

## LOW ENERGY LASER LIGHT (632.8 NM) SUPPRESSES AMYLOID- $\beta$ PEPTIDE-INDUCED OXIDATIVE AND INFLAMMATORY RESPONSES IN ASTROCYTES

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**Abstract**—Oxidative stress and inflammation are important processes in the progression of Alzheimer's disease (AD). Recent studies have implicated the role of amyloid  $\beta$ -peptides (A $\beta$ ) in mediating these processes. In astrocytes, oligomeric A $\beta$  induces the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes resulting in its activation to produce anionic superoxide. A $\beta$  also promotes production of pro-inflammatory factors in astrocytes. Since low energy laser has previously been reported to attenuate oxidative stress and inflammation in biological systems, the objective of this study was to examine whether this type of laser light was able to abrogate the oxidative and inflammatory responses induced by A $\beta$ . Primary rat astrocytes were exposed to Helium–Neon laser ( $\lambda$ =632.8 nm), followed by the treatment with oligomeric A $\beta$ . Primary rat astrocytes were used to measure A $\beta$ -induced production of superoxide anions using fluorescence microscopy of dihydroethidium (DHE), assembly of NADPH oxidase subunits by the colocalization between the cytosolic p47<sup>phox</sup> subunit and the membrane gp91<sup>phox</sup> subunit using fluorescent confocal microscopy, phosphorylation of cytosolic phospholipase A<sub>2</sub> cPLA<sub>2</sub> and expressions of pro-inflammatory factors including interleukin-1 $\beta$  (IL-1 $\beta$ ) and inducible nitric-oxide synthase (iNOS) using Western blot Analysis. Our data showed that laser light at 632.8 nm suppressed A $\beta$ -induced superoxide production, colocalization between NADPH oxidase gp91<sup>phox</sup> and p47<sup>phox</sup> subunits, phosphorylation of cPLA<sub>2</sub>, and the expressions of IL-1 $\beta$  and iNOS in primary astrocytes. We demonstrated for the first time that 632.8 nm laser was capable of suppressing cellular pathways of oxidative stress and inflammatory responses critical in the pathogenesis in AD.

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**Abbreviations:** AD, Alzheimer's disease; A $\beta$ , amyloid  $\beta$ -peptide; BCA, bicinchoninic acid; BSA, bovine serum albumin; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HFIP, hexafluoro-2-propanol; IL-1 $\beta$ , interleukin-1 $\beta$ ; iNOS, inducible nitric-oxide synthase; LED, light-emitting diode; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

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This study should prove to provide the groundwork for further investigations for the potential use of laser therapy as a treatment for AD. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** phospholipase A<sub>2</sub>, interleukin-1 $\beta$ , iNOS, NADPH oxidase, oxidative stress, inflammation.

Oxidative stress and inflammation have been implicated in many neurodegenerative diseases including Alzheimer's disease (AD) (Behl et al., 1994; Simonian and Coyle, 1996; Behl and Holsboer, 1998; Heneka and O'Banion, 2007). Oxidative stress induced by overproduction of reactive oxygen species (ROS) causes damage of basic components in cells, such as lipids (Cini and Moretti, 1995), DNA (Mecocci et al., 1994) and proteins (Smith et al., 1991). In most cell systems, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Abramov et al., 2004) and mitochondrial abnormalities (Hirai et al., 2001) are two important sources of ROS. NADPH oxidase is comprised of six subunits (Groemping and Rittinger, 2005; Mizrahi et al., 2006) and its activation is mediated by translocation of the cytosolic subunits (p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup> and the GTPase Rac) to the plasma membrane subunits (gp91<sup>phox</sup> and p22<sup>phox</sup>) (Groemping and Rittinger, 2005; Mizrahi et al., 2006). Activation of NADPH oxidase in astrocytes and microglia results in increased production of superoxide anions, which are toxic to neighboring neurons in AD brains (Shimohama et al., 2000; Qin et al., 2002; Abramov et al., 2004). Oxidative stress also triggers critical downstream pathways including activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), an enzyme responsible for membrane integrity (You et al., 2005; Huber et al., 2006; Sun et al., 2007; Shelat et al., 2008). Consistent with this line of evidence, we have reported that amyloid  $\beta$ -peptide (A $\beta$ ) activates NADPH oxidase to induce ROS and activation of cPLA<sub>2</sub> in primary rat astrocytes (Zhu et al., 2006). Activated cPLA<sub>2</sub>, in turn, targets mitochondria, resulting in mitochondrial dysfunction and further overproduction of ROS (Zhu et al., 2006). Based on these previous studies, it is reasonable to suggest two major mechanisms for A $\beta$  to induce ROS production, initially from NADPH oxidase and subsequently from mitochondria through cPLA<sub>2</sub> activation.

There is compelling evidence that inflammation plays a vital role in pathogenesis of AD (Heneka and O'Banion, 2007). A $\beta$  triggers expressions of inflammatory factors including interleukin-1 $\beta$  (IL-1 $\beta$ ) and inducible nitric-oxide synthase (iNOS) in glial cells (Akama and Van Eldik, 2000; White et al., 2005). IL-1 $\beta$  is a critical inflammatory cytokine

in AD (Griffin and Mrak, 2002) since it can stimulate production of iNOS and other inflammatory cytokines (Lee et al., 1993; Blom et al., 1997).

Low-level laser has been reported to attenuate oxidative stress and inflammation (Karageuzyan et al., 1998; Sakurai et al., 2000; Freitas et al., 2001; Fillipin et al., 2005; Abdel et al., 2007; Lim et al., 2007; Aimbire et al., 2008; Boschi et al., 2008; Hammer et al., 2008; Giuliani et al., 2009). Although the mechanism is yet to be fully understood, this type of light therapy has been used to help tissue repair and wound healing in *in vivo* models (Whelan et al., 2001, 2003; Albertini et al., 2007; Correa et al., 2007; Viegas et al., 2007; Aimbire et al., 2008; Reis et al., 2008) and rescue neurons from neurotoxic injuries (Wong-Riley et al., 2005; Liang et al., 2006), implying a variety of promising clinical applications. In this study, we hypothesize that laser has the capability of suppressing A $\beta$ -induced oxidative stress and inflammation in astrocytes, the most abundant cell type in the brain. We tested the effects of low-level laser light at 632.8 nm on A $\beta$ -induced ROS production through the activation of NADPH oxidase, and its downstream pathways involving phosphorylation of cPLA<sub>2</sub> and expression of inflammatory factors including IL-1 $\beta$  and iNOS. Information derived from this study should prove to provide groundwork for further investigations on the potential application of laser therapy as a treatment for AD.

## EXPERIMENTAL PROCEDURES

### Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) with high glucose, Ham's F-12 medium, fetal bovine serum (FBS), dihydroethidium (DHE) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), hexafluoro-2-propanol (HFIP) and poly-D-lysine were from Sigma-Aldrich (St. Louis, MO, USA). A $\beta$ <sub>1–42</sub> was from American Peptide (Sunnyvale, CA, USA). gp91 ds-tat Peptide 2, a peptide inhibitor of NADPH oxidase, was from anaSpec (Fremont, CA, USA). Goat polyclonal anti-gp91<sup>phox</sup> and rabbit polyclonal anti-p47<sup>phox</sup> were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein-donkey anti-goat antibody, and Texas Red-sheep anti-rabbit antibody were from Abcam (Cambridge, MA, USA).

### Cell culture

Primary cortical astrocytes were obtained using a standard stratification/cell-shaking procedure from newborn rat brains. Following the procedure from our previous studies yielded confluent mixed glial cultures within 7–9 days, after which the flasks were shaken at 180 rev./min at room temperature (25 °C) for 3 h to remove microglial cells (Zhu et al., 2005, 2006). The purity of these primary rat astrocyte cultures was >95% verified by anti-gial fibrillary acidic protein labeling (data not shown). Astrocytes were cultured onto 35 mm dishes or coverslips coated with poly-D-lysine (0.4 mg/mL) and fed every 48 h with fresh DMEM culture medium supplemented with 10% FBS. Cells were maintained at 37 °C in a 5% CO<sub>2</sub> humidified incubator.

### Preparation of A $\beta$ <sub>1–42</sub>

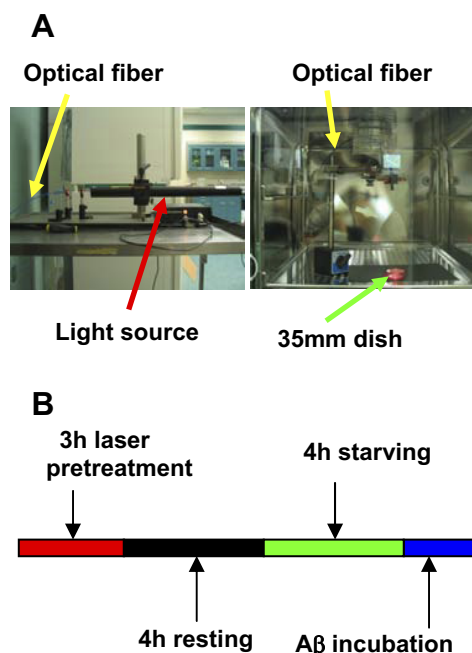
A $\beta$ <sub>1–42</sub> (1 mg) in the powder form was dissolved in 200  $\mu$ l of HFIP, and the solution was aliquoted into Eppendorf tubes, and after

removing HFIP using a speed vacuum apparatus, samples were stored at –20 °C until use. The A $\beta$  film left in the tube was resuspended in 2  $\mu$ l DMSO and further diluted in 98  $\mu$ l Ham's F-12 medium to make a 100  $\mu$ M A $\beta$ <sub>1–42</sub> solution. The solution was then sonicated for 1 min and further diluted in DMEM to the final concentration of 5  $\mu$ M for treatments.

### Laser irradiation protocol and A $\beta$ treatment

The source of light for irradiation was a helium–neon laser ( $\lambda$ =632.8 nm) with an output power of 15 mW. The light source was placed outside of incubator and an optical fiber was used to guide the laser from above onto a 35 mm dish covered with a plastic lid inside the incubator (Fig. 1A). Coverslips coated with poly-D-lysine were in 35 mm dishes if immunostaining was needed for confocal microscopy. The spot size was adjusted to cover the whole dish with area of 10 cm<sup>2</sup>. The irradiation power density was 1.5 mW/cm<sup>2</sup>. In this study, the irradiation time was 3 h. The total energy was 162 J and energy density was 16.2 J/cm<sup>2</sup>. Both cell morphology and trypan blue exclusion assay showed that laser irradiation did not cause any ill-effect to cell viability (data not shown). In addition, no detectable change in culture medium temperature was observed during laser irradiation.

Fig. 1B describes the laser irradiation protocol. Briefly, for the group with laser pretreatment and A $\beta$  treatment, astrocytes in 35 mm dishes and DMEM with phenol red and 10% FBS were



**Fig. 1.** Laser irradiation set-up and protocol. (A) Laser irradiation set-up. Helium–Neon laser ( $\lambda$ =632.8 nm) light source with the power 15 mW was placed outside of incubator (A, left). Optical fiber was used to guide laser irradiation onto a 35 mm dish in the incubator (A, right). (B) Laser irradiation protocol. For the group with laser pretreatment and A $\beta$  treatment, astrocytes in 35 mm dishes and DMEM with 10% FBS were pretreated with laser for 3 h (red). After allowing cells to rest for another 4 h (black), cells were serum starved for 4 h by replacing the DMEM containing 10% FBS with serum-free DMEM (green). Cells were then incubated with A $\beta$  (5  $\mu$ M) (blue). For control group, astrocytes were starved for 4 h and incubated with serum-free DMEM. For the group with A $\beta$  treatment alone, astrocytes were starved for 4 h and then incubated with A $\beta$  in DMEM. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

pretreated with laser for 3 h. After allowing cells to rest for another 4 h, cells were serum starved for 4 h by replacing DMEM containing 10% FBS with serum-free DMEM. Cells were then incubated with A $\beta$  (5  $\mu$ M) for 2 h for measuring phosphorylation of cPLA $_2$ , superoxide anion production, and colocalization between p47<sup>phox</sup> and gp91<sup>phox</sup>, and for 16 h for measuring IL-1 $\beta$  and iNOS. For control group, astrocytes were starved for 4 h and incubated in serum-free DMEM. For the group with A $\beta$  treatment alone, astrocytes were starved for 4 h and then incubated with A $\beta$  in a CO $_2$ -incubator without laser installed. For the group with treatment with NADPH oxidase inhibitor (gp91 ds-tat Peptide 2), the inhibitor (1  $\mu$ M) was added into culture medium 1 h before A $\beta$  treatment.

### Reactive oxygen species measurement

To quantify superoxide anion production induced by A $\beta$  in astrocytes, cells were starved for 4 h, followed by incubation of cells with both A $\beta$  (5  $\mu$ M) and DHE (20  $\mu$ M) for 2 h. DHE is a cell permeable fluorescent probe. Upon oxidation by superoxide anion, it binds to DNA in nuclei and becomes highly fluorescent (Chapman et al., 2005). Fluorescent intensity measurement of DHE was performed at room temperature using a Nikon TE-2000 U fluorescence microscope with a 20 $\times$  objective lens. Images were acquired using a CCD camera controlled by a computer running MetaVue imaging software (Universal Imaging, PA, USA). The fluorescent intensity of DHE per cell was measured. Background subtraction was done for all images prior to data analysis.

### Confocal immunofluorescence microscopy

After treatments, cells were fixed with 4% paraformaldehyde at 37  $^{\circ}$ C for 30 min. PBS containing 5% BSA was then applied to cells for 1 h to block nonspecific bindings. To label gp91<sup>phox</sup> at the cell surface, goat polyclonal anti-gp91<sup>phox</sup> (1:200 dilution) was added and incubated at 4  $^{\circ}$ C without permeabilization. To label p47<sup>phox</sup>, cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Rabbit polyclonal anti-p47<sup>phox</sup> (1:200 dilution) in PBS with 1% BSA was then added and incubated at 4  $^{\circ}$ C overnight. This was followed by fluorescent labeling with secondary antibodies (1:500 dilution) at room temperature for 1 h. The secondary antibodies for gp91<sup>phox</sup> and p47<sup>phox</sup> were fluorescein-donkey anti-goat antibody, and Texas Red-sheep anti-rabbit antibody, respectively. Secondary antibodies did not show immunostaining in the absence of the primary antibody (data not shown). Confocal immunofluorescence microscopy was performed with an Olympus FV1000 confocal inverted microscope (Tokyo, Japan). Confocal images were acquired with a 60 $\times$ , numerical aperture 1.2 oil immersion objective lens for colocalization studies between NADPH oxidase subunits, gp91<sup>phox</sup> and p47<sup>phox</sup>. Background subtraction was done for all images before analysis. Colocalization images were obtained by suppressing all colors, except yellow, in superimposed images using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The colocalization of p47<sup>phox</sup> with gp91<sup>phox</sup> was quantified by normalizing the intensity of yellow by the intensity of gp91<sup>phox</sup>.

### Western blot analysis

After treatments, the total protein concentration of cell lysate was determined by BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacture's instruction. Equivalent amounts of protein from each sample (e.g., 40  $\mu$ g) were diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and were incubated overnight at 4  $^{\circ}$ C in 3% (w/v) BSA with 0.02% (w/v) sodium azide in TBST with p-cPLA $_2$  or cPLA $_2$  antibodies (1:1000 dilution; Cell

Signaling Technology, Beverly, MA, USA), anti-IL-1 $\beta$  antibody (1:1000 dilution; R&D Systems, Minneapolis, MN, USA), anti-iNOS antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and  $\beta$ -actin antibody (Sigma, St. Louis, MO, USA). Membranes were washed three times during a 15-min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL, USA) to visualize bands. The protein bands detected on x-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

Data were presented as mean $\pm$ SD from at least three independent experiments. Analysis was carried out with one-way ANOVA, followed by Bonferroni's post hoc tests. Values of  $P < 0.05$  are considered to be statistically significant.

## RESULTS

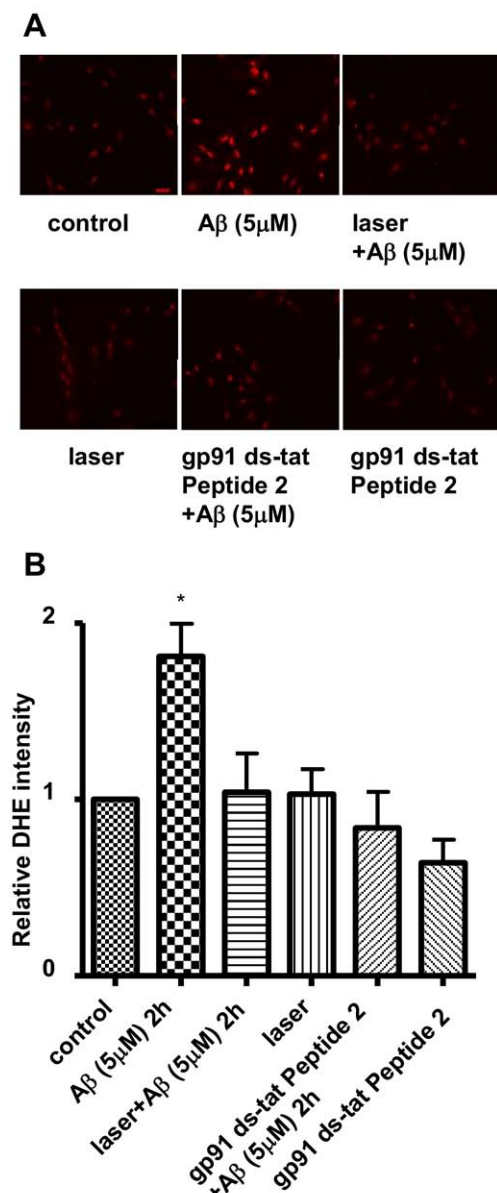
### Laser light suppressed A $\beta$ -induced superoxide production in primary astrocytes

In order to investigate the effects of laser light on A $\beta$ -induced superoxide production, primary rat cortical astrocytes were fluorescently labeled with DHE. We found that stimulation of astrocytes by A $\beta$  for 2 h led to an increase in superoxide anions, as indicated by a significant increase in DHE fluorescent intensity, whereas pretreatment of cells with laser abrogated an A $\beta$ -mediated increase in superoxide anions (Fig. 2). Astrocytes exposed to laser without A $\beta$  treatment showed no effect on DHE intensity (Fig. 2). To verify this technique of measurement for superoxide anions, we demonstrated that NADPH oxidase inhibitor (gp91 ds-tat Peptide 2) suppressed A $\beta$ -mediated increase in DHE intensity. The inhibitor alone had no effect on DHE intensity. In sum, A $\beta$ -induced ROS production in astrocytes through NADPH oxidase activation was suppressed by laser irradiation.

### Laser light suppressed A $\beta$ -induced assembly of NADPH oxidase subunits in primary astrocytes

Since ROS production through NADPH oxidase is preceded by translocation of cytosolic subunits to bind with membrane subunits, we tested the effects of laser on colocalization between cytosolic p47<sup>phox</sup> and membrane-associated gp91<sup>phox</sup> after treating astrocytes with A $\beta$ . Confocal immunofluorescence microscopy of gp91<sup>phox</sup> and p47<sup>phox</sup> subunits showed that stimulation of astrocytes by A $\beta$  for 2 h led to a significant increase in colocalization between these two subunits, while laser pretreatment suppressed the colocalization to the control level (Fig. 3). These results suggest that laser attenuates A $\beta$ -induced NADPH oxidase activation by inhibiting the assembly of its subunits. Astrocytes exposed to laser without A $\beta$  treatment showed no effect on colocalization (Fig. 3). To validate the fluorescent confocal microscopy method for measurement of the colocalization between these two subunits, we dem-





**Fig. 2.** Laser (632.8 nm) suppressed Aβ-induced ROS production in astrocytes. (A) Representative images of astrocytes labeled with dihydroethidium (DHE). Compared to control (A, upper, left), Aβ increased superoxide production (A, upper, middle). Pretreatment with laser suppressed superoxide production induced by Aβ (A, upper, right), but laser alone did not induce superoxide production (A, lower, left). gp91 ds-tat Peptide 2 (NADPH oxidase inhibitor) suppressed Aβ-induced superoxide production in astrocytes (A, lower, middle), but the inhibitor alone did not induce superoxide production (A, lower, right). Scale bar: 40 μm. (B) Quantitative analysis of DHE intensity shows that laser (632.8 nm) suppressed Aβ-induced superoxide production in astrocytes. Pretreatment with laser without Aβ treatment did not affect DHE intensity. gp91 ds-tat Peptide 2 suppressed Aβ-induced increase in DHE intensity, but the inhibitor alone did not affect DHE intensity. Data are expressed as percentages of control and mean ± SD from four independent experiments with three replicates per experiment (\*  $P < 0.05$ ). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

onstrated that NADPH oxidase inhibitor (gp91 ds-tat Peptide 2) suppressed Aβ-mediated increase in colocalization (Fig. 3). The inhibitor alone had no effect on the colocalization. These data suggest that laser attenuates Aβ-induced ROS production through inhibiting NADPH oxidase activation.

To rule out the possibility that laser and Aβ might have affected the expressions of p47<sup>phox</sup> and gp91<sup>phox</sup>, which may also cause changes in the colocalization between p47<sup>phox</sup> and gp91<sup>phox</sup>, Western blot analysis showed that laser and Aβ treatments did not change the total expressions of p47<sup>phox</sup> and gp91<sup>phox</sup> in astrocytes (Fig. 4).

#### Laser suppressed Aβ-induced phosphorylation of cPLA<sub>2</sub> in primary astrocytes

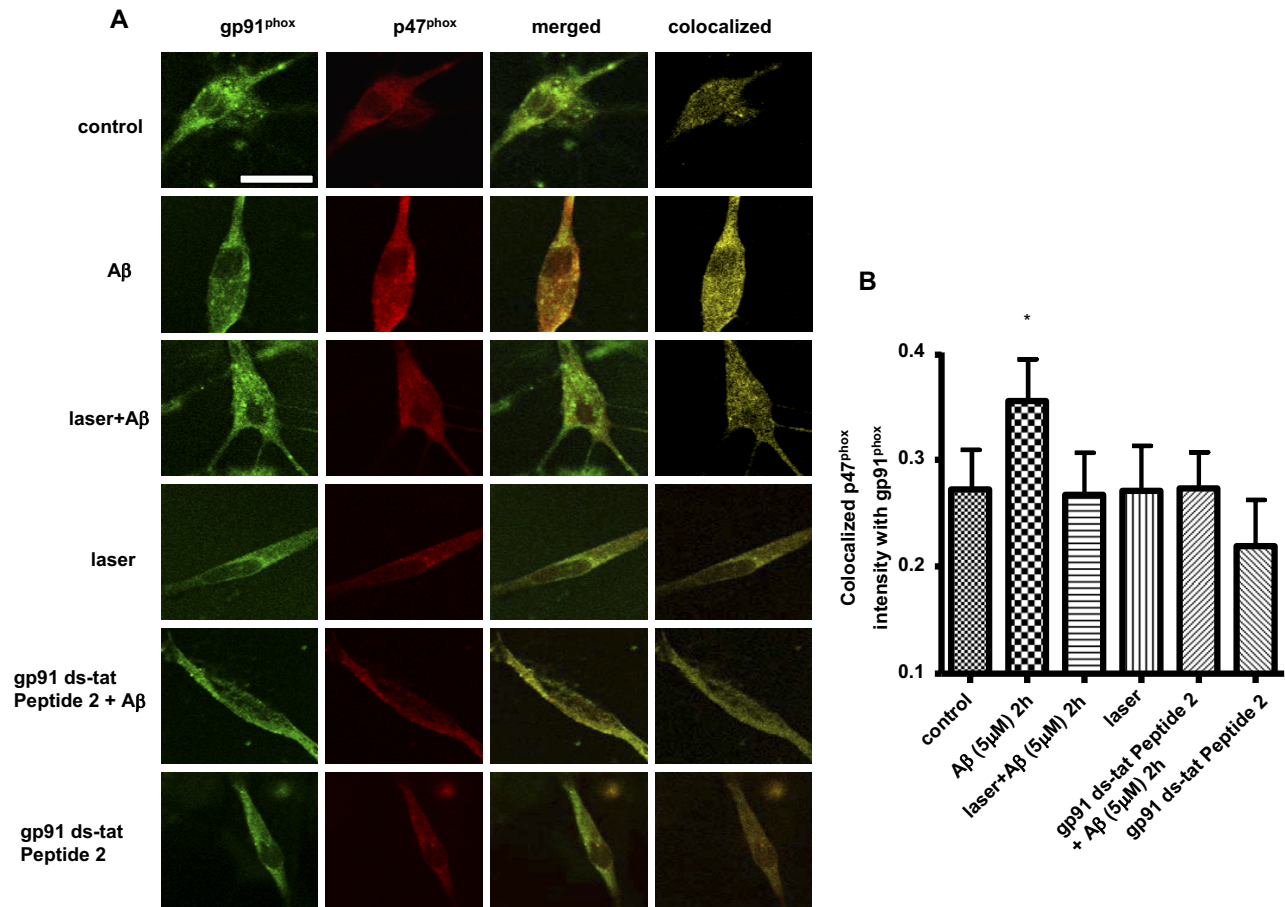
ROS from NADPH oxidase can stimulate downstream signaling pathways including activation of mitogen-activated protein kinase (MAPK) which further leads to phosphorylation of cPLA<sub>2</sub> (Shelat et al., 2008). p-cPLA<sub>2</sub> targets mitochondria and triggers further ROS production from mitochondria (Zhu et al., 2006). Since our data suggested that laser suppressed Aβ-induced ROS production through inhibiting NADPH oxidase activation, we hypothesized that laser also suppresses Aβ-induced phosphorylation of cPLA<sub>2</sub>. In support of our hypothesis, Western blot analysis of p-cPLA<sub>2</sub> and cPLA<sub>2</sub> showed that stimulation of astrocytes by Aβ for 30 min led to a significant increase in phosphorylation of cPLA<sub>2</sub>, while laser pretreatment suppressed the phosphorylation to the control level (Fig. 5). Astrocytes exposed to laser without Aβ treatment exerted no effect on phosphorylation of cPLA<sub>2</sub> (Fig. 5).

#### Laser suppressed Aβ-induced expression of pro-inflammatory factors in primary astrocytes

AD is associated with increased inflammatory responses (Griffin et al., 1998), such as increase in IL-1β (Zhu and Qian, 2006). Since there is evidence that low-level light can offer anti-inflammatory effects (Sakurai et al., 2000; Freitas et al., 2001; Lim et al., 2007; Aimbire et al., 2008; Boschi et al., 2008; Hammer et al., 2008), we tested whether laser was capable of suppressing Aβ-induced inflammation in astrocytes. Western blot analyses of IL-1β and iNOS showed that stimulation of astrocytes by Aβ for 16 h led to a significant increase in the expressions of IL-1β and iNOS, while laser pretreatment suppressed the expressions to the control level (Fig. 6). Astrocytes exposed to laser without Aβ treatment showed that laser itself had no effect on expressions of these inflammatory factors (Fig. 6).

## DISCUSSION

The present study demonstrated, for the first time, the ability of laser light at  $\lambda = 632.8$  nm to suppress Aβ-induced ROS production and inflammatory response in primary rat astrocytes. Specifically, we demonstrated the ability of this laser to suppress Aβ-induced ROS production, colocalization between NADPH oxidase subunits gp91<sup>phox</sup> and

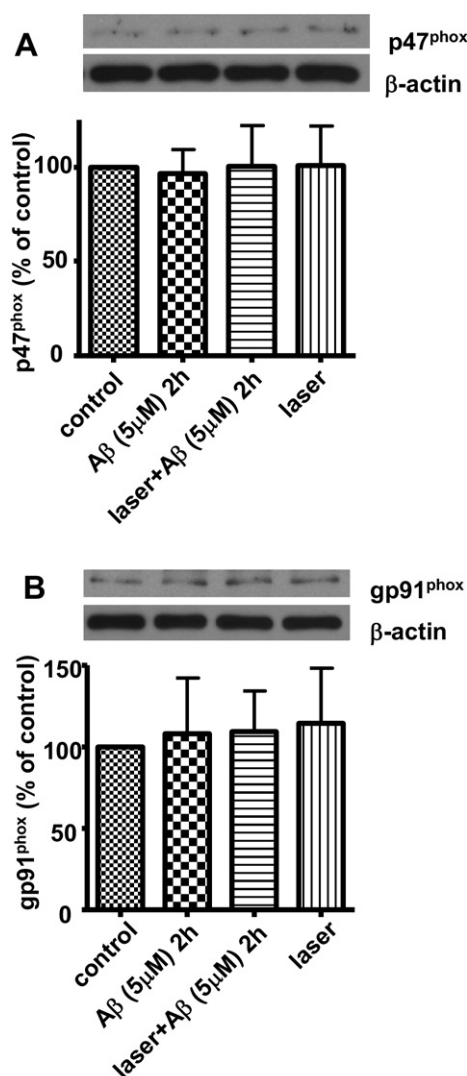


**Fig. 3.** Laser (632.8 nm) suppressed A $\beta$ -induced colocalization between NADPH oxidase subunits gp91<sup>phox</sup> and p47<sup>phox</sup> in astrocytes. (A) Representative confocal images of gp91<sup>phox</sup> (green) and p47<sup>phox</sup> (red) in astrocytes. The images of colocalization (yellow) were obtained by superimposing confocal images of corresponding p47<sup>phox</sup> (red) and gp91<sup>phox</sup> (green) followed by suppressing all colors except yellow. Scale bar: 10  $\mu$ m. (B) Quantitative analysis of colocalization between gp91<sup>phox</sup> and p47<sup>phox</sup> shows that laser (632.8 nm) suppressed A $\beta$ -induced colocalization. Pretreatment with laser without A $\beta$  treatment did not affect colocalization. gp91 ds-tat Peptide 2 (NADPH oxidase inhibitor) suppressed A $\beta$ -induced colocalization, but the inhibitor alone did not affect colocalization. Around 120 cells were used to do quantification for each group. Data are expressed as percentages of control and mean  $\pm$  SD from four independent experiments with three replicates per experiment (\*  $P < 0.05$ ). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

p47<sup>phox</sup>, phosphorylation of cPLA<sub>2</sub>, and expression of pro-inflammatory factors including IL-1 $\beta$  and iNOS.

AD is a prominent neurodegenerative disease affecting a large proportion of the aging population (Hebert et al., 2003). Although oxidative stress has been implicated in the progression of this disease, the mechanism for production of ROS has not been clearly elucidated (Gupta et al., 1991; Smith et al., 1991, 1996; Behl et al., 1994; Mecocci et al., 1994; Yan et al., 1994; Cini and Moretti, 1995; Forster et al., 1996; Simonian and Coyle, 1996; Mecocci et al., 1997; Behl and Holsboer, 1998; Schippling et al., 2000; Hamilton et al., 2001; Lovell and Markesbery, 2007). Our previous studies have shown that A $\beta$  triggered NADPH oxidase activation to induce ROS and subsequently phosphorylated cPLA<sub>2</sub> in primary astrocytes (Zhu et al., 2006). Furthermore, cPLA<sub>2</sub> was shown to cause a decrease in mitochondrial membrane potential ( $\Delta\Psi_m$ ) and result in more ROS production (Zhu et al., 2006). Interestingly, low-level laser ( $\lambda = 635$  nm) has been reported to increase  $\Delta\Psi_m$  and ATP synthesis (Bortoletto et al., 2004). Another study

showed that low-level infrared laser protected PC12 cells against oxidative stress through its ability to modulate  $\Delta\Psi_m$  (Giuliani et al., 2009). In fact, low-level laser therapy has been reported to prevent oxidative stress in rat traumatized Achilles tendon (Fillipin et al., 2005) and Duchenne muscular dystrophy patients (Abdel et al., 2007). Our data showed that 632.8 nm laser suppressed A $\beta$ -induced ROS production (Fig. 1) through inhibiting colocalization of NADPH oxidase subunits, gp91<sup>phox</sup> and p47<sup>phox</sup> (Fig. 2). It is known that the interactions of gp91<sup>phox</sup> with the cytosolic components result in a conformational change in gp91<sup>phox</sup>, which enables the electron flow from NADPH to oxygen and the generation of superoxide anions (Han et al., 1998; Diebold and Bokoch, 2001). In this study, our results lead to a new hypothesis that low-energy laser light may be capable of causing a conformational change in gp91<sup>phox</sup>, which inhibits the interaction of gp91<sup>phox</sup> with the cytosolic components and the generation of superoxide anions. More investigations are needed to further our understanding in the mechanism of inhibiting the assembling of

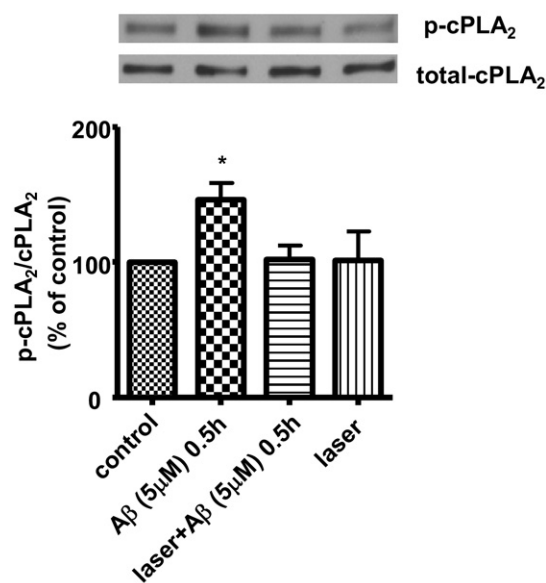


**Fig. 4.** Laser (632.8 nm) and Aβ did not alter the expression of p47<sup>phox</sup> and gp91<sup>phox</sup> in astrocytes. (A) Western blot analysis shows that laser (632.8 nm) and Aβ did not alter the expression of p47<sup>phox</sup> in astrocytes. (B) Western blot analysis shows that laser (632.8 nm) and Aβ did not alter the expression of gp91<sup>phox</sup> in astrocytes. Data are expressed as percentages of control and mean ± SD from three independent experiments with three replicates per experiment.

gp91<sup>phox</sup> with the cytosolic components by low-energy laser light. Consistent with previous studies reporting that phosphorylation of cPLA<sub>2</sub> is one of the downstream pathways of NADPH oxidase (Zhu et al., 2006; Shelat et al., 2008), our data also showed that Aβ-induced p-cPLA<sub>2</sub> was suppressed by laser pretreatment (Fig. 3). Since cPLA<sub>2</sub> is associated with perturbation of mitochondria  $\Delta\Psi_m$ , results from this study suggested that laser pretreatment reduced ROS from NADPH oxidase and subsequently ROS-induced mitochondrial dysfunction through cPLA<sub>2</sub>.

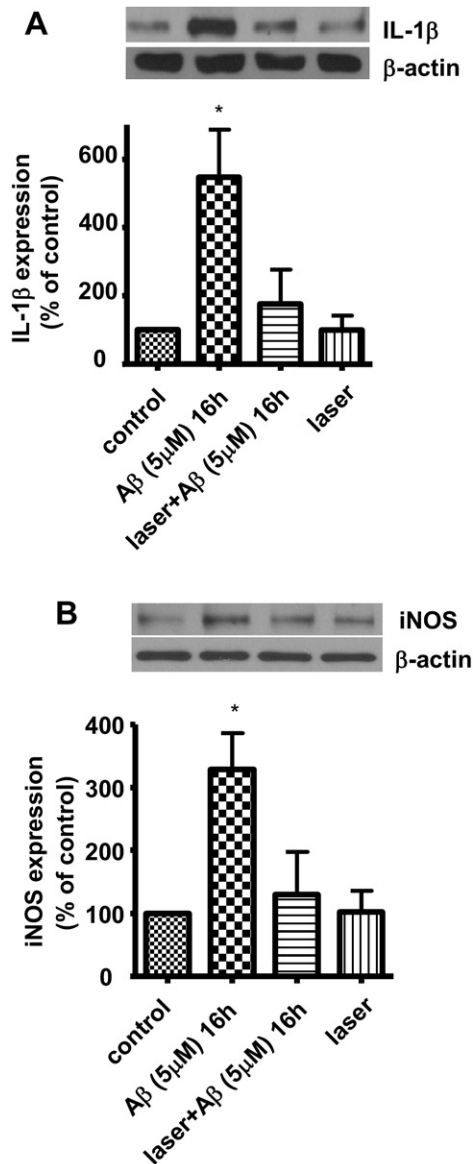
Inflammation is an early event of AD and plays critical roles in disease development (Tuppo and Arias, 2005; Wyss-Coray, 2006; Cameron and Landreth, 2010; McNaull et al., 2010). The major players involved in the inflammatory process in AD are microglia and astrocytes (McGeer

and McGeer, 1995, 2001; Combs, 2009). In AD, Aβ plaques are surrounded by activated microglia and astrocytes (Wallace et al., 1997; Yin et al., 2006; Bolmont et al., 2008; Koenigsnecht-Talboo et al., 2008; Yan et al., 2009) which have been shown to secrete many pro-inflammatory molecules, such as cytokines, chemokines, prostaglandins, complement proteins and complement inhibitors (McGeer and McGeer, 1995, 2001; Zhang et al., 2009). Among these pro-inflammatory molecules, IL-1β is an important regulator of inflammatory cascades and plays a key role in AD pathogenesis (Griffin and Mrak, 2002). IL-1β is capable of stimulating astrocytes to produce additional pro-inflammatory cytokines such as IL-6, another inflammation marker associated with neurodegeneration (Blom et al., 1997; Fiebich et al., 1998; Griffin et al., 1998). Furthermore, IL-1β triggers activation of NFκB (Cao et al., 1996; Song et al., 1997; Baeuerle, 1998) which is necessary for Aβ-stimulated iNOS induction in astrocytes (Akama et al., 1998; Akama and Van Eldik, 2000). iNOS is responsible for production of nitric oxide (Lee et al., 1993; Weldon et al., 1997) which may be detrimental to neurons (Hu et al., 1997). Low-level laser attenuates inflammation (Sakurai et al., 2000; Freitas et al., 2001; Lim et al., 2007; Aimbire et al., 2008; Boschi et al., 2008; Hammer et al., 2008) and helps tissue repair and wound healing (Whelan et al., 2001, 2003; Albertini et al., 2007; Correa et al., 2007; Viegas et al., 2007; Reis et al., 2008). However, the effects of laser on Aβ-induced inflammation in astrocytes have not been studied. Here we showed that laser pretreatment suppressed Aβ-induced the expression of IL-1β in astrocytes (Fig. 4A). Consistent with the notion that Aβ stimulation of iNOS in astrocytes is IL-1β dependent (Akama and Van Eldik, 2000), laser also attenuated iNOS induction



**Fig. 5.** Laser (632.8 nm) suppressed Aβ-induced phosphorylation of cPLA<sub>2</sub> in astrocytes. Western blot analysis shows that laser (632.8 nm) suppressed Aβ-induced phosphorylation of cPLA<sub>2</sub> in astrocytes. Data are expressed as percentages of control and mean ± SD from four independent experiments with three replicates per experiment (\*  $P < 0.05$ ).





**Fig. 6.** Laser (632.8 nm) suppressed A $\beta$ -induced expressions of IL-1 $\beta$  and iNOS in astrocytes. (A) Western blot analysis shows that laser (632.8 nm) suppressed A $\beta$ -induced expression of IL-1 $\beta$  in astrocytes. (B) Western blot analysis shows that laser (632.8 nm) suppressed A $\beta$ -induced expression of iNOS in astrocytes. Data are expressed as percentages of control and mean  $\pm$  SD from four independent experiments with three replicates per experiment (\*  $P < 0.05$ ).

in astrocytes (Fig. 4B). Since IL-1 $\beta$  is a key player in AD pathogenesis and iNOS activity has detrimental effects on neurons, 632.8 nm laser may also protect neurons against inflammation in AD brain.

Previous studies suggest that low-energy light exerts beneficial effects through modulating gene expressions (Shefer et al., 2002; Eells et al., 2004). Brief treatment with 670 nm light-emitting diode (LED) regulates expression of genes encoding DNA repair protein, antioxidant enzymes and molecular chaperons in retinas of methanol intoxicated rats (Eells et al., 2004). Low-energy laser irradiation (632.8 nm, 4.5 mW, 3 s) also increases the expression of

anti-apoptotic protein Bcl-2 and reduces the expression of pro-apoptotic protein Bax in muscle cell cultures (Shefer et al., 2002).

There has been a large body of compelling evidence suggesting that biological effects of lights could be used to treat neurodegenerative conditions, although the mechanism has yet to be fully understood. *In vitro*, LED attenuates apoptosis in PC12 cells after exposure to A $\beta_{25-35}$  (Duan et al., 2003). LED also increases survival and ATP content of neurons and decreases oxidative stress after rotenone-induced toxicity (Liang et al., 2008). In fact, LED also promotes neurite outgrowth of rat cortex in tissue culture (Wollman and Rochkind, 1998). *In vivo*, near-infrared light exerts neuroprotective effects against rotenone-induced neurotoxicity in rats (Rojas et al., 2008), and these effects were supported by assessing behavioral, morphological and neurochemical changes (Rojas et al., 2008). LED has been shown to induce central and peripheral nerve regeneration after trauma (Anders et al., 1993; Byrnes et al., 2005) and reduce neuroinflammation in rats (Byrnes et al., 2005). In this study, we demonstrated that 632.8 nm laser suppressed A $\beta$ -induced ROS production and inflammation in primary astrocytes. Although we did not investigate the dose response by varying irradiance (W/cm<sup>2</sup>) and energy density (J/cm<sup>2</sup>), it is important to note that a biphasic dose response has been demonstrated many times in low level laser therapy research (Huang et al., 2009). These dose dependence studies applied irradiance ranging from 0.7 to 40 mW/cm<sup>2</sup> and energy density ranging from 0.18 to 9 J/cm<sup>2</sup>. While the irradiance (1.5 mW/cm<sup>2</sup>) was applied in this study, the energy density (16.2 J/cm<sup>2</sup>) was outside the range of those in previous studies from others. Therefore, one might consider optimizing the use of light by applying various irradiance, and exposure time. Another parameter for optimization is the wavelength, because a longer wavelength is known for a deeper tissue penetration. For example, 810 nm LED light enhances mitochondrial metabolism (Trimmer et al., 2009), and transcranial infrared laser (805 nm) therapy has been reported to improve clinical rating scores after embolic strokes in rabbits (Lapchak et al., 2004). This study serves as a proof-of-principle to provide insights into potential applications of laser therapy for attenuating oxidative and inflammatory responses in AD.

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