHODS

2.1 Cell isolation and culture
NF-kB luciferase reporter mouse embryonic fibroblasts (Luc MEF) were isolated from embryos removed from pregnant HLL mice between day 13 and day 15 with 0.05% trypsin. The cells were then cultured in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin and stored in 37° C incubator. For all the experiments, cells were grown to at most 80% confluence before seeding to 96 well plates and only the cells between passage 3 and passage 8 were employed.

2.2 Laser irradiation
The in vitro experiments were conducted with a diode laser (Model D030-MM-FCTS/B, Opto Power Corp., Tucson, AZ), which emits 810nm near infrared radiation. 1 mW to 30 mW powers were generated for 7 seconds to 5 minutes to deliver different energy densities, including 0.003, 0.03, 0.3, 3 and 30 J/cm2. 1000 to 3000 cells were seeded into each well of 96 well plates the night before the experiment. Additional overnight incubation with 0.2% FBS DMEM might be needed for certain assays.

2.3 Cell-Titer Glo Assay for ATP
After irradiation, at the various time points, cells in each well were first lysed with 50μL cell lysis buffer and the plate was placed on shaker for 2 minutes to ensure completely release of ATP. 5μL were then transferred to 96 well plates with black wall and clear bottom for BCA assay for protein concentration measurement, while the rest cell lysates were transferred to 96 well plates with white wall and clear bottom for ATP measurement. 100 μL of Cell-Titer Glo Assay (Promega, Madison, WI) was added into each sample and wait about 5 minutes for stabilizing the luminescence signal. The luminescence was measure by a luminometer. Reading time was set to be 10 seconds when the signal is significantly stronger than background noise, and each sample was read twice. Another plate of cells incubated in DMEM with 5mM L-deoxyglucose (DOG) and 0.05% sodium azide for two hours before laser irradiation, which served as negative control to the laser induced ATP synthesis.

2.4 Luciferase Assay for NF-kB activation

Luc MEF was chosen to test the NF-kB activation. Overnight incubated 96 well plates with 3000 to 5000 cells in each well were prepared. At various time points after low level light irradiation, the medium was removed and cells were washed twice before adding 50µL lysis buffer. 45µL lysates were transferred to white wall, clear bottom 96 well plates for chemiluminescence and the rest of lysate was for BCA assay. Luciferase enzymatic activities in Luc MEFs were measured by a luminometer immediately after adding 100 µL Steady-Glo Luciferase Assay substrates (Promega, Madison, WI) into each sample. Read each well twice, set the reading time to be 5 seconds and the background signal was subtracted from the reading to yield the final luminescence. The intensity of luciferase luminescence corresponded to the level of NF-kB activation.

2.5 Microscopic Imaging of ROS production

Cells plated in glass-bottom dishes were incubated in 37° C incubator overnight, washed three to four times by PBS, and then changed the medium to 0.2% FBS DMEM for at least 12 hour incubation before the experiments. After experiments, add 1mM dichlorodihydrofluorescein (DCFDA) (Molecular Probes, Inc, Invitrogen) into the medium and waited for 20 minutes. The DCFDA and MitoTracker Red fluorescence was observed in Zeiss Axiovert 100 TV Microscope with FITC filter. H_2O_2 induced ROS fluorescence was used to determine the exposure time and gamma parameters for all the samples.

2.6 Western Blots

The antibodies to the phospho I-kB and phosphor NF-kB were applied to indicate the time course of release of NF-kB from I-kB induced by the laser. 1.0-2.0 x 10^6 cells were seeded in 100mm petri dishes with 10% DMEM, and the cells were incubated in 0.2% DMEM for an additional day. The lysates were prepared after irradiation at the different time point. TNF-alpha was used as a positive control. The cells were incubated in the TNF-alpha containing medium for 15 min before the lysate preparation.

2.7 Statistical Analysis

All chemiluminescence readings were normalized to total protein measured by Bradford technique (BCA, Pierce Biotechnology Inc.). All assays were performed in duplicate, and each sample was read twice and took the average. Then we used Excel software to perform Single-Factor ANOVA to evaluate the statistical significance of experimental results ($p < 0.05$).

3   RESULTS

3.1 Laser induced ATP synthesis

To test if mitochondria in the Luc MEF respond to low level laser irradiation, we first irradiated the cells with 5 different fluences and measured ATP levels over time. The results showed no significant difference after 0.003 J/cm^2, but fluences of 0.03 J/cm^2 and 0.3 J/cm^2 induced ATP increase by 10% and 60% respectively, and the ATP increase reached a plateau after 3 J/cm^2 and 30 J/cm^2 (Figure11B). The ATP increase reached a peak immediately after 5 minute irradiation and gradually decreased back to the baseline in 4 hours (Figure1A). Then we applied the same protocol to the cells pre-incubated 2 hours in NaN_3/Deoxyglucose (DOG) in DMEM and measured ATP synthesis immediately after irradiation (Figure 1C). NaN_3 + DOG not only lowered the baseline but also made the cells non-responsive to the laser. Since NaN_3 interacts with cytochrome c oxidase in the electron transport chain, ATP depletion induced by NaN_3 + DOG indicates that laser increased ATP synthesis by upregulating mitochondrial respiratory. On the other hand, when we applied the same protocol to the cells pre-incubated in PBS for 2 hours, we observed that ATP synthesis induced by laser showed no significant difference from the cells in DMEM (Figure 1C). This result implies that the ATP up-regulation by the laser is independent of the extracellular glucose level.
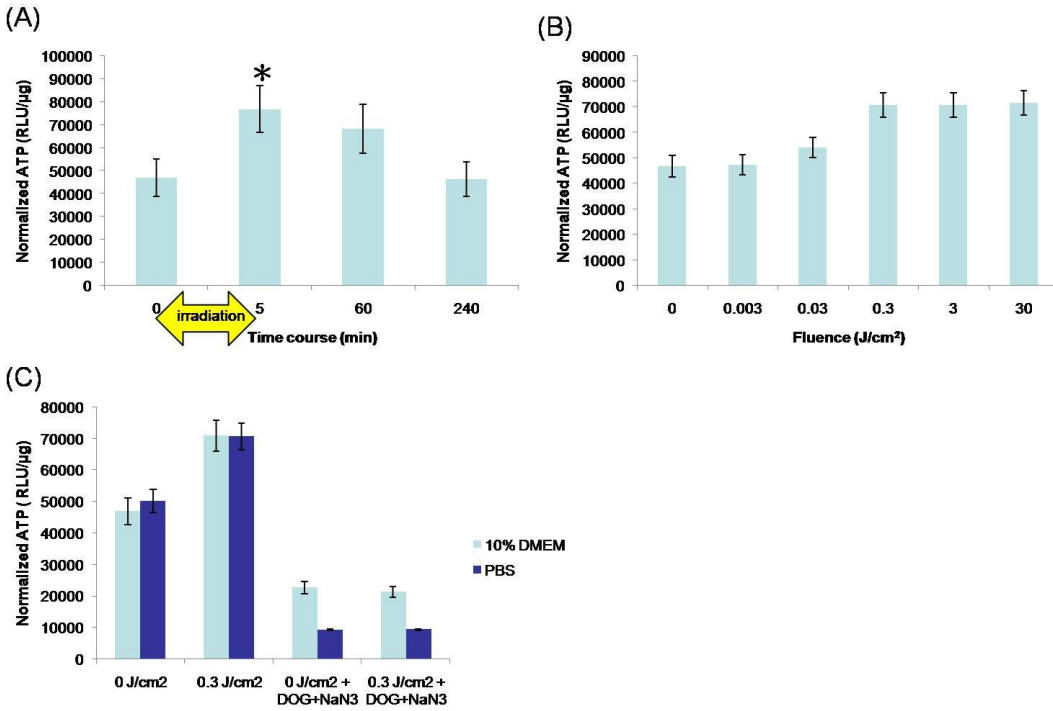
Figure 1. (A)Time course of ATP synthesis induced by 0.3 J/cm^2. (B) ATP synthesis immediately after laser irradiation. (C) Effects of PBS and NaN$_3$/DOG on ATP synthesis.

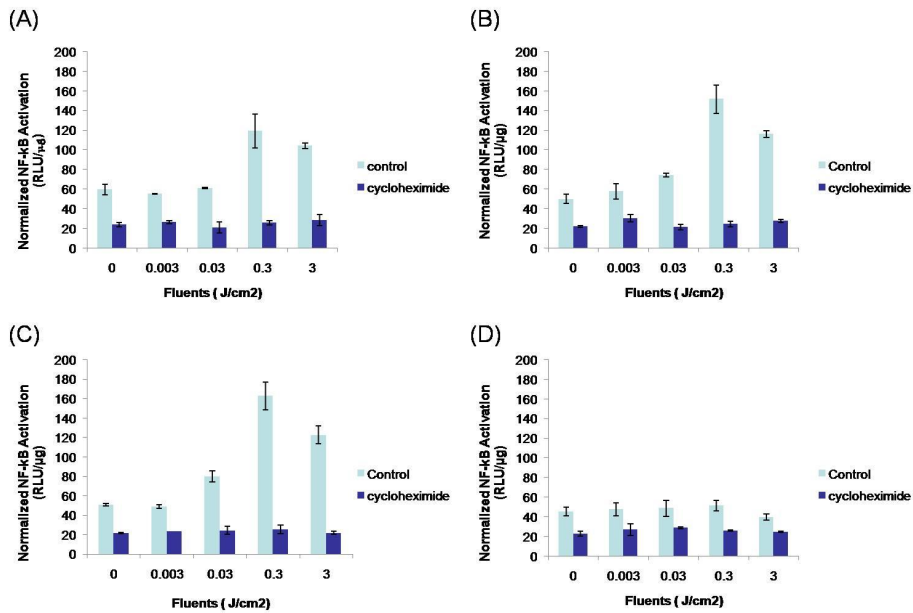## 3.2 NF-kB activation at different times after low-level laser

Figure 2. Effects of cycloheximide in laser induced NF-kB activation at (A) 1 hour, (B) 6 hours, (C) 10 hours, and (D) 24 hours.

We irradiated cells with the same power densities and measured luciferase response over 24 hours (Figure 2, A to D). We observed NF-kB luciferase response increase in 6 hours with 0.03 J/cm^2 and higher but no significant difference with 0.003 J/cm^2. Moreover, the luminescence was first detectable in 1 hour, reached a steady peak at 6 hour and returned to the base line at 24 hours. 0.3 J/cm^2 showed the strongest signal at 6 hours, but luminescence slowly decreased when the fluence went higher.

The luminescence increase measured was proposed to be due to the upregulation of luciferase enzyme expression activated by NF-kB transcription factor.. If the laser irradiation does increase luciferase luminescence, then an inhibitor of protein translation should abrogate the effect. We pre-incubated Luc MEF with 10 μM cycloheximide for 2 hours, irradiated with same energy densities and measured the luminescence at 1 hour, 6 hour, 10 hour and 24 hours. We observed that after cycloheximide treatment, luminescence in all fluences decreased to a steady level, about 30% of base level (Figure 2, A to D). The luminescence increase seen after fluences ≥ 0.03 J/cm^2 at 1 hour did not occur after cycloheximide treatment, which indicated that laser induced NF-kB activation and upregulated luciferase translation within 1 hour after irradiation. In summary, laser with 0.03 J/cm^2 or higher induced NF-kB activation, which upregulated luciferase expression.

3.3 Laser upregulates ROS production

We hypothesized that low level laser generates reactive oxygen species, which serve as intermediate signaling molecules leading to the phosphorylation of I-kB. Phosphorylated I-kB allows the release of free NF-kB to migrate into the nucleus and enhance transcriptions of many genes. Furthermore, ROS level is highly regulated by intracellular superoxide dismutase (SOD), which converts ROS to hydrogen peroxide. Increase in hydrogen peroxide concentration should be detectable after laser irradiation if ROS participates in the laser induced NF-kB activation pathway. We used DCFDA to detect hydrogen peroxide concentration after various laser fluences and several mitochondrial inhibitors, including antimycin A, paraquat (Sigma-Aldrich) and rotenone (Sigma-Aldrich), which served as positive controls. The microscopy images, comparing with the dark control, showed that laser irradiated MEF cells emitted stronger green fluorescent (Figure 3).
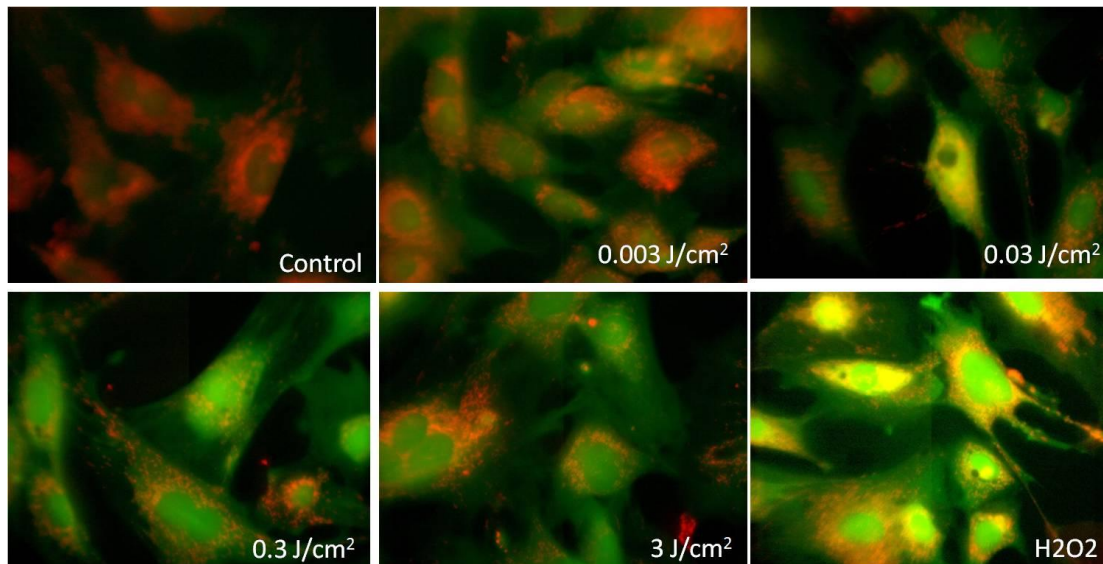

Figure 3. DCFDA fluorescent microscopy images with MitoTracker Red counter stain. Exposure time for both probes was set to 300ms.

3.4 Mitochondrial inhibitors increase ROS and NF-kB activation but not ATP

We tested three mitochondrial inhibitors that have all been reported to induce intracellular ROS by blocking the normal flow of electrons in the mitochondrial respiratory chain. We reasoned that if the mechanism of NIR light activation of NF-kB was via generation of ROS, then these mitochondrial inhibitors should be able to activate NF-kB as well. Preincubation of Luc MEF with each mitochondrial inhibitor for 2 hours and measurement of the luciferase luminescence at the 6 hour time point (Figure 4A). We observed that all the inhibitors generated higher luminescence, which indicated that mitochondrial inhibitors activated NF-kB to a higher degree. In addition, paraquat and rotenone showed similar NF-kB activation perhaps due to the fact that they are both mitochondrial I inhibitors, and antimycin A, the complex III inhibitor, induced the strongest activation among all the inhibitors.

To examine if mitochondrial inhibitors affect mitochondria through similar mechanisms as the light, we measured ATP change in the cells with presence of inhibitors. Although mitochondrial inhibitors activated NF-kB in a similar fashion to light, they depleted mitochondrial respiration by cutting electron transport at various sites and consequently reduce ATP synthesis (Figure4C). Complex I inhibitors, paraquat and rotenone both reduced ATP to the similar level. On the other hand, antimycin A, a complex III inhibitor depleted about 60% ATP, the greatest depletion among the three.

3.5 Effects of ROS scavengers in NF-kB activation

So far we have observed ROS production in laser irradiation and NF-kB activation by mitochondrial inhibitors. To further support that ROS participates in the NF-kB activation, we incorporated antioxidants, N-acetyl-L-cysteine (NAC) and Ascorbic Acid into the system. If ROS is an intermediate molecule in the NF-kB activation, adding ROS scavengers into the system would reduce the activation. The results showed that antioxidants reduced the laser effect on NF-kB activation but had no significant effects on the dark control (Figure 4B).
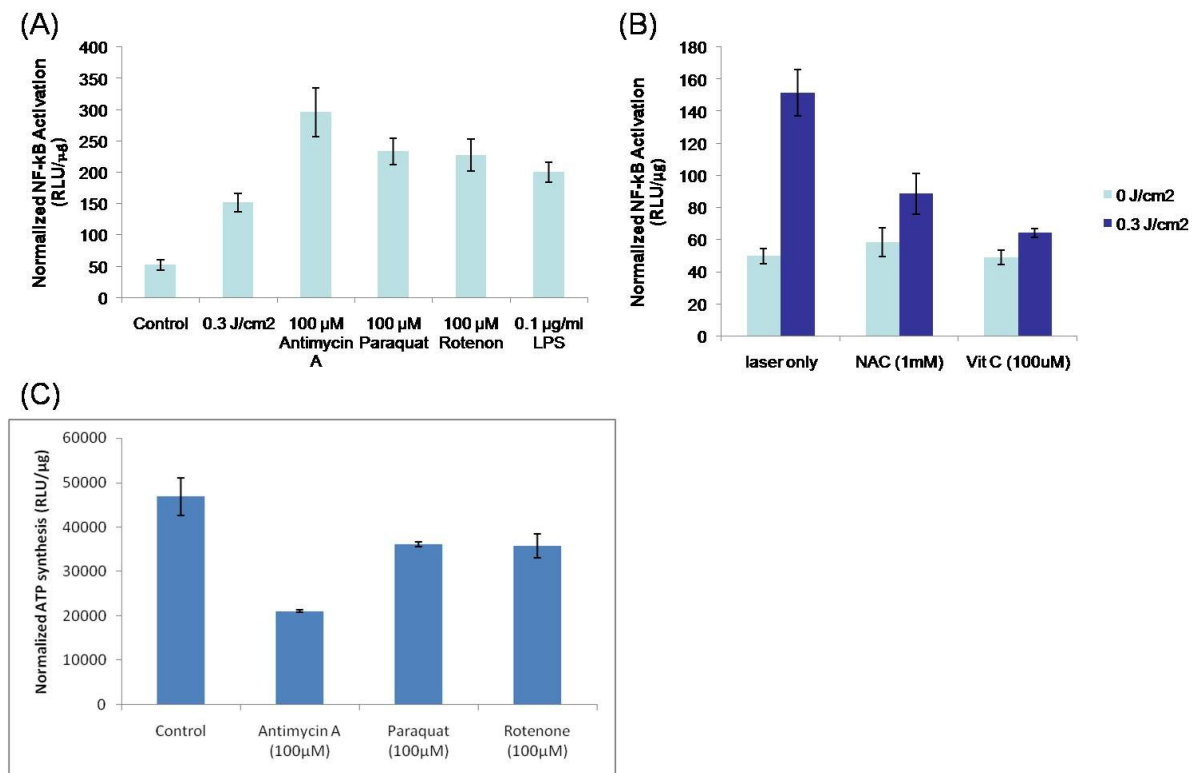

Figure 4. (A) NF-kB activation by mitochondrial inhibitors at 6 hour time point. (B) Effects of Antioxidants on laser induced NF-kB activation. (C) ATP synthesis induced by mitochondrial inhibitors.
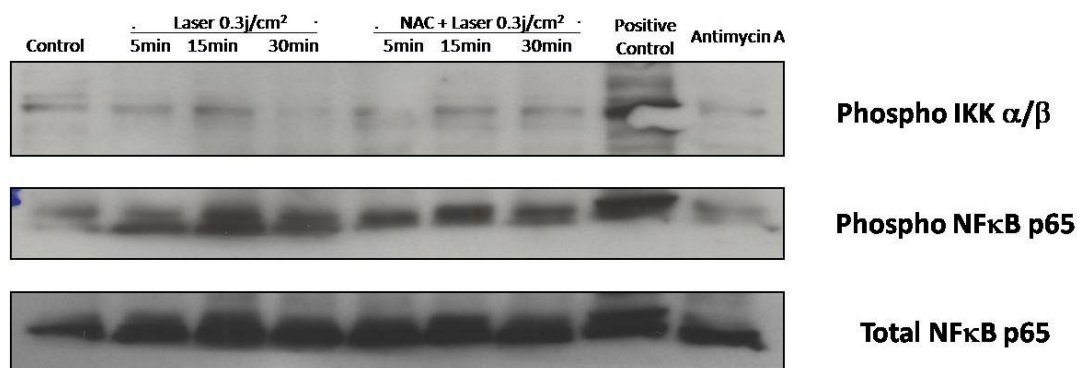
3.5 ROS activates NF-kB via I-kB phosphorylation

Figure 5. Western Blot for NFκB activation by Laser.

Next we investigated the phosphorylation of IKK and NF-kB by the Western blot. It showed that both IKK α/β and NF-kB were phosphorylated immediately after irradiation and reached a peak in 15 minutes, and disappeared in 30 minutes. (Figure 5) Moreover, NAC did not show much difference from the laser activation in both phospho-IKK and phospho-NF-kB. In addition, antimycin A did not show similar phosphorylation as the laser or TNFα, which implied that antimycin A might activates NF-kB through an alternative pathway.

4   DISCUSSION

This report has demonstrated for the first time that low levels of near-infrared laser light can activate NF-kB in MEF cells. We confirmed reports from other laboratories that LLLT can induce expression of ROS in illuminated cells. Our results suggest that activation of NF-kB after 810-nm laser light is likely to be mediated via ROS generation. The fact that the addition of anti-oxidants abrogates the activation of NF-kB provides additional evidence that ROS are involved in the activation of NF-kB.

A delivered energy dose of 0.003 J/cm^2 did not activate NF-kB, but increasing fluences showed activation with a maximum effect at 0.3 J/cm^2, and somewhat lower activation levels at higher fluences. Nevertheless the very wide range of fluences (3 orders of magnitude between 0.03 and 30 J/cm^2) that gave positive activation suggests that the NF-kB activation phenomenon is more akin to a switch being turned on after a minimum light dose greater than 0.003 J/cm^2 has been given. The literature contains many examples of a biphasic dose response after LLLT both in vitro and in vivo. The so-called Arndt-Schultz curve of energy dose versus response that is applied to LLLT [25], states that low doses of energy stimulate cell and tissue processes, while high energy doses reverse the stimulation and lead to inhibition. The biphasic dose-response curve is usually displayed with a relatively sharp peak for the maximum effect level, while our data show a very broad peak of energy versus response.

The time course of the laser-induced NF-kB activation we measured appeared to be different from that observed in activation of NF-kB by LPS [26]. At the 6 hour time point, NF-kB activation reached a peak and remained fairly steady at the 10 hour time point, while reducing to nothing at 24 hour time point. . Moreover, we also demonstrated that preincubation of cells with cycloheximide before irradiation strongly reduced the effect of the laser at all time points. This experiment suggested that translation of luciferase enzymes was actually increased by NF-kB activation induced by light.

It has been recently discovered that there are (at least) two NF-kB activation pathways [27].. The canonical NF-kB signaling pathway activated in response to infections (toll like receptor signaling) and cytokines is based on degradation of IkB inhibitors. This pathway depends on the IkB kinase (IKK), which contains two catalytic subunits, IKKα and IKKβ. IKKβ is essential for inducible IkB phosphorylation and degradation, whereas IKKα is not.

IKKα is involved in processing of the NF-kB2 (p100) precursor. IKKα preferentially phosphorylates NF-kB2, and this activity requires its phosphorylation by upstream kinases, one of which may be NF-kB-inducing kinase (NIK). IKKα is therefore a pivotal component of a second NF-kB activation pathway based on regulated NF-kB2 processing rather than IkB degradation.

Considering the fact that NF-kB is known to be a redox-sensitive nuclear transcription factor [15], we tested whether ROS were induced by light in MEF cells and therefore whether ROS could act as a signaling intermediate in the NF-kB activation pathway. The primary ROS generated in the mitochondrial electron transport chain is superoxide, which is destroyed by superoxide dismutase (SOD). SOD converts superoxide to a freely diffusible molecule, hydrogen peroxide that is capable of oxidizing the intracellular probe dichlorodihydrofluoroscein (DCDHF). DCDHF fluorescence as measured by fluorescence microscopy and spectrofluorimetry further confirmed ROS production after irradiation.

Mitochondrial inhibitors (i.e. compounds that inhibit various units of the respiratory chain) are known to induce expression of ROS. Both paraquat and rotenone that inhibit complex I, and to an even greater extent antimycin A that inhibits complex II produced ROS in Luc MEF cells. Furthermore, we demonstrated that mitochondrial inhibitors have similar effects in Luc MEF cells as 810 nm laser, which led us to the hypothesis that light might activate NF-kB via ROS production. We tested our hypothesis with ROS scavengers, including N-acetyl-L-cysteine and ascorbic acid. It is interesting to note that NAC and ascorbic acid modified light effect but had no significant effect on the dark control at 6 hours after irradiation. This result plus increased DCFDA fluorescence in the cells supports our hypothesis that ROS might be signaling molecules in the laser induced NF-kB activation.

NF-kB could be released from the IKK by phosphorylation of serine or tyrosine aminoacids on the IKK. The results of western blot showed that phosphor Ser-IKK was present immediately after irradiation of 0.3 J/cm^2 and disappeared in 20 minutes. This result further supports the finding that 0.3 J/cm^2 was sufficient to initiate the signaling pathway. However, though antimycin A also activates NF-kB, it seems likely it did not activate through the same pathway as laser or TNFα. It might imply that different concentration of ROS can activate NF-kB in different signaling pathways. In addition, NAC did not shown completely abrogation of the phospho-IKK, which was consistent with the luciferase results that NAC could not completely block the laser effect.

Recent work has identified protein kinase D (PKD, formerly known as PKCν) as a mitochondrial sensor of oxidative stress [28]. It was reported that low dose hydrogen peroxide (250-nM) led to tyrosine phosphorylation at Tyr463 on PKD and the enzyme's consequent activation [29]. This activation of PKD then led to activation of IKKβ, followed by IkB degradation and NF-kB activation [30]. The pathway did not depend on IKKα nor on NF-kB inducing kinase (NIK) [29].

Karu *et al.* proposed a novel mitochondrial signaling pathway in mammalian cells initiated by red and near-IR light in vitro in 2004 [31]. Recently, Schroeder *et al.* discovered that IR light could initiate a cellular signaling response in normal human skin fibroblasts. All these data support the proposed model that mitochondria can communicate with other cellular organelles via specific signaling mechanisms [32]. Numerous reports indicate that light could regulate gene expression via mitochondrial mechanisms. For example, Hu *et al.* [33] showed that He-Ne laser illumination increased mitochondrial membrane potential together with ATP synthesis in melanoma cells. They also found upregulation of cytochrome c oxidase activity, increased phosphorylation of Jun N-terminal kinase (JNK) and later, activated activator protein-1 (AP-1), which led to increased cell proliferation.

While altering mitochondrial electron transport using inhibitors of respiration leads to increased ROS production and to NF-kB activation, light has different effects on ATP synthesis from mitochondrial inhibitors. He-Ne laser irradiation in isolated mitochondria has been shown to promote O$_2$ consumption by cytochrome c oxidase and electron transport activities [5]. We observed that ATP concentration in Luc MEF cells was increased with a peak immediately after irradiation and gradually decreased back to the baseline in 6 hours. Preincubation of cells with NaN$_3$ and deoxyglucose (a regimen designed to deplete ATP and mimic hypoxia [34]) before irradiation strongly abrogated the increased ATP synthesis induced by light. The results implied that inhibiting cytochrome c oxidase with azide ions blocks the light effect on the mitochondrial respiratory chain. On the other hand, mitochondrial inhibitors, including antimycin A, paraquat and rotenone, all reduced the ATP concentration in the cells.

In conclusion, our experiments demonstrated that low-level 810 nm laser activates NF-kB in the MEF cells faster than lipopolysaccharide activation via Toll-like receptors. ROS produced by irradiation plays a key role in the signaling pathway of light induced NF-kB activation. In addition, unlike mitochondrial inhibitors, light also upregulates ATP synthesis by increasing cytochrome c oxidase activities.

5   ACKNOWLEDGEMENTS

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