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Low-Power Laser Irradiation Activates Src Tyrosine Kinase Through Reactive Oxygen Species-Mediated Signaling Pathway

JUNTAO ZHANG, DA XING,* AND XUEJUAN GAO

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou, China

Low-power laser therapy in medicine is widespread but the mechanisms are not fully understood. It has been suggested that low-power laser irradiation (LPLI) could induce photochemical reaction and activate several intracellular signaling pathways. Reactive oxygen species (ROS) are considered to be the key secondary messengers produced by LPLI. Here, we studied the signaling pathway mediated by ROS upon the stimulation of LPLI. Src tyrosine kinases are well-known targets of ROS and can be activated by oxidative events. Using a Src reporter based on fluorescence resonance energy transfer (FRET) and confocal laser scanning microscope, we visualized the dynamic Src activation in Hela cells immediately after LPLI. Moreover, Src activation by LPLI was in a dose-dependent manner. The increase of Src phosphorylation at Tyr416 was detected by Western blotting. In the presence of vitamin C, catalase alone, or the combination of catalase and superoxide dismutase (SOD), the activation of Src by LPLI is significantly abolished. In contrast, Gö6983 loading, a PKC inhibitor, did not affect this response. Treatment of Hela cells with exogenous H_2O_2 also resulted in a concentration-dependent activation of Src. These results demonstrated that it was ROS that mediated Src activation by LPLI. Cellular viability assay revealed that laser irradiation of low doses ($\leq$25 J/cm^2) promoted Hela cells viability while high doses impaired. Therefore, LPLI induces ROS-mediated Src activation which may play an important role in biostimulatory effect of LPLI.

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Low-power laser irradiation (LPLI) by helium–neon (He–Ne) laser has been found to modulate various biological processes in cell culture and animal models. This phenomenon of photobiomodulation has been widely applied in the treatment of skeletal muscle regeneration (Bibikova and Oron, 1993; Ben-Dov et al., 1999; Shefer et al., 2001, 2003), the wound healing (Conlan et al., 1996; Hawkins et al., 2005), skin wound care (De Araújo et al., 2007) and dental treatment (Jakse et al., 2007; Saygun et al., 2007). In addition, intense pulsed light, as a new, effective and non-ablative therapy method for damaged and aged skin, has satisfied application in aesthetic surgery (Bjerring et al., 2004). Experimental evidences show that LPLI can promote cell proliferation (Ben-Dov et al., 1999; Vinck et al., 2003; Yu et al., 2003), movement (Haas et al., 1990; Hiroki et al., 2003; Yu et al., 2003) and attachment (Karu and Pyatibrat, 2001; Feist et al., 2003; Karu et al., 2003) and so on. However, the molecular mechanism associated with the stimulatory effect of photo irradiation has not been clarified. One possibility is that the light energy is absorbed in intracellular chromophores and converted to metabolic energy, since cellular ATP levels increase almost twofold after He–Ne laser irradiation (Karu and Pyatibrat, 1995). Some studies have reported laser irradiation affects Ca^{2+} exchange through cell membrane, causing transient increase of intracellular Ca^{2+} concentration (Lavi et al., 2003). Consistent with Ca^{2+} increase, our previous study has indicated that PKC kinases are also activated in human lung adenocarcinoma cells stimulated by LPLI (Gao et al., 2006). Other reports have showed that LPLI induce the phosphorylation of mitogen-activated protein kinase (MAPK/ERK; Shefer et al., 2001). All these studies suggest that LPLI modulates diverse signaling pathways and ultimately affects the cell physiological processes.

The early signaling events of mammalian cells upon LPLI irradiation may be the burst of reactive oxygen species (ROS) generated from the photosensitization of endogenous cell chromophores, such as cytochromes, flavins, and NADPH (Karu, 1999; Alexandratou et al., 2002; Jou et al., 2002; Godley et al., 2005). Some reports have suggested that flavin and NADPH oxidases contribute to ROS production induced by violet and blue light (Huang et al., 2001; Eichler et al., 2005), while cytochrome C oxidase may be as a photoacceptor of monochromatic red to IR irradiation and a contributor to ROS generation (Karu, 1999; Pastore et al., 2000). It has been suggested that ROS, which have been presented as an important secondary messenger required for the activation of many signal molecules in various signal pathways, may play key roles in light/tissue interaction (Alexandratou et al., 2002; Jou et al., 2002; Matsui et al., 2007).

It is widely recognized that ROS as physiological regulators can up-regulate the activity of various protein kinases. For example, ROS has been shown to mediate the activation of the epidermal growth factor (EGF) in lysophophatidic acid-treated

Abbreviations: AA, ascorbic acid; CCK-8, Cell Counting Kit-8; CFP and YFP, cyan and yellow fluorescent protein; DHA, dehydroascorbic acid; EGF, epidermal growth factor; ROS, reactive oxygen species; FRET, fluorescence resonance energy transfer; LPLI, low-power laser irradiation; PKCs, protein kinase Cs; PMA, Phorbol 12-myristate 13-acetate; PTPs, protein tyrosine phosphatases; SOD, superoxide dismutase.

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*Correspondence to: Da Xing, MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China. E-mail: xingda@scnu.edu.cn

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Hela cells (Cunnick et al., 1998), the activation of Janus kinases in platelet-derived growth factor-treated Rat-1 cells (Simon et al., 1998), and the activation of Akt in angiotensin II-treated vascular smooth muscle cells (Ushio-Fukai et al., 1999). Non-receptor tyrosine kinases, particularly the Src kinases, are now widely recognized to be activated by oxidative events, and this process is involved in the activation of many downstream kinases such as the mitogen-activated protein kinase (MAPK) family, Akt, protein kinase C, and the EGF receptor (Stocker and Keaney, 2004).Src family kinases play key roles in regulating signal transduction by a diverse set of cell surface receptors, inducing tyrosine phosphorylation of many signaling and cytoskeletal proteins. They are critically involved in fundamental cellular processes, including cell proliferation, attachment, migration and survival (Parsons and Parsons, 2004). The activity of the Src family of tyrosine kinases is tightly controlled by inhibitory phosphorylation of a carboxy-terminal tyrosine residue (Tyr527 in chicken c-Src). This phosphorylation induces an inactive conformation in the kinase through the subsequent intramolecular binding with its SH2 domain. The closed/inactive state is converted to the open/active state through Tyr527 dephosphorylation. This conformational change promotes autophosphorylation at Tyr416 which, in turn, leads to enhanced Src kinase activity and to its complete activation (Brown and Cooper, 1996; Xu et al., 1997). Beside the well known C-terminal tyrosine dephosphorylation activation system, Src is proposed to possess redox switch. It has been recently reported that Src is directly oxidized by the burst ROS during integrin-mediated cell adhesion and the oxidation, which involves two sulfhydryl groups, namely, Cys245 in the SH2 domain and Cys487 in the kinase domain, leads to the enhanced activation of c-Src (Giannoni et al., 2005). However, whether Src has regulatory role in LPLI-modulated physiological process remains largely unexplored, so it is important to dig out whether and how Src is activated following LPLI.

In this study, we investigated the dynamic change of Src activity using FRET-imaging technique and its relationship with ROS production in living Hela cells treated with LPLI. Our results suggested that LPLI induced ROS-mediated activation of Src in a dose-dependent and time-related manner. Our findings provided a possible mechanism for the low-power laser therapy effects.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). EGF was purchased from PeproTech (Rocky Hill, NJ). PMA, dehydroascorbic acid (DHA), catalase and SOD (SOD1, Cu/Zn isoform) was purchased from Sigma (St. Louis, MO). Gö6983 was from Qiagen (Darmstadt, Germany). Anti-phospho-Src (Tyr416) antibody was from Upstate Biotechnology and anti-Src antibody was from Cell Signaling (Beverly, MA). IRDye 800CW anti-rabbit IgG (Rockland, Gilbertsville, PA), Alexa Fluor 680_goat anti-Mouse IgG (MolecularProbes, Eugene, OR). H2DCFDA and Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lipofectamine™ 2000 Reagent was purchased from Invitrogen (Carlsbad, CA). DNA Extraction kit was purchased from Qiagen (Valencia, CA). 4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) was from Invitrogen. PULSin™ was from PolyPlus Transfection (Illkirch, France).

Cell culture and cell transfection

Hela cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum (Sijiqing, Hangzhou, China), 50 units/ml penicillin, and 50 g/ml streptomycin in 5%CO_2, 95% air at 37°C in humidified incubator. Plasmids DNA of Src reporters (presented by Prof. Shu Chien, University of California, San Diego) were transfected into Hela cells by Lipofectamine™ 2000 reagent according to the manufacturer's protocol. The Src reporter is composed of CFP, the SH2 domain, a flexible linker, the Src substrate peptide and YFP. Before Src phosphorylation, the juxtaposition of cyan and yellow fluorescent proteins (CFP and YFP) allows to yield a high fluorescence resonance energy transfer (FRET). On Src phosphorylation, the substrate peptide can bind to the phosphopeptide-binding pocket of the SH2 domain and separate YFP from CFP, thus decreasing the FRET (Fig. 1; Wang et al., 2005).

Confocal laser scanning microscopes (LSM) and imaging analysis

Hela cells transfected Src reporter were grown in 35 mm culture dishes which were plated on the chamber of LSM with 5% CO_2 at 37°C using the temperature regulator (Tempcontrol 37-2 digital, Zeiss, Jena, Germany). FRET was performed on a commercial Laser Scanning Microscope (LSM510/ConfoCor2) combination system (Zeiss) in the dark. For excitation, the 458 nm line of a Ar-Ion laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT458), and focused through a Zesss C-Apochromat 40×, NA1.3 objective onto the sample. The emission fluorescence was split by a second dichroic mirror (secondary beam splitter NFT515) into two separate channels: the 470-500 nm bandpass (CFP channel) and the 530 nm longpass (YFP channel). Digital image analysis for quantitative evaluation was performed using Zeiss Rel3.2 image processing software (Zeiss). After choosing the whole area of observed cells, the average fluorescence intensities of CFP and YFP channels and the background were obtained. The background-subtracted fluorescence intensity of YFP divided by background-subtracted fluorescence intensity of CFP is the ratio of YFP/CFP. For each experiment, the ratio of YFP/CFP over time was normalized to 1 at zero time point. The cell images are presented in pseudocolor to highlight the changes in the ratio of YFP/CFP fluorescence intensity over time.

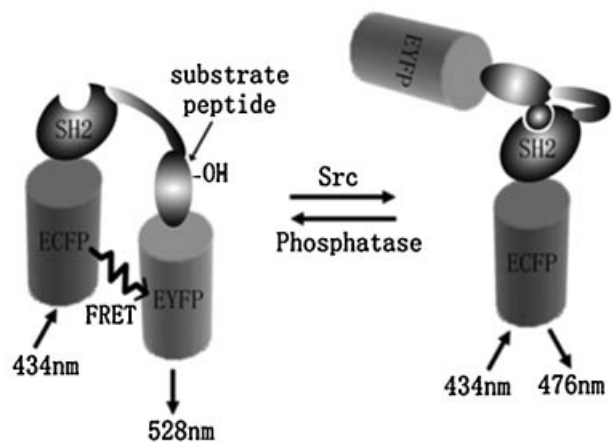

Fig. 1. Schematic diagram of Src reporter. The Src reporter is composed of CFP, the SH2 domain, a flexible linker, the Src substrate peptide and YFP. On Src phosphorylation, the substrate peptide can bind to the phosphopeptide-binding pocket of the SH2 domain and separate YFP from CFP, thus decreasing the FRET. Since it does not contain the full Src sequence, but a Src substrate, it monitors the activation of endogenous Src in live cells.

Delivery of catalase and SOD into Hela cells

Hela cells were grown in 35 mm culture dish or in 6-cm petri dish and 24 h later, subjected to protein delivery assay using PULSin™, a delivery reagent for catalase and SOD according to the manufacturer's instructions. Cells were washed three times with PBS before addition of the PULSin/protein mixture (for the delivery of catalase alone: 4 μl PULSin™ per 1 μg catalase; for the delivery of the combination of catalase and SOD: 6 μl PULSin™ per 1 μg catalase and 1 μg SOD). After 4 h of incubation at 37°C and 5% CO_2, the cells were washed twice with PBS and cultured in fresh DMEM medium again. Cells were stimulated with LPLI or EGF 36 h later.

Western blotting

For Western blotting assay, the cells were plated in 6-cm petri dishes. Briefly, cells were washed three times with precooled PBS before being scraped. The treated or untreated cells were lysed in a buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 100 mM PMSF and 1× protease inhibitor cocktail set I (Calbiochem, La Jolla, CA). The samples were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Roche, Mannheim, Germany). The resulting membrane was blocked with 5% skim milk, incubated with a designated primary antibody and the secondary antibody. The signals were detected with an ODYSSEY® Infrared Imaging System (LI-COR, Lincoln, NE). The following antibodies were used for immunoblot: anti-phospho-Src (Tyr416), Src antibody, IRDye® 800 CW anti-rabbit IgG, Alexa Fluor 680® goat anti-Mouse IgG.

LPLI on Hela cells

The experiment was conducted as described in our previous work (Gao et al., 2006). After 36–48 h of serum starved with 0.5% FBS, the cells were irradiated with He–Ne laser (632.8 nm, spot diameter 0.635 cm; HN-1000, Guangzhou, China) for 0.8, 1.33, 2.66, 4, 6.66, or 13.33 min in the dark, with the corresponding fluences of 3, 5, 10, 15, 25, and 50 J/cm^2 respectively (light guide tip output 25.2 mW, power density 64.6 mW/cm^2).

EGF, PMA, and H_2O_2 treatment

After 36–48 h of serum starved with 0.5% FBS, Hela cells transfected with Src reporter or non-transfected were treated with EGF (50 ng/ml) or H_2O_2 (25, 50, 100, and 200 μM) or PMA (50 ng/ml), diluted in PBS. Before and after treatment, cells were observed and photographed using the LSM microscope.

Assay of intracellular ROS

H2DCFDA is a ROS-sensitive probe that can be used to detect ROS production in living cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihydrofluorescein derivatives H2DCF. H2DCF oxidation yields a fluorescent adduct, dichlorofluorecein (DCF) that is trapped inside the cell. Cells were incubated at 37°C with 10μM H2DCFDA for 3 min and then fluorescence was recorded using LSM. DCF fluorescence was excited by 488 nm using the argon laser, the beam splitter was NFT490 nm and the emission was detected using a 530-nm longpass filter. Ten cells were randomly selected from the images and the fluorescence intensity was then measured for each cell, and the relative fluorescence intensity was taken as the average of the 10 values.

Vitamin C uptake

Cells were incubated for 30 min in incubation buffer (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4) supplemented with 4 mM DHA, the oxidized form of vitamin C. After loading, the cells were washed twice with PBS and incubated in fresh medium containing 0.5% FCS.

Cell viability assay

Hela cells were cultured in 96-well microplates at a density of 4×10^3 cells/well and each well contained 100 μl culture medium. After 36–48 h serum starvation with 0.5% FCS, the grouped cells were irradiated with He–Ne laser in the dark at indicated fluences respectively or added with different concentrations of H_2O_2. The interval wells were filled with ink in order to minimize the scattered or reflected light. Twelve hours after the treatments, cells were incubated with CCK-8 solution (10 μl/well) for 2 h. Viability determination was based on the bioconversion of the tetrazolium compound by intracellular dehydrogenase into formazan, as determined by absorbance at 450 nm using an Infinite 2000 plate reader (TECAN, Mönnedorf, Switzerland). Cell relative viability was expressed as the ratio of the treated viability compared with the control viability ($=1$). The value is directly proportional to the number of viable cells in a culture medium and the cell proliferation.

Statistical analysis

All assays were performed at least three times. All the error bars represented SEM ($n \geq 3$). For statistical evaluation Student's paired *t*-test was used and significance was defined as $P < 0.05$.

Results

Dose- and time-dependent activation of Src kinases stimulated by LPLI

A powerful method for imaging and quantifying of spatio-temporal activation of Src in living cells is the transfection of cells with FRET reporter for Src. We transfected genetically encoded Src reporter into Hela cells to assess the effect of LPLI on Src activity. As shown in Figure 2A, 5 J/cm^2 LPLI induced the transient decrease of YFP/CFP emission ratio, which indicated the Src activation. It should be noted that laser irradiation began but not ended at time zero, so the possible change of Src activity during the period of irradiation could be recorded. During this response, Src activity peaked at 3 min and then restored to nearly basal level by 10 min and remained relatively invariant thereafter. Src activation resulted in preferential phosphorylation of Src reporter in the cytosol than the nucleus. Increasing the illuminating energy up to 25 J/cm^2 caused a stronger activation of Src which lasted over 30 min. The data in Figure 2B showed the emission intensity of CFP fluorescence, YFP fluorescence and the ratio of YFP/CFP in response to LPLI (25 J/cm^2). The emission increased in the CFP channel but decreased in the YFP channel (Fig. 2B), which implied that Src was activated. To investigate the relationship between the dose of LPLI and Src activation, the effects of 3, 10, 15, and 50 J/cm^2 LPLI were also studied. LPLI (3 and 10 J/cm^2) resulted in similar degree of Src activation to 5 J/cm^2 LPLI. The graphs of Figure 2C revealed the dose-dependent activation of Src by LPLI. Low doses caused weak and transient activation and high doses induced strong and sustained activation. While inducing the highest increase of Src activity, 50 J/cm^2 laser irradiation in some cases caused slight cell contraction, probably due to toxic effect of high-dose laser irradiation (data not show). Pretreatment of cells with PP1, a specific inhibitor of Src kinase, completely inhibited the activation of Src by various doses of LPLI (as shown in Fig. 2C, represented by 25 J/cm^2 LPLI + PP1).

To confirm that the activation of Src by LPLI, is due to the photochemical reaction but not to the possible thermal effect caused by laser irradiation, we measured the temperature rise using a thermal imaging systems (FLIR System's Thermal CAM™ P65 with a thermal sensitivity of 0.08°C at 30°C) and the results showed that the temperature rise caused even by the highest dose we used, 50 J/cm^2, is about 0.5°C. So we raised the temperature by 0.7° (to 37.7°C) using the temperature regulator of confocal laser scanning microscope and treated

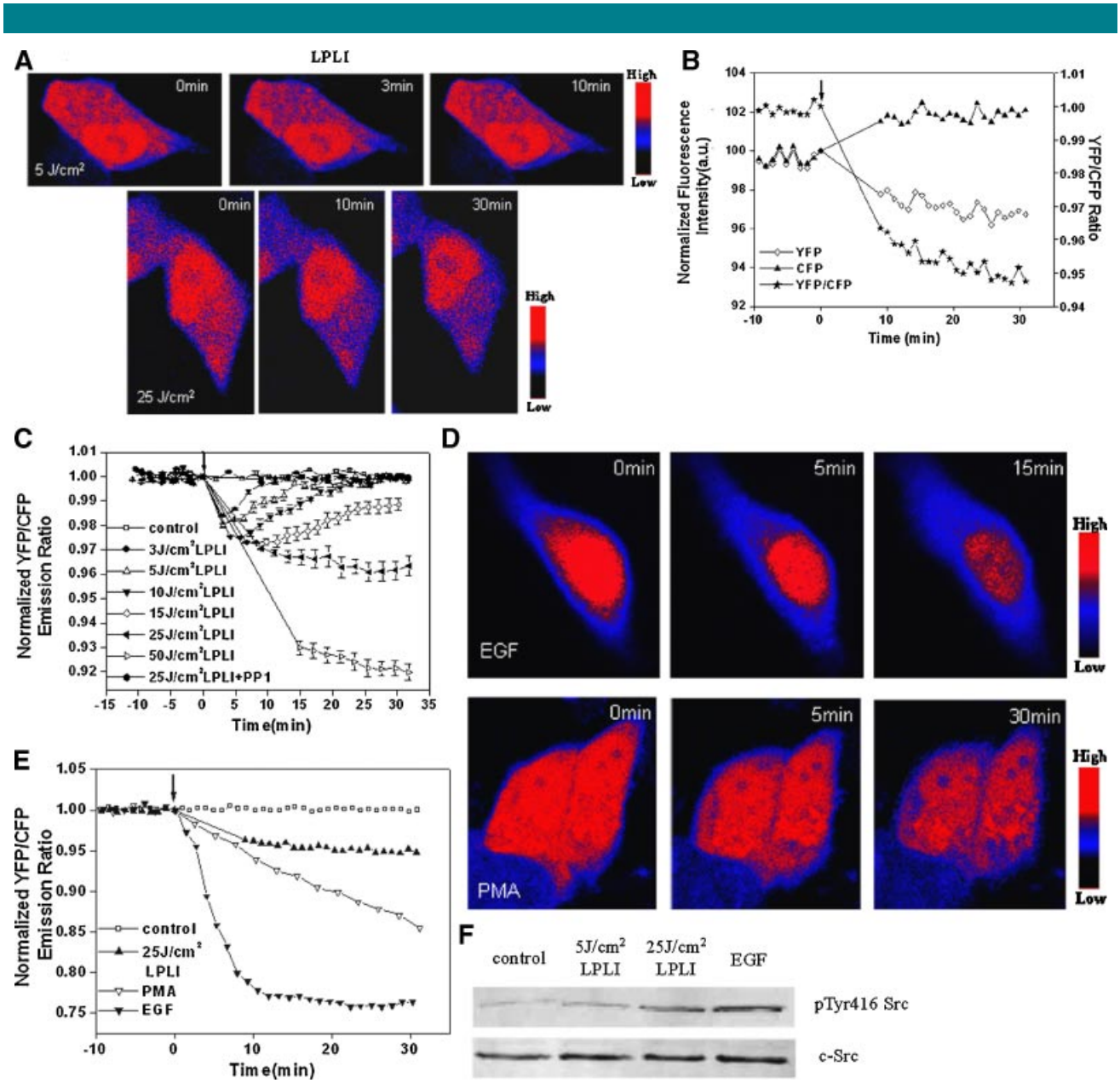

Fig. 2. Dynamics of Src activity induced by LPLI, EGF and PMA in Hela cells. A: The pseudocolor image series of YFP/CFP emission ratio at the indicated times after the stimulation of 5 and 25 J/cm^2 LPLI. B: The time courses of YFP emission intensity, CFP emission intensity and YFP/CFP emission ratio of Src reporter in response to 25 J/cm^2 LPLI. C: The time courses of YFP/CFP emission ratio of Src reporter in response to different doses of LPLI (3, 5, 10, 15, 25, 50, or 25 J/cm^2 after 1 h preincubation with 10 μM PP1). D: Pseudocolor images of YFP/CFP emission ratio of Src reporter in response to EGF and PMA. E: The comparison of the degree of Src activation by 25 J/cm^2 LPLI with EGF and PMA. F: Hela cells were treated with 5 J/cm^2 LPLI, 25 J/cm^2 LPLI, EGF or kept as control. Five minutes after stimulation, cells were subjected to Western blotting assay. Cell lysates from various samples were probed for phospho-Src (Tyr416) (upper) and Src (to show comparable protein loading among various samples, lower). Values are mean ± SEM of at least three separate experiments expressed as the percentage of the pretreatment level. (Laser was applied at time 0.)

Hela cells with 37.7°C for 10 min to investigate whether this temperature rise could activate Src. The result showed that this temperature rise led to no change of Src activity (data not show).

To validate the effective measurement of Src activity by Src reporter in the process stimulated by LPLI, we monitored the activation process of Src induced by EGF and PMA using Src reporter as positive controls (Wang et al., 2005; Walker et al., 2007). As shown in Figure 2D,E, EGF stimulation led to a rapid and robust change of FRET, with 24% emission ratio decrease of YFP to CFP, consistent with previous work (Wang et al., 2005). By contrast, PMA induced a gradual FRET decrease, which lasted for a longer time over 1 h. The corresponding changes of FRET ratio of Src reporter of the cells treated with EGF and PMA were consistent with that for LPLI treatment (Fig. 2D,E). These results demonstrated that the Src reporter was phosphorylated when the cells were treated with EGF and PMA or LPLI, indicating Src activation during these processes.

Since the FRET effect of Src reporter was only determined by the balance between Src kinases and protein tyrosine

phosphatases (PTPs), we next explored whether LPLI increased Src phosphorylation at Tyr416 which was indicative of Src activation by Western blot assay using anti-phospho-Src (Tyr416) antibody and anti-c-Src antibody. As shown in Figure 2F, the result demonstrated that the phosphorylation level of Src was evidently improved by LPLI in a dose-dependent manner at 5 min after the stimulation of 5 J/cm^2, 25 J/cm^2 LPLI and EGF. Thus, it can be concluded that LPLI induced the activation of Src which was dependent on the accumulated dose of laser irradiation and partially due to the direct phosphorylation of Src at Tyr416.

Intracellular ROS production induced by LPLI

ROS levels could be elevated by visible light, and it has been suggested that the production of ROS is responsible for the biostimulative effect of LPLI (Callaghan et al., 1996; Grossman et al., 1998). Thus, we explored whether LPLI stimulated the generation of ROS in Hela cells by using the oxidation-sensitive fluorescence probe H2DCF-DA. Our interest being in the early signaling events, so we focused on the 5-min time point after stimulation with LPLI. The fluorescence images were collected by laser confocal microscope. As shown in Figure 3A, in Hela

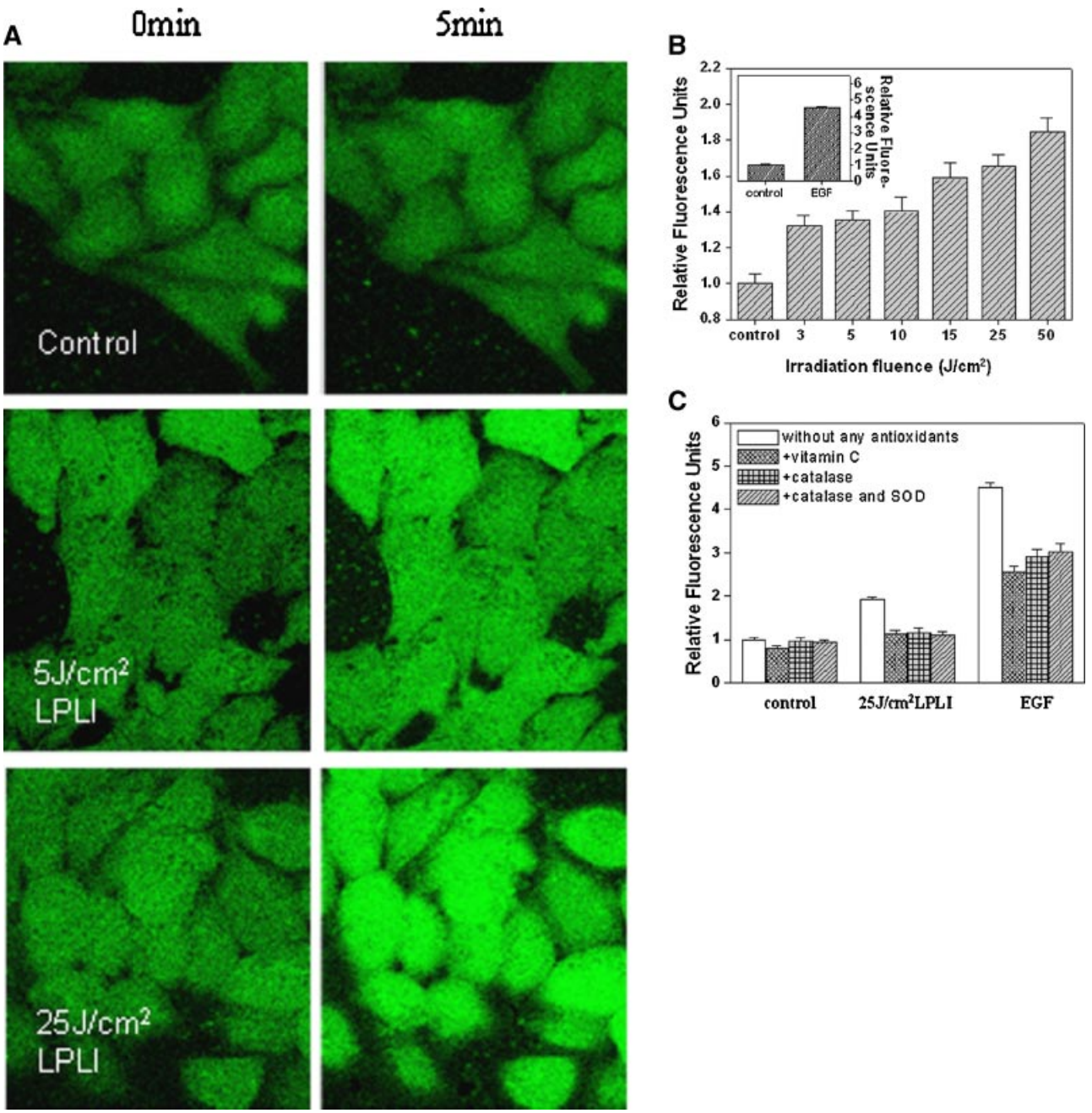

Fig. 3. ROS generation induced by LPLI. A: The DCF fluorescence images of Hela cells stimulated with 5 or 25 J/cm^2 LPLI were recorded with confocal laser scanning microscope at indicated times. B: Histograms indicate the values of the rise of intracellular DCF fluorescence intensity at 5 min after treatment with different doses of LPLI as well as EGF. C: Vitamin C, catalase or catalase plus SOD inhibited ROS production by the stimulation of LPLI or EGF. Values were all from the images recorded by confocal laser scanning microscope. Values are mean ± SEM. (Laser was applied at time 0.)

cells treated with 5 and 25 J/cm^2 LPLI, ROS were increased apparently in a dose-dependent manner compared with control cells. Similar effects were noted in cells stimulated with other doses of laser irradiation (as plotted in Fig. 3B). As a positive comparison of ROS production by LPLI, EGF induced a burst of ROS in Hela cells which increased by more than two times within 5 min (Fig. 3B, inset).

It was reported that antioxidants could eliminate light effect (Grossman et al., 1998; Karu and Pyatibrat, 2001), thus we tested the effect of various antioxidants on ROS production induced by LPLI. Vitamin C is a broad-spectrum antioxidant and could effectively quench ROS, including that induced by granulocyte macrophage–colony-stimulating factor (GM-CSF), ligand of FAS receptor (CD9) (Guaiquil et al., 2001; Cárcamo et al., 2002; Perez-Cruz et al., 2003). The results showed that in the presence of vitamin C (loaded by 4 mM DHA), the level of ROS generation by LPLI stimulation decreased nearly to the basal level (as represented by 25 J/cm^2 LPLI + vitamin C in Fig. 3C), while the generation of ROS induced by EGF decreased approximately 50% (Fig. 3C). By protein transfection technique, we delivered catalase alone or the combination of catalase and SOD into Hela cells 36 h before LPLI or EGF stimulation. The results showed that catalase alone or the combination of catalase and SOD significantly prevented the increase of DCF fluorescence upon the stimulation of LPLI and partially reduced the intensity of DCF fluorescence after EGF stimulation (Fig. 3C).

The activation of Src by LPLI can be inhibited by ROS scavengers, but not by the inhibitor of PKCs

Since ROS could function as an activator of Src, we explore the effect of these ROS scavengers on Src activation by LPLI. As shown in Figure 4A (upper row) and Figure 4B, in cells loaded with vitamin C we found that there was no detectable change of FRET ratio after the stimulation of 25 J/cm^2 LPLI, indicating that vitamin C prevented Src activation. The similar results were noted when doses of LPLI changed to 3, 5, 10, 15, or 50 J/cm^2 (data not shown). As to the stimulation of 50 J/cm^2 LPLI, the activity of Src still showed some degree of increase in the presence of vitamin C, though much lower than that of the control (stimulated with LPLI in the absence of vitamin C (data not show)).

To further investigate whether H_2O_2 or $O_2^{-\cdot}$ contributed to the activation of Src by LPLI, we delivered catalase or the combination of catalase and SOD into Hela cells 36 h before LPLI stimulation and the phosphorylation level of Src was analyzed by Western blotting at 5 min after the stimulation of 25 J/cm^2 LPLI. The results in Figure 4C revealed that H_2O_2 played an important role in LPLI/Src signaling pathway. However, it was still not clear whether $O_2^{-\cdot}$ also took part in the activation of Src because the phosphorylation level of Src in sample treated with catalase combined with SOD showed no significant difference with the sample treated with catalase alone. Taken together, the results implied that ROS acted as an important mediator of Src activation by LPLI.

It has been indicated that ROS are necessary for optimal tyrosine phosphorylation of the EGF receptor as well as phospholipase C-g1 in EGF-induced signaling pathway (Bae et al., 1997). To confirm the role of ROS in Src activation, Figure 4A (lower row) and Figure 4B indicated that stimulation of cells in the presence of vitamin C also led to a dampening of EGF signaling, where a marked reduction in Src phosphorylation was clearly evident. In addition to the quantitative effect, a delay in kinetics of phosphorylation was also discernable.

Our recent work proved that PKCs were also activated by LPLI (Gao et al., 2006) and several PKC isoforms have been shown to lie upstream of where Src is activated (Tatin et al., 2006; Walker et al., 2007). To investigate whether PKCs contribute to Src activation, we treated Hela cells with PKC inhibitor Gö6983 before LPLI stimulation. The results showed that Gö6983 had no effect on Src activation by different doses of LPLI (as represented by 25 J/cm^2 LPLI + Gö6983 in Fig. 4C,D), indicating that Src activation occurred in a PKC-independent way.

Kinetics and concentration dependence of exogenous H_2O_2 to activate Src

To directly test the effect of ROS on Src activation, Hela cells were treated with exogenous H_2O_2 at a low to moderate concentration (25–200 μM). Results showed that 100 μM H_2O_2 induced the evident FRET decrease of Src reporter, indicating the significant activation of Src (Fig. 5A). The kinetics of Src activation by H_2O_2 was also found to be concentration-dependent and showed a long-lasting increase of Src activity which may be due to the permanent existence of exogenous H_2O_2 (Fig. 5B). H_2O_2 treatment (25 μM) resulted in a decrease in FRET ratio reaching to 3.3% at 30 min, similar to that of 15 J/cm^2 LPLI, and 50μM H_2O_2 reaching to 4.3% similar to that of 25 J/cm^2 LPLI. Treating cells with 200 μM H_2O_2 resulted in a severe oxidative stress because the cells showed contraction, indicating oxidative damage while Src was dramatically activated (data not show).

The effects of LPLI and exogenous H_2O_2 on the viability of Hela cells

Because Src plays important roles in multicellular behavior such as cell growth, proliferation, migration and attachment, we examined whether LPLI exerts long-term effects on Hela cells viability after Src-mediated signal transduction events. After 36–48 h serum starvation, Hela cells were stimulated with various doses of LPLI and subjected to CCK-8 assay 12 h after laser irradiation. The results in Figure 6A indicated that LPLI of low doses leaded to the different extent increase of cell viability. Compared with the control group, significant differences in cell viability were observed for the groups treated with 3, 5, and 25 J/cm^2 LPLI. This beneficial effect seemed reaching its maximum at a dose between 5 and 15 J/cm^2, because it became declined at 25 J/cm^2. Increasing the laser dose up to 50 J/cm^2 evidently decreased the cell viability and caused cell morphologic damage.

To assess the role of ROS in LPLI effect, we directly measured the influence of different concentrations of exogenous H_2O_2 on cell viability. The data in Figure 6B showed that cell viability were effectively improved by H_2O_2 within the range of low concentrations, and there was significant increase in cell viability in groups treated with 50 and 100 μM H_2O_2 compared to the control group ($P < 0.05$). Increasing the concentration up to 200 μM H_2O_2 markedly weaken cell viability. Comparing these results with those obtained with LPLI indicated that H_2O_2 can mimic the effect of LPLI and the production of ROS may be a key contributor to the effect of laser irradiation.

Discussion

In the present study, the dynamics of Src activity in LPLI-illuminated Hela cells and its relationship with the production of intracellular ROS are investigated. LPLI was found to induce the activation of Src which occurred in a ROS-dependent manner. The results contribute to the general idea of ROS as key secondary messengers during LPLI-induced biological effect.

Previous studies have showed that LPLI promotes cell proliferation, movement and attachment. The molecular mechanism about the stimulatory effect of LPLI on these cell behaviors remains much unclear, especially about the latter two. As an important mediator of signal transduction, Src

tyrosine kinases are critically involved in the control of cytoskeletal organization and are required for the regulation of fundamental cellular processes including cell proliferation, cell shape, adhesion, migration and survival. So we explored the possibility that LPLI modulated these cell behaviors through regulation of Src activity. The present study indicates LPLI can induce Src activation which is in a dose-dependent manner. LPLI induced a rapid Src activation within 3 min. At constant power density, short-time irradiation only induced transient and weak activation while long-time irradiation caused sustained and strong activation of Src.

Src activation by LPLI was demonstrated to depend on intracellular ROS production. This conclusion is based on the following observations: (a) LPLI increased intracellular ROS level; (b) ROS scavengers could inhibit Src activation by LPLI; (c) exogenous H_2O_2 could mimic the effect of LPLI on Src activity.

LPLI can induce intracellular ROS generation which is in a dose-dependent manner. It is believed that ROS are produced through activation of endogenous photosensitizers. Therefore, the resulting ROS are spread all over the cell in contrast to photodynamic therapy treatment in which ROS are produced in subcellular locations based on the photosensitizer localization (such as plasma membrane, mitochondria). ROS as common secondary messengers play important roles in photosignal transduction. It is found that $[Ca^{2+}]_i$ elevation follows $O_2^{-\cdot}$ and H_2O_2 formation in cardiomyocytes stimulated with

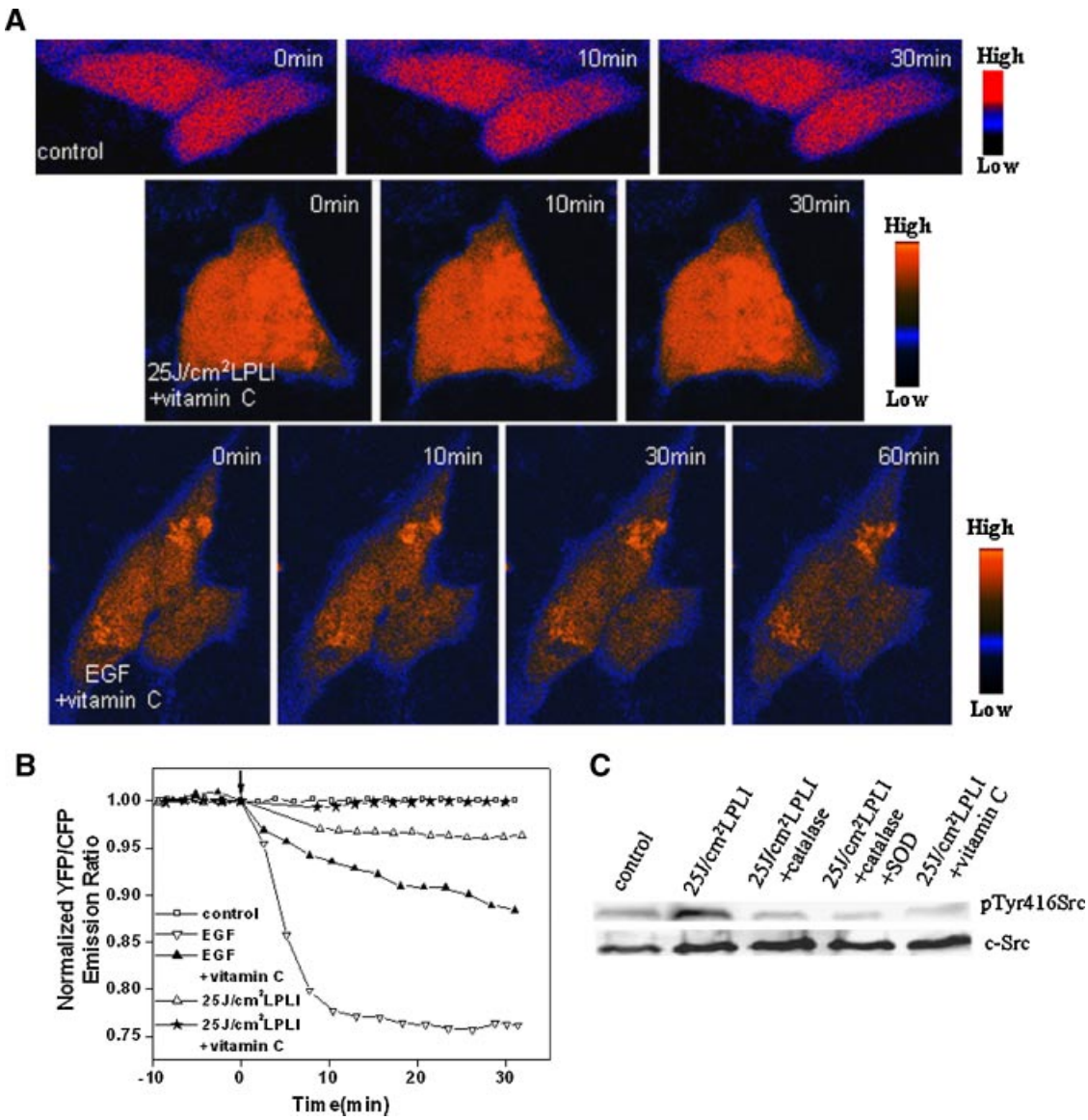

Fig. 4. LPLI-induced Src activation inhibition by antioxidants, but not by PKC inhibitor Gö6983. A: The pseudocolor image series of YFP/CFP emission ratio in Hela cells treated with 25 J/cm^2 LPLI and EGF in the presence of vitamin C. B: The quantitative time courses of the CFP/YFP emission ratio in Hela cells treated with 25 J/cm^2 LPLI and EGF with or without the presence of vitamin C. C: Hela cells were stimulated with 25 J/cm^2 LPLI 36 h after the mock transfection or the transfection of catalase or the combination of catalase and SOD or 30 min loading of vitamin C. Five minutes after the stimulation, Hela cells were subjected to Western blotting assay. Cell lysates from various samples were probed for phospho-Src (Tyr416) (upper) and Src (to show comparable protein loading among various samples, lower). D: The pseudocolor image series of the CFP/YFP emission ratio with or without the presence of PKC inhibitor Gö6983. E: The quantitative time courses of the CFP/YFP emission ratio in Hela cells treated with 25 J/cm^2 LPLI with or without the presence of PKC inhibitor Gö6983. Values are mean ± SEM of three separate experiments. (Laser was applied at time 0.)

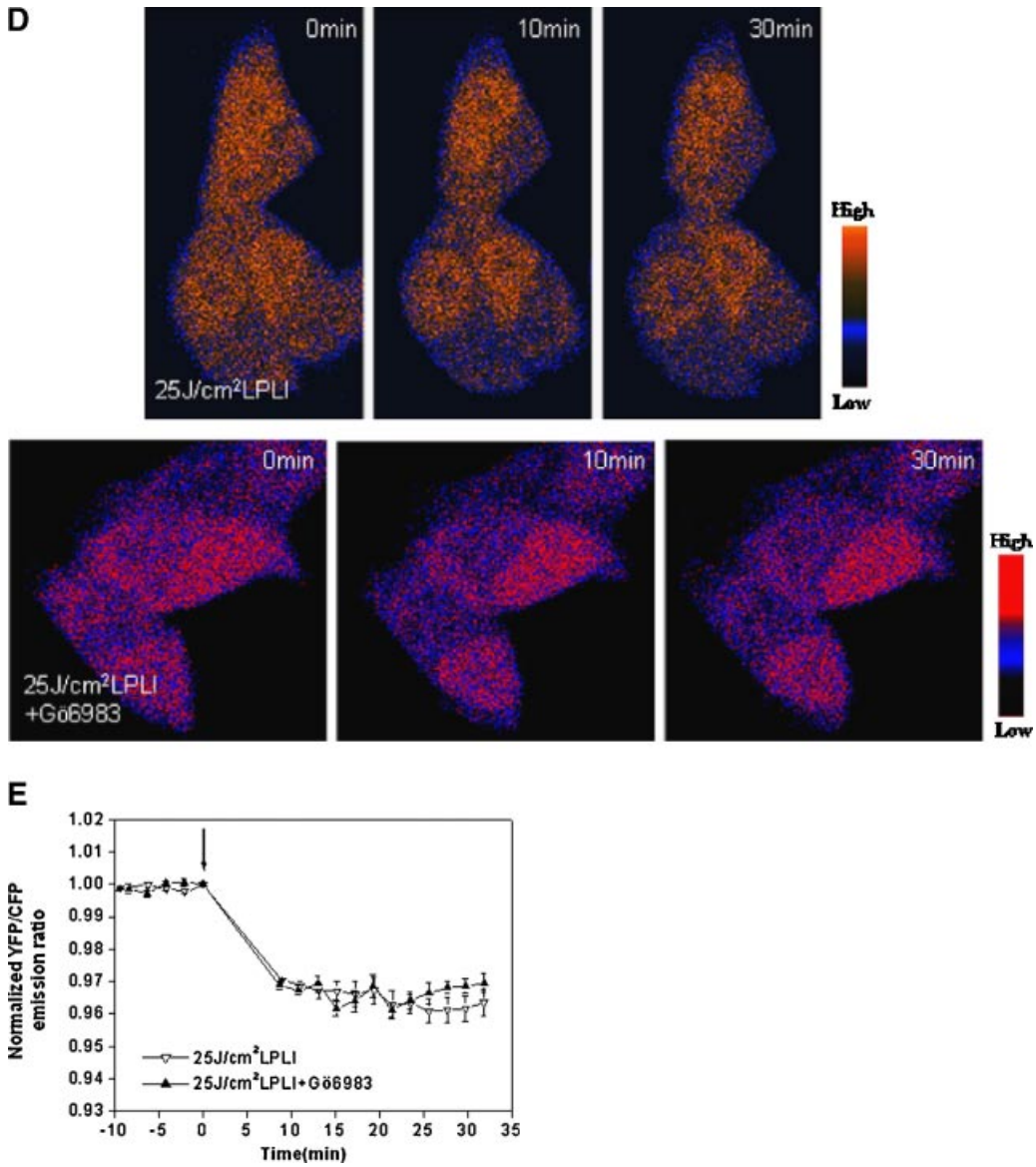

Fig. 4. (*Continued*)

low-energy visible light (Lavi et al., 2003). In UV-induced signal pathways ROS were reported to mediate Akt activation (Huang et al., 2001).

As an important antioxidant that protects organism from oxidative stress, vitamin C is transported into most cells in the oxidized form DHA and is rapidly reduced inside the cell and accumulated as AA which is a strong redoxing substance. Our data showed that loading Hela cells with vitamin C by incubation 4 mM DHA completely inhibits LPLI-induced ROS production as well as Src activation. It has been reported that incubation of cells with 500 μM DHA leaded to intracellular accumulation of vitamin C up to 5 mM which evidently inhibited GM-CSF-induced ROS production. Therefore, it can be postulated that intracellular accumulation of vitamin C supplied as 4 mM DHA was sufficient to completely scavenge ROS produced by LPLI. Previous work have reported that the inhibition of NF-kB activation in enthanol-treated embryo and Jak-2 activation in response to GM-CSF stimulation by vitamin C also showed correlationship with its inhibitory effect on ROS production (Cárcamo et al., 2002; Perez-Cruz et al., 2003). Treatment with catalase, the specific antioxidant for H_2O_2, further demonstrated that H_2O_2 is one type of free radicals that is responsible for Src activation by LPLI. This result supported the opinion that H_2O_2, which is a relatively stable species of free radicals and can freely diffuse through cellular membranes, might function as an ideal physiological regulator of signaling (Finkel, 2003). The results of antioxidant treatment confirmed our hypothesis that Src activation by LPLI occurred in a ROS-dependent way.

Consistent with a potential role of ROS in LPLI-triggered signal transduction, exogenous H_2O_2 was also found to activate Src in a dose-dependent manner. Low concentration of H_2O_2 increased the activity of Src to a degree much similar to that of moderate doses of LPLI such as 15 and 25 J/cm^2. Treatment of platelets with H_2O_2 also results in a time- and concentration-dependent activation of Src (Rosado et al., 2004).

The mechanism of Src activation by LPLI-induced ROS is still unclear. Up-regulation of Src activity by ROS is partially

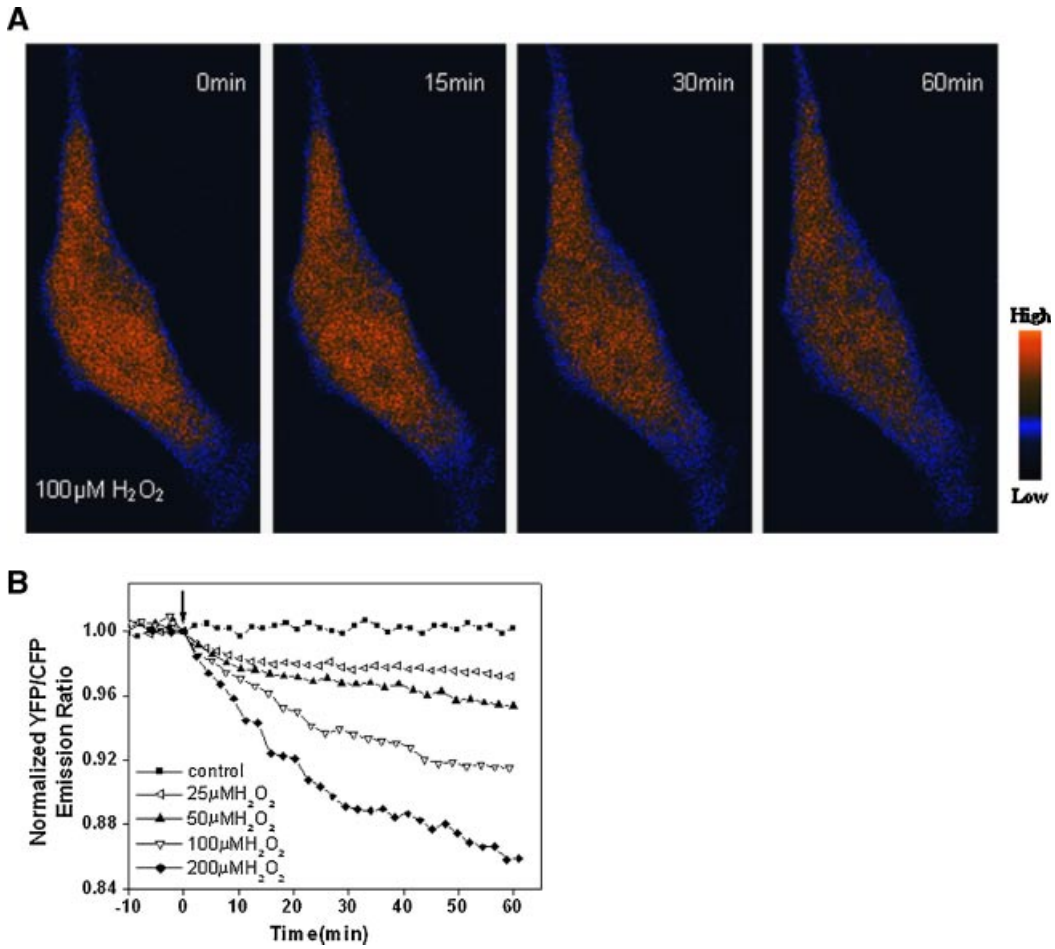

Fig. 5. Activation of Src by exogenous H_2O_2. A: The pseudocolor images CFP/YFP emission ratio after 100 μM H_2O_2 treatment. B: The quantitative time courses of the CFP/YFP emission ratio after 25, 50, 100, or 200 μM H_2O_2 treatment or mock treatment (images not shown). (H_2O_2 was applied at time 0.)

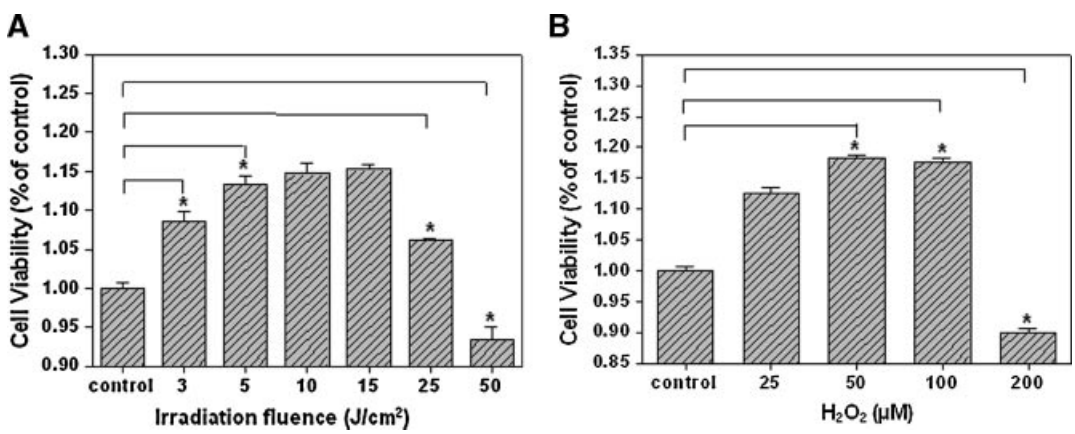

Fig. 6. The effects of LPLI or exogenous H_2O_2 on the viability of Hela cells. Hela cells were serum-starved for 36–48 h, and then subjected to laser irradiation (A) or 25–200 μM H_2O_2 stimulation (B). After stimulation, Hela cells were maintained cultured in 0.5% FBS for 12 h. Then cell viability assays were performed using CCK-8; $^*P < 0.05$.

explained by the ability of ROS to inactivation of PTPs or the ability of ROS to oxidize thiol groups in Src. Inactivation of PTPs has been proposed as the mechanism of UV-induced signal transduction, including receptor activation (Grob et al., 1999). It is well known that inhibition of PTPs induced by ROS help to shift the balance in favor of protein tyrosine phosphorylation (Chiarugi and Cirri, 2003; Singh et al., 2005). Transient inactivation of the receptor-coupled PTP by ROS induced upon the activation of B lymphocyte antigen receptor (BCR) is considered sufficient to activate the Src family kinases Lyn (Singh et al., 2005). Besides PTP inhibition, ROS can directly oxidize specific cysteines in Src resulting in a conformation change that is necessary for Src activation. This redox regulation is suggested to be an important feature of Src function (Giannoni et al., 2005). Similar cysteine modification by NO resulting in *S*-nitrosylation is also found to activate Src both in vivo and in vitro (Akhand et al., 1999).

Low doses of laser irradiation induce small amount of ROS generation which may only cause temporal imbalances of redox homeostasis and lead to transient activation of Src. In contrast, high energy of laser irradiation results in large amounts of ROS production which may subject the cells to chronic oxidative stress (Dröge, 2002). This chronic oxidative stress can give rise to dysregulation of redox state and sustained activation of Src. The pleotropic function of Src on fundamental cellular processes underscores the importance of Src activation in LPLI beneficial biostimulation effects. The activation of Src under the condition of high doses of laser irradiation may provide survival signals against oxidative stress-induced apoptosis.

Though we reasoned that there may be crosstalk between PKCs and Src in LPLI-triggered signaling pathways, the PKC inhibitor Gö6983 exerts no effect on Src activation, suggesting that Src activation is independent of PKCs. There were two possibility: (1) The two pathways ROS/Src and ROS/Ca^{2+}/PKCs might be relatively independent; (2) The two pathways might have other potential mechanisms of crosstalk. Anyway, this result strengthened our conclusion that ROS played a crucial role in LPLI-induced Src activation.

Previous studies have reported that LPLI activates MAPK/ERK and Akt (Shefer et al., 2001, 2003), but the mechanism remains unclear. In response to oxidant stress, both MAPK/ERK and Akt function as downstream of Src (Kitagawa et al., 2002; Zhougang and Schnellmann, 2004; Wang et al., 2007). Thus, our findings may provide a possible mechanism involving LPLI/ROS/Src/ERK and LPLI/ROS/Src/Akt pathways.

How will activated Src play roles in LPLI stimulatory effect? On the basis of published reports on the regulatory role for Src, it can be postulated that Src phosphorylates structural proteins such as VE-cadherin and cortacin which will affect cell adhesion and movement; Src also can activate transcriptors, such as NF-kB and STAT-3, and protein kinases, such as EGFR, ERK1/2, Akt and FAK, eventually influencing long-term cell behaviors such as proliferation and survival.

Integration of our findings with published reports allows us to propose a model about LPLI-induced signal transduction involving Src (Fig. 7).

We conclude that LPLI stimulates Src activation which is mediated by ROS. This finding provides evidence regarding the important role of ROS as a secondary messenger in the stimulatory effect of LPLI. Src activation evoked by low-power photoirradiation might explain previous obtained results such as the LPLI-enhanced migration and proliferation of melanocytes during the repigmentation of segmental-type vitiligo (Yu et al., 2003), increased attachment of Hela cells to glass matrix (Karu and Pyatibrat, 2001), and accelerated adhesion and growth of cultured human gingival fibroblasts on periodontally involved root surfaces (Feist et al., 2003).

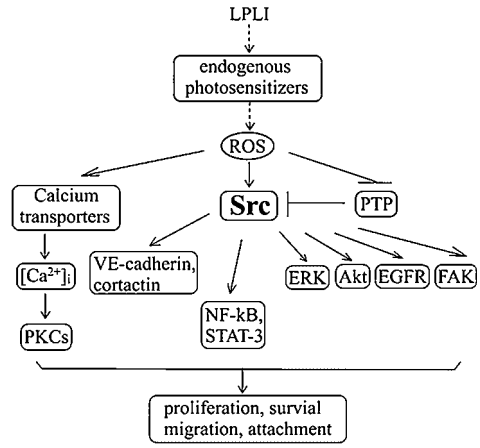

Fig. 7. A model of the signaling pathways of Src activation. Broken lines mean that the downstream changes produced by the upstream do not be proved clearly. Real lines mean that the downstream effects induced by the upstream have been proved already.

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Literature Cited

Akhand AA, Pu M, Senga T, Kato M, Suzuki H, Miyata T, Hamaguchi M, Nakashima I. 1999. Phosphorylation of c-Src on tyrosine 527 by another protein tyrosine kinase. J Biol Chem 274:25821–25826.

Alexandratou E, Yova D, Handris P, Kletsas D, Loukas S. 2002. Human fibroblast alterations induced by low power laser irradiation at the single cell level using confocal microscopy. Photochem Photobiol Sci 1:547–552.

Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. J Biol Chem 272:217–221.

Ben-Dov A, Shefer G, Irinitchev A, Wernig A, Oron U, Halevy O. 1999. Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. Biochim Biophys Acta 1448:372–380.

Bibikova A, Oron U. 1993. Promotion of muscle regeneration in the toad (*Bufo viridis*) gastrocnemius muscle by low-energy laser irradiation. Anat Rec 235:374–380.

Bjerring P, Christiansen K, Troilius A, Dierickx C. 2004. Facial photo rejuvenation using two different intense pulsed light (IPL) wavelength bands. Lasers Surg Med 34:120–126.

Brown MT, Cooper JA. 1996. Regulation, substrates and functions of Src. Biochim Biophys Acta 1287:121–149.

Callaghan G, Riordan C, Gilmore WS, McIntyre IA, Allen JM, Hannigan BM. 1996. Reactive oxygen species inducible by low-intensity laser irradiation alter DNA synthesis in the haemopoietic cell line U937. Lasers Surg Med 19:201–206.

Cárcamo JM, Bórquez-Ojeda O, Golde DW. 2002. Vitamin C inhibits granulocyte macrophage-colony-stimulating factor-induced signaling pathways. Blood 99:3205–3212.

Chiarugi P, Cirri P. 2003. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. Trends Biochem Sci 28:509–514.

Conlan MJ, Rapley JW, Cobb CM. 1996. Biostimulation of wound healing by low-energy laser irradiation. A review. J Clin Periodontol 23:492–496.

Cunnick JM, Dorsey JF, Standley T, Turkson J, Kraker AJ, Fry DW, Jove R, Wu J. 1998. Role of tyrosine kinase activity of epidermal growth factor receptor in the lysophosphatidic acid-stimulated mitogen-activated protein kinase pathway. J Biol Chem 273:14468–14475.

De Araújo CE, Ribeiro MS, Favaro R, Zezell DM, Zorn TM. 2007. Ultranstructural and autoradiographical analysis show a faster skin repair in HeNe laser-treated wounds. J Photochem Photobiol B 86:87–96.

Dröge W. 2002. Free radicals in the physiological control of cell function. Physiol Rev 82:47–95.

Eichler M, Lavi R, Shainberg A, Lubart R. 2005. Flavins are source of visible-light-induced free radical formation in cells. Lasers Surg Med 37:314–319.

Feist IS, De Micheli G, Carneiro SR, Eduardo CP, Miyagi S, Marques MM. 2003. Adhesion and growth of cultured human gingival fibroblasts on periodontally involved root surfaces treated by Er:YAG laser. J Periodontol 74:1368–1375.

Finkel T. 2003. Oxidant signals and oxidative stress. Curr Opin Cell Biol 15:247–254.

Gao XJ, Chen TS, Xing D, Wang F, Pei YH, Wei XB. 2006. Single cell analysis of PKC activation during proliferation and apoptosis induced by laser irradiation. J Cell Physiol 206:441–448.

Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P. 2005. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. Mol Cell Biol 25:6391–6403.

Godley BF, Shamsi FA, Liang FQ, Jarrett SG, Davies S, Boulton M. 2005. Blue light induces mitochondrial DNA damage and free radical production in epithelial cells. J Biol Chem 280:21061–21066.
Grob S, Knebel A, Tenev T, Neininger A, Gaestel M, Herrlich P, Böhmer FD. 1999. Inactivation of protein-tyrosine phosphatases as mechanism of transducion. J Biol Chem 274:26378–26386.
Grossman N, Schneid N, Reuveni H, Halevy S, Lubart R. 1998. 780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: Involvement of reactive oxygen species. Laser Surg Med 22:212–218.
Guaiquil V, Vera J, Golde D. 2001. Mechanism of vitamin C inhibition of cell death induced by oxidative stress in glutathione-depleted HL-60 cells. J Biol Chem 276:40955–40961.
Haas AF, Isseroff RR, Wheeland RG, Rood PA, Graves PJ. 1990. Low-energy helium-neon laser irradiation increases the motility of cultured human keratinocytes. J Invest Dermatol 94:822–826.
Hawkins D, Houreld N, Abrahamse H. 2005. Low level laser therapy (LLLT) as an effective therapeutic modality for delayed wound healing. Ann NY Acad Sci 1056:486–493.
Hiroki D, Isamu S, Kenji N, Hideo T, Hideol T, Michisaburol T, Kimiharu A, Toshihiro N, Shigehisa Y. 2003. Low-power laser accelerates cell motility of gingival keratinocytes in vitro. Ohu Univ Dent J 30:141–147.
Huang CS, Li JX, Ding M, Leonard SS, Wang LY, Castranova V, Vallyathan V, Shi XL. 2001. UV induces phosphorylation of protein kinase B (Akt) at Ser-473 and Thr-308 in mouse epidermal Cl 41 cells through hydrogen peroxide. J Biol Chem 276:40234–40240.
Jakse N, Payer M, Tangl S, Berghold A, Kirmeier R, Lorenzoni M. 2007. Influence of low-level laser treatment on bone regeration and osseointegration of dental implants following sinus augmentation. An experimental study on sheep. Clin Oral Implants Res 18:517–524.
Jou MJ, Jou SB, Chen HM, Lin CH, Peng T. 2002. Critical role of mitochondrial reactive oxygen species formation in visible laser irradiation-induced apoptosis in rat brain astrocytes (RBA-1). J Biomed Sci 9:507–516.
Karu T. 1999. Primary and secondary mechanisms of action of visible to near-IR irradiation on cells. J Biochem Photobiol B: Biol 49:1–17.
Karu TI, Pyatibrat LV. 1995. Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro. J Biochem Photobiol B: Biol 27:219–223.
Karu TI, Pyatibrat LV. 2001. Cell attachment modulation by radiation from a pulsed light diode ($\lambda = 820$ nm) and various chemicals. Lasers Surg Med 28:227–236.
Karu TI, Pyatibrat LV, Kalendo GS. 2003. Photobiological modulation of cell attachment via cytochrome c oxidase. Photochem Photobiol Sci 3:211–216.
Kitagawa D, Tanemura S, Ohata S, Shimizu N, Seo J, Nishitai G, Watanabe T, Nakagawa K, Kishimoto H, Wada T, Tezuka T, Yamamoto T, Nishina H, Katada T. 2002. Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src-dependent epidermal growth factor receptor phosphorylation. J Biol Chem 277:366–371.
Lavi R, Shainberg A, Friedmann H, Shneyvays V, Rickover O, Eichler M, Kaplan D, Lubart R. 2003. Low energy visible light induces reactive oxygen species generation and stimulates an increase of intracellular calcium concentration in cardiac cells. J Biol Chem 278:40917–40922.
Matsui S, Tsujimoto Y, Matsushima K. 2007. Stimulatory effects of hydroxyl radical generation by Ga-Al-As laser irradiation on mineralization ability of human dental pulp cells. Biol Pharm Bull 30:27–31.
Parsons SJ, Parsons JT. 2004. Src family kinases, key regulators of signal transduction. Oncogene 23:7906–7909.
Pastore D, Greco M, Passarella S. 2000. Specific helium-neon laser sensitivity of the purified cytochrome c oxidase. Int J Radiat Biol 76:863–870.
Perez-Cruz I, Carcamo JM, Golde DW. 2003. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. Blood 102:336–343.
Rosado JA, Redondo PC, Salido GM, Gómez-Arteta E, Sage SO, Pariente JA. 2004. Hydrogen peroxide generation induces pp60src activation in human platelets. J Biol Chem 297:1665–1675.
Saygun I, Karacay S, Serdar M, Ural AU, Sencimen M, Kurtis B. 2007. Effects of laser irradiation on the release of basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1), and receptor of IGF (IGFBP3) from gingival fibroblasts. Lasers Med Sci 23:211–215.
Shefer G, Oron U, Irintchev A, Wernig A, Helevy O. 2001. Skeletal muscle cell activation by low-energy laser irradiation: A role for the MAPK/ERK pathway. J Cell Physiol 187:73–80.
Shefer G, Itamar B, Oron U, Halevy O. 2003. Low-energy laser irradiation enhances de novo protein synthesis via its effects on translation-regulator proteins in skeletal muscle myoblasts. Biochim Biophys Acta 1593:131–139.
Simon AR, Rai U, Fanburg BL, Cochran BH. 1998. Activation of the JAK-STAT pathway by reactive oxygen species. Am J Physiol 275:1640–1652.
Singh DK, Kumar D, Siddiqui Z, Basu SK, Kumar V, Rao KVS. 2005. The strength of receptor signaling is centrally controlled through a cooperative loop between Ca^{2+} and an oxidant signal. Cell 121:281–293.
Stocker R, Keaney JF, Jr. 2004. Role of oxidative modifications in atherosclerosis. Physiol Rev 84:1381–1478.
Tatin F, Varon C, Génot E, Moreau V. 2006. A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. J Cell Sci 119:769–781.
Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. 1999. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 274:22699–22704.
Vinck EM, Cagnie BJ, Cornelissen MJ, Declercq HA, Cambier DC. 2003. Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. Lasers Med Sci 18:95–99.
Walker VG, Ammer AG, Cao ZX, Clump A, Jiang BH, Kelley LC, Weed SA, Zot H, Flynn DC. 2007. PI-3-kinase activation is required for PMA directed activation of c-Src by AFAP-110. Am J Physiol Cell Physiol 293:C119–C132.
Wang YX, Botvinick EL, Zhao YH, Berns MW, Usami S, Tsien RY, Chien S. 2005. Visualizing the mechanical activation of Src. Nature 434:1040–1045.
Wang YJ, Hunter MG, Marsh CB. 2007. Understanding the mechanisms by which reactive oxygen species (ROS) activate mononuclear phagocytes in inflammatory diseases of the lung. FASEB J 21:871–872.
Xu W, Harrison SC, Eck MJ. 1997. Three-dimensional structure of the tyrosine kinase c-Src. Nature 385:595–602.
Yu HS, Wu CS, Yu CL, Kao YH, Chiou MH. 2003. Helium-neon laser irradiation stimulates migration and proliferation in melanocytes and induces repigmentation in segmental-type vitiligo. J Invest Dermatol 120:56–64.
Zhougang S, Schnellmann RG. 2004. H_2O_2-induced transactivation of EGF receptor requires Src and mediates ERK1/2, but not Akt, activation in renal cells. Am J Physiol Renal Physiol 286:F858–F865.