JOURNAL OF CELLULAR PHYSIOLOGY 206:441–448 (2006)

Single Cell Analysis of PKC Activation During Proliferation and Apoptosis Induced by Laser Irradiation

XUEJUAN GAO,[1] TONGSHENG CHEN,[1] DA XING,[1]* FANG WANG,[1] YIHUI PEI,[1] AND XUNBIN WEI[2]

[1]*Institute of Laser Life Science, South China Normal University, Guangzhou, China*

[2]*Wellman Laboratories of Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston*

Laser irradiation has been shown to trigger cellular proliferation and apoptosis in various cell types. Studying the signaling pathways involved in the laser irradiation is important for understanding these processes. In present study, to monitor the protein kinase Cs (PKCs) activity in living cells in real time, we transfected and screened human lung adenocarcinoma cells (ASTC-a-1) stably expressing C kinase activity reporter (CKAR) constructed based on fluorescence resonance energy transfer (FRET) technique. The CKAR is a specific, reversible reporter of phosphorylation by PKCs and it can monitor the ongoing balance between PKCs and phosphatases. The increasing dynamics of PKCs activity is monitored during cell proliferation induced by low-power laser irradiation (LPLI) (0.8 J/cm^2) in serum-starved ASTC-a-1 cells stably expressing CKAR reporter using FRET imaging on laser scanning confocal microscope and using spectrofluorometric analysis on a luminescence spectrometer, respectively. However, the decreasing dynamics of PKCs activity has been monitored in real time using FRET imaging for the cells treated with high fluence LPLI (60 J/cm^2), which was previously found to induce cell apoptosis. Taken together, LPLI induces the ASTC-a-1 cell proliferation by specifically activating PKCs. However, PKCs activity decreases during cell apoptosis induced by high fluence LPLI. Our results indicate that PKCs play an important role in the laser irradiation-induced biological effects. J. Cell. Physiol. 206: 441–448, 2006.
© 2005 Wiley-Liss, Inc.

Laser irradiation has a significant role in triggering cell proliferation, differentiation, and apoptosis in various cell types (Bibikova and Oron, 1993; Barushka et al., 1995; Conlan et al., 1996). The promotive effects of low-power laser irradiation (LPLI) on cell proliferation have been described in several cell types (Boulton and Marshall, 1986; Spivak et al., 1992), but the mechanism of cellular proliferation has not been fully understood. One possibility is that the laser energy is absorbed in intracellular chromophores and converted to metabolic energy (Belkin et al., 1988; Conlan et al., 1996; Karu, 1999). Cellular ATP levels increase almost twofold after He-Ne laser irradiation in cultured HeLa cells (Karu et al., 1995). It has been shown that LPLI may produce reactive oxygen species (ROS), which might be beneficial for the skin and sperm cells (Grossman et al., 1998). Other studies demonstrate that LPLI induces cell proliferation via phosphoinosotide 3-kinase/protein kinase B (PI3K/PKB) and Ras/Raf/ERK signaling transduction pathways (Shefer et al., 2003). One of the phenotypic changes observed in cells after LPLI treatment is the increase of intracellular Ca^{2+} concentration (Breitbart et al., 1996; Alexandratou et al., 2002). In addition, the increases of Ca^{2+} concentration and diacylglycerol (DG) in the cytoplasm have also been observed in the process of respiratory burst of neutrophils induced by LPLI (Duan et al., 2001). These studies show the roles for both DG and Ca^{2+} signaling in cell proliferation induced by LPLI. It is well known that protein kinase Cs (PKCs) can be activated by DG and/or regulated by Ca^{2+} (Braun et al., 2005). It has also been speculated that LPLI affected Ca^{2+}-dependent-PKC signal transduction pathways (Ben-Dov et al., 1999). In view of these studies, PKCs may be involved in the regulation of cell proliferation induced by LPLI.

However, high fluence LPLI inhibites cell viability (Hilf et al., 1986; Schaffer et al., 1997; Ocana-Quero et al., 1998). The phenomenon is consistent with our recent results that high fluence LPLI treatment induced ASTC-a-1 cells apoptosis when light irradiation fluence exceeded 60 J/cm^2, and the cell apoptosis was dependent on the dosage of laser irradiation (Wang et al., 2005). It is known that apoptosis is an important process. Various extracellular stimuli activate related intracellular signaling pathways, which induce the activation of caspases, resulting in cell apoptosis (Verheij et al., 1996).

PKCs are involved in different, even opposite functions, including the cellular proliferation, tumor promotion, differentiation, and apoptosis of many types of cells (Musashi et al., 2000; Braun et al., 2005). PKC was first described as a Ca^{2+} activated, phospholipid-dependent

Abbreviations: CCK-8, Cell Counting Kit-8; CFP and YFP, cyan and yellow fluorescent protein; CKAR, C kinase activity reporter; DG, diacylglycerol; FRET, fluorescence resonance energy transfer; LPLI, low-power laser irradiation; PI3K, phosphoinosotide 3-kinase; PKB, protein kinase B; PKCs, protein kinase Cs; PLC, phospholipase C; PMA, Phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TPKs, tyrosine protein kinases.

Xuejuan Gao and Tongsheng Chen contributed equally to this study.

Contract grant sponsor: National Natural Science Foundation of China; Contract grant numbers: 60378043, 30470494; Contract grant sponsor: Natural Science Foundation of Guangdong Province; Contract grant numbers: 015012, 04010394.

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

Received 21 March 2005; Accepted 27 June 2005

DOI: 10.1002/jcp.20484

© 2005 WILEY-LISS, INC.

serine/threonine protein kinase (Takai et al., 1977; Nishizuka, 1988). The 13 members of the PKC family can be grouped into three major classes: Ca^{2+}-dependent classical PKCs, Ca^{2+}-independent novel PKCs, and Ca^{2+}- and lipid-independent atypical PKCs (Jaken and Parker, 2000; Newton, 2001). The Ca^{2+}-dependent classical PKCs may be involved in the regulation of cellular proliferation induced by LPLI. The activation of PKCs during apoptosis has been studied extensively (Musashi et al., 2000). However, the involvement of PKCs during apoptosis induced by high fluence LPLI has not been observed. Therefore, it isn't clear whether the Ca^{2+}-controlled PKCs are involved in the cell proliferation induced by LPLI and apoptosis induced by high fluence LPLI.

Fluorescence resonance energy transfer (FRET) is a nonradiative transfer of energy between two fluorophores that are placed in close vicinity and in a proper relative angular orientation. FRET technique has been widely used to study protein–protein interactions in living cells with improved spatial and temporal resolution, distance range, and sensitivity (Ting et al., 2001; Zhang et al., 2002; and Gaits and Hahn, 2003; reviewed in Sekar and Periasamy, 2003). Variants of green fluorescent protein (GFP) have provided genetically encoded fluorophores that serve as donor and/or acceptor in FRET (Heim and Tsien, 1996; Mitra et al., 1996; Mizuno et al., 2001). FRET technique has been used to develop genetically encoded fluorescent probes for various cellular functions (reviewed in Sekar and Periasamy, 2003; Nagai et al., 2004). For example, Ca^{2+} probe was generated recently based on FRET technique to visualize subcellular Ca^{2+} dynamics with better spatial and temporal resolution (Nagai et al., 2004); CY-PKCδ, a genetically encoded chimera of native PKC delta fused to cyan and yellow fluorescent protein (CFP and YFP), had potential for high throughput screening (HTS) of prospective PKCδ ligands within the context of cell type (Braun et al., 2005); SCAT3 was constructed to monitor the spatio-temporal dynamics of caspase-3 activity in living cells (Kiwamu et al., 2003). With the FRET technique, we recently found in living cells that caspase-3 was activated and related to the subsequent apoptosis induced by high fluence LPLI (Wang et al., 2005).

In order to monitor the dynamics of PKCs activity, C kinase activity reporter (CKAR) has been constructed based on FRET technique, which comprises CFP, the FHA2 phospho-peptide-binding domain, a PKC substrate sequence, and YFP (Violin et al., 2003). CKAR is successfully designed to be a specific, reversible reporter of phosphorylation by PKCs and it could monitor the ongoing balance between PKCs and phosphatases (Violin et al., 2003). When phosphorylated, the substrate sequence binds the FHA2 phospho-peptide-binding domain, reversible by phosphatases, and this conformational change results in a change in FRET (Violin et al., 2003).

In this study, we have investigated the dynamics of PKCs activity during cell proliferation induced by LPLI and during cell apoptosis induced by high fluence LPLI, respectively. Our studies reveal that LPLI could activate PKCs and then induce human lung adenocarcinoma cells (ASTC-a-1) proliferation, and that high fluence LPLI could inactivate PKCs, which further verified that high fluence LPLI could induce cell apoptosis (Wang et al., 2005). Our results indicate that PKCs play an important role in the laser irradiation-induced biological effects.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lipofectamine™ Reagent was purchased from Invitrogen (Carlsbad, CA). DNA Extraction kit was purchased from Qiagen (Valencia, CA). Gö 6983 was purchased from Merck (Darmstadt, Germany).

Cell culture

ASTC-a-1 cells were grown in DMEM supplemented with 15% fetal calf serum (FCS), and cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2).

Cell preparation and LPLI on ASTC-a-1 cells

ASTC-a-1 cells were cultured in DMEM supplemented with 1% serum at a density of 4×10^3 cells/well in 96-well microplates. Twenty-four hours after culture, the cells were divided into four groups and each group was irradiated with He-Ne laser (632.8 nm, 5 mw, spot diameter 0.635 cm; HN-1000, Guangzhou, China) in the dark at fluence of 0, 0.5, 0.65, and 0.8 J/cm^2, respectively. The interval wells were filled with ink in order to minimize the light reflections. After irradiation, 96-well microplates were returned to the incubator for a further culture at 37°C, 5% CO_2. The irradiation was performed on monolayer cells. In all cases, control non-irradiated cells were kept in the same conditions as the treated cells.

Cell viability assays

Cell viability was assessed with CCK-8 2 and 5 days after the laser irradiation, respectively. At the indicated times, CCK-8 was added to the cells and incubated for 1.5 h. OD_{450}, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China). The value is directly proportional to the number of viable cells in a culture medium and the cell proliferation.

Cell transfection and screening

Plasmid DNA of CKAR (presented by Prof. Alexandra C. Newton of California University) was transfected into ASTC-a-1 cells by using Lipofectamine™ reagent. The cells stably expressing CKAR reporter were screened with 0.8 mg/ml G418, and positive clones were picked up with micropipettes. CKAR reporter was constructed by fusing of a CFP and a YFP with a specialized linker that contains the PKC substrate sequence. Before PKCs activation, CFP and YFP are placed in close vicinity. Energy can be transferred directly from CFP to YFP, so fluorescence emitted from YFP can be detected when CFP is excited. Once PKCs are activated, the linker will change in conformation. CFP separates from YFP, so the FRET effect of CKAR must decrease effectively (Violin et al., 2003).

Laser scanning microscopes (LSM)

FRET was performed on a commercial Laser Scanning Microscopes (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-Ion Laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT458), and focused through a Zeiss C-Apochromat 20×, NA 0.4 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). For intracellular measurements, the desired measurement position was chosen in the LSM image. To quantify the results, the images of CFP and YFP emission intensities were processing with Zeiss Rel3.2 image processing software (Zeiss, Germany).

LPLI and FRET imaging

ASTC-a-1 cells stably expressing CKAR reporter were grown on a 35-mm culture dish. To measure the FRET effect in living cells, the culture dish was placed on the stage of the LSM microscope. After 24 h of serum deprivation, the cells

were irradiated with He-Ne laser (632.8 nm, 5 mw) at fluence of 0.8 J/cm^2 in the dark or treated with 1μM Gö 6983, a specific inhibitor of PKC (Violin et al., 2003), or treated with DMSO (vehicle for Gö 6983). During He-Ne laser irradiation and imaging process, cells were maintained at 37°C using the temperature regulator (Tempcontrol 37–2 digital, Zeiss) in the whole experiment process. Before and after irradiation treatment, temperature elevations of culture liquid were less than 0.2°C and the images of YFP and CFP emission intensities were obtained by performing LSM microscope.

PMA stimulating and FRET imaging

After 6 h of serum deprivation, ASTC-a-1 cells stably expressing CKAR reporter were treated with PMA, PKC activator (Johnson et al., 2002), diluted in DMSO. Before and after treatment with PMA, cells were observed and photographed using the LSM microscope, and the images of YFP and CFP channels and the FRET images of CKAR were recorded.

Spectrofluorometric analysis of PKCs activity induced by both LPLI and PMA in living cell

ASTC-a-1 cells stably expressing CKAR reporter were grown in DMEM supplemented with serum free for 24 h. Then, the cells were irradiated with He-Ne laser (632.8 nm, 5 mw) at fluence of 0.8 J/cm^2 in the dark. After irradiation, the cells were immediately transferred into a quartz cuvette. The quartz cuvette was then placed inside the sample holder of a luminescence spectrometer (LS55, PerkinElmer, USA). The fluorescence emission spectra from 0 to 50 min after LPLI treatment were obtained by performing a spectrum scanning analysis of the luminescence spectrometer. The excitation wavelength was 434 ± 5 nm, the excitation slit was 10 nm, the emission slit was 15 nm, and the scanning speed was 200 nm/sec. The fluorescence emission spectra from 0 to 50 min of the same generation of cells were recorded in the similar ways after PMA treatment. The corresponding background spectra of cell-free culture medium were subtracted.

High fluence LPLI and FRET imaging

ASTC-a-1 cells stably expressing CKAR reporter, without serum deprivation, were irradiated with He-Ne laser (632.8 nm, 40 mw) at fluence of 60 J/cm^2 in the dark or treated with 1 μM Gö 6983. The subsequent cell imaging and data analysis were the same as LPLI.

Statistics analysis

Data from cell counts are expressed as means ± SD of three independent experiments. For statistical evaluation Student's paired t-test was used and significance was defined as $P < 0.05$.

RESULTS

Real-time monitoring of PKCs activation induced by LPLI in single living cell

In order to study the dynamics of PKCs activation in single living cell, we transfected and screened the ASTC-a-1 cells stably expressing CKAR. The fluorescence images of ASTC-a-1 cell stably expressing CKAR are shown in Figure 1A (left column).

To monitor the effects of LPLI on PKCs activity in living cells, ASTC-a-1 cells stably expressing CKAR reporter were treated with LPLI (0.8 J/cm^2). It has been long known that growth factor depletion inhibits cell-cycle and differentiation. The biostimulatory effects of LPLI could be more pronounced in cells with a low metabolic rate (Karu et al., 1995; reviewed in Karu, 1999). In this experiment, we used ASTC-a-1 cells that were rendered quiescent by serum starvation for 24 h. The real-time monitoring of PKCs activation was performed on LSM microscope, and the CFP and YFP emissions were imaged in Figure 1A. The baseline fluorescence intensities had no changes (data not shown), which are the same as that of control experiments in Figure 1D. The emission increased in the CFP channel but decreased in the YFP channel (Fig. 1A), which implied that PKCs were activated. The activated PKCs phosphorylated the substrate sequence of CKAR reporter and resulted in conformational change and the decrease in FRET of the CKAR (Violin et al., 2003). The Figure 1B was the quantification of fluorescence intensities of two channels after LPLI treatment, which showed the increased emission of CFP channel and the decreased emission of YFP channel. Thus the ratio of YFP/CFP emission or the FRET ratio decreased upon activation of PKCs with LPLI (Fig. 1C). Note that the data in Figure 1D was plotted as the ratio of YFP emission (which decreases as FRET decreases) to CFP emission (which increases as FRET decreases) and the baseline FRET ratio didn't change. The increase in FRET ratio of the starved cells after the specific PKC inhibitor Gö 6983 treatment was shown in Figure 1D, opposite to the decrease of LPLI treatment. Treatment of the cells with DMSO (vehicle for Gö 6983) could not cause a change in FRET ratio (Fig. 1D). The increased FRET ratio of the starved cells after Gö 6983 treatment demonstrated that Gö 6983 further inhibited PKCs activity (Fig. 1D). These results indicate that CKAR is a specific reporter for PKCs activation (Violin et al., 2003; Fig. 2) and that LPLI activate PKCs in living cells.

Real-time monitoring of PKCs activation after PMA treatment in single living cell

To confirm the above results that PKCs could be activated by LPLI in living cells, we investigated the FRET changes of CKAR when ASTC-a-1 cells stably expressing CKAR reporter were treated with PMA after 6 h of serum deprivation. The tumor-promoting properties of PMA exemplify the cellular consequences of tipping the balance of cellular signals excessively toward phosphorylation (Violin et al., 2003). PMA can activate the PKC (Johnson et al., 2002), and the activated PKC will phosphorylate the substrate sequence between CFP and YFP, which results in conformational change and the decrease in FRET of the CKAR (Violin et al., 2003). The fluorescence images of CFP and YFP channels after PMA treatment were recorded (data not shown). The CFP emission intensity increased, and the YFP emission intensity decreased, thus the YFP/CFP emission intensity, FRET ratio images, decreased (Fig. 2A). The corresponding changes of CFP and YFP fluorescence intensities and FRET ratio of CKAR of the cells treated with PMA were consistent with that for LPLI treatment (Fig. 2B and C). These results demonstrated that the CKAR probe was phosphated when the cells were treated with PMA or LPLI (0.8 J/cm^2), which indicated that PKCs were activated during the two processes.

Spectrofluorometric analysis of PKCs activation induced by both LPLI and PMA in living cells

In order to further demonstrate PKCs activation induced by LPLI, we used a spectrometer to measure the changes of FRET effects of CKAR in response to LPLI and PMA treatment. ASTC-a-1 cells stably expressing CKAR reporter after 24 h of serum deprivation were treated with LPLI (0.8 J/cm^2) and PMA, respectively. The emission spectra of the CKAR were measured in living cells for control and LPLI/PMA treatment (Fig. 3). After PMA treatment, the activated PKCs phosphorylated CKAR and resulted in a decrease in FRET, thus the emission peak of CFP (476 nm) increased gradually, and the emission peak of YFP (527 nm) decreased gradually (Fig. 3B). The same

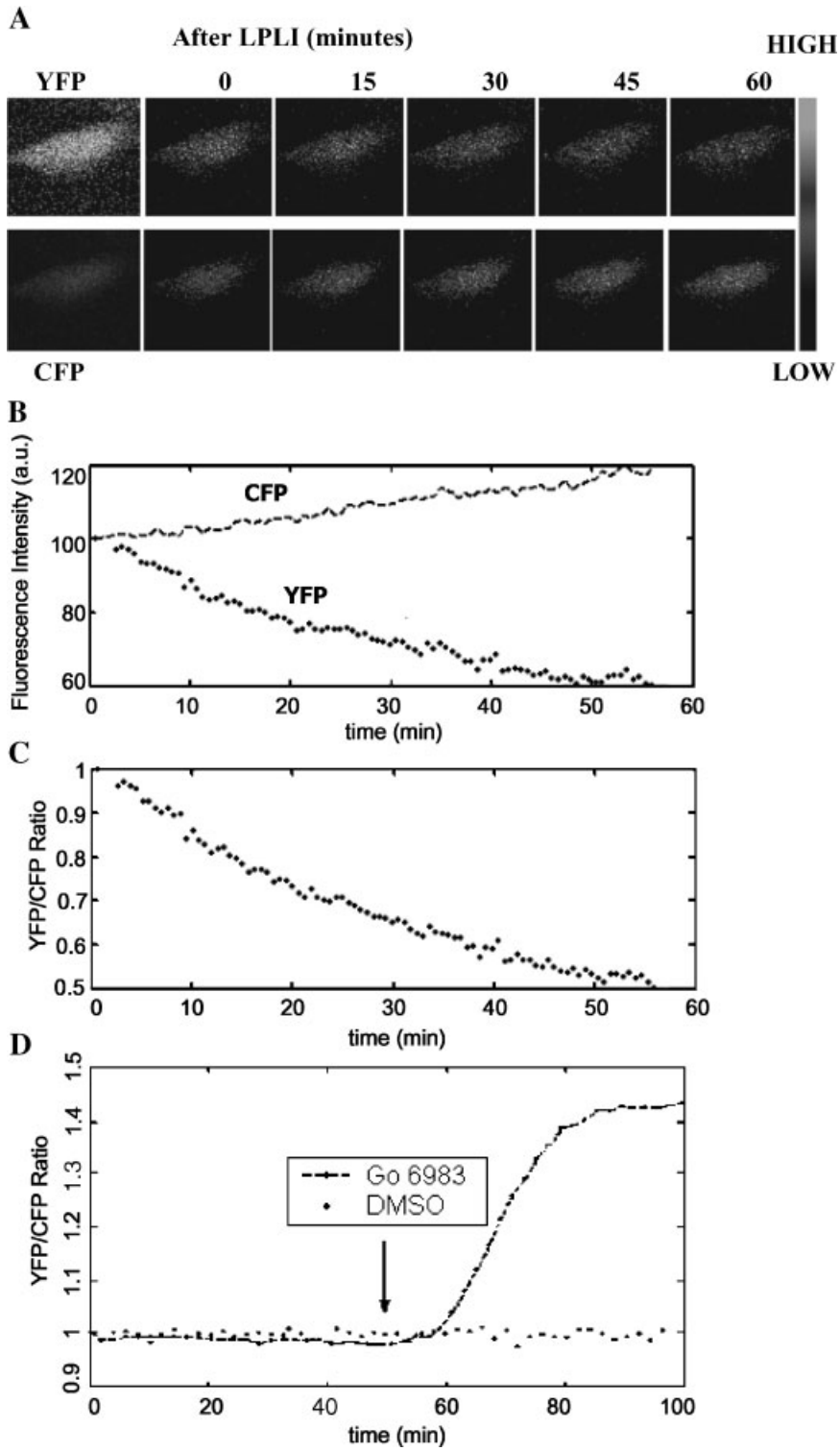

Fig. 1. Dynamics of PKCs activity induced by LPLI in single living cell. **A**: Fluorescence image series of CFP and YFP emission intensities after LPLI treatment. The left column is the fluorescence image, and the others are pseudocolor images. The cells were starved for 24 h and then irradiated at fluence of 0.8 J/cm^2 with He-Ne laser (632.8 nm, 5 mw). The fluorescence images of CFP and YFP channels excited by Ar-Ion laser (458 nm) were recorded with LSM microscope and processed with pseudocolor technique. **B**: Dynamics of YFP emission intensities and CFP emission intensities corresponding to the images of YFP and CFP in A. **C**: Dynamic FRET changes of CKAR representing the PKCs activity according to the data in B. **D**: CKAR is a specific reporter for PKCs activation. The cells were starved for 24 h and treated with the specific PKC inhibitor Gö 6983 or treated with DMSO (vehicle for Gö 6983) after the baseline fluorescence of CFP and YFP emissions was recorded. The FRET ratio was processed with the same method as C. Results represent one of three replicates.

generation of cells was treated with LPLI (Fig. 3A) and the results were consistent with that when the cells were treated with PMA. These results demonstrated that activated PKCs by PMA or LPLI phosphorylated CKAR and resulted in the decrease of FRET, which were consistent with the results from imaging study. These data further indicate that the PKCs activity increases during LPLI treatment.

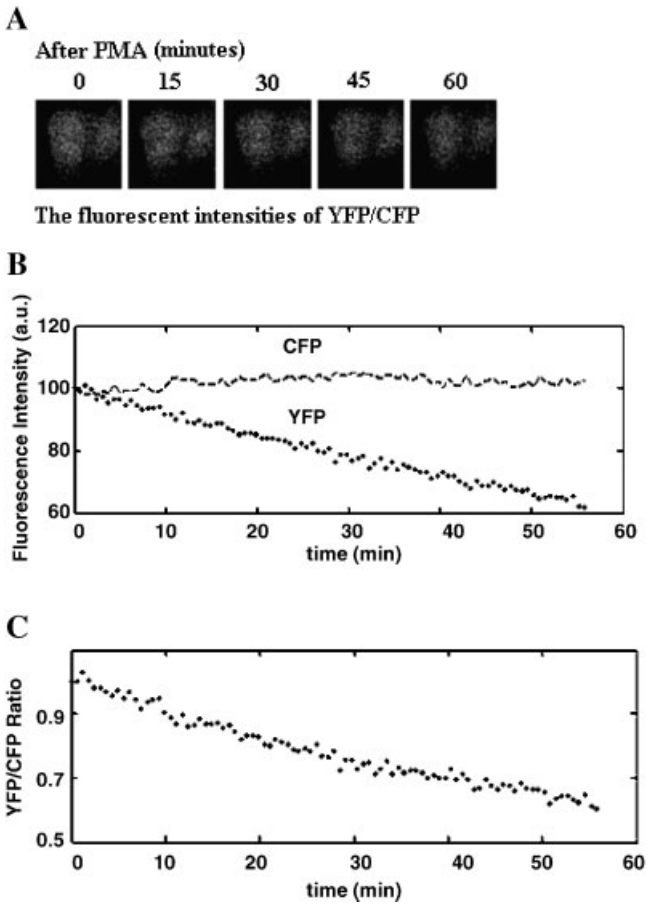

Fig. 2. Dynamics of PKCs activity induced by PMA in single living cell. **A**: Dynamic FRET ratio images of CKAR after PMA treatment. The cells were starved for 6 h and then treated with PMA. The images of fluorescence intensities (YFP/CFP) excited by Ar-Ion laser (458 nm) were recorded with LSM microscope and processed with pseudocolor technique. **B**: Dynamics of YFP emission intensities and CFP emission intensities. **C**: Dynamic FRET changes of CKAR representing the PKCs activity according to the data in B. Results represent one of three replicates.

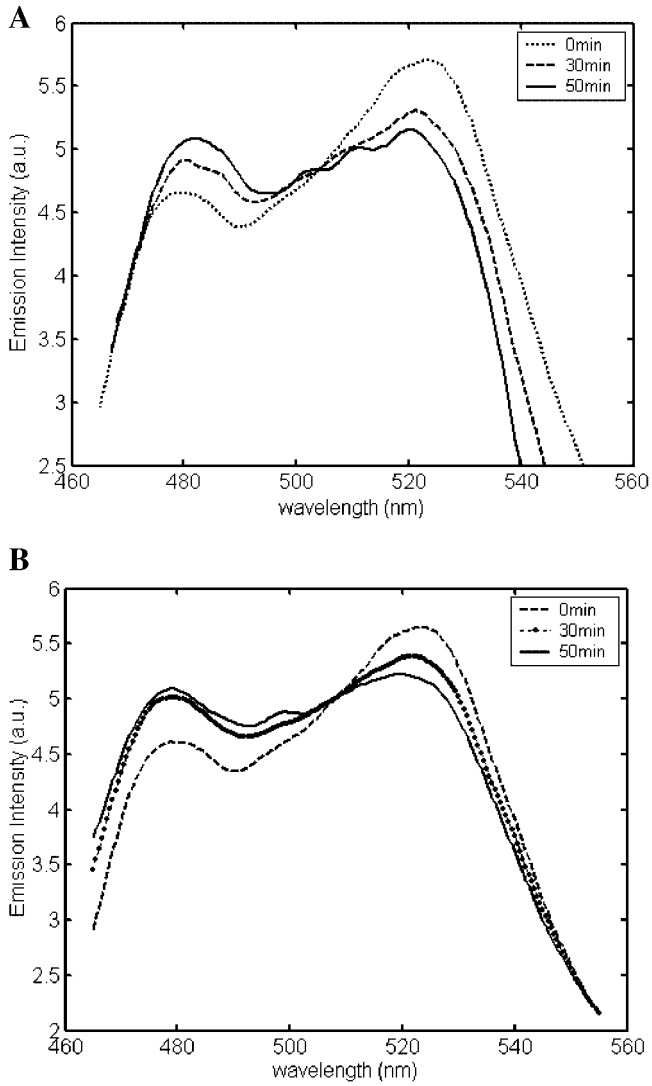

Fig. 3. Spectrofluorometric analysis of PKCs activation induced by both PMA and LPLI in living cells. **A**: The emission spectra of CKAR after 0, 30, and 50 min of LPLI treatment. ASTC-a-1 cells stably expressing CKAR reporter were starved in DMEM for 24 h and then irradiated with He-Ne laser (632.8 nm, 5 mw) at fluence of 0.8 J/cm^2. The cells were excited at the excitation wavelength of CFP (434 ± 5 nm), resulting in a CFP emission peak (476 nm) and YFP emission peak (528 nm) caused by FRET from CFP. And the fluorescence emission spectra of YFP and CFP were obtained by performing a spectrum scanning analysis of the luminescence spectrometer. **B**: The emission spectra of CKAR after 0, 30, and 50 min of PMA treatment. The fluorescence emission spectra of CKAR of the same generation of cells were recorded as in A after PMA treatment.

Real-time monitoring of PKCs activation induced by high fluence LPLI in single living cell

To monitor the effects of high fluence LPLI on PKCs activation in living cell, ASTC-a-1 cells stably expressing CKAR reporter were treated with high fluence LPLI (60 J/cm^2). We performed the real-time monitoring of PKCs activation in single living cell on LSM microscope. The results showed that the baseline fluorescence intensities decreased a little (data not shown), which are the same as that of control experiments in Figure 4C. The fluorescence intensity of YFP increased and the intensity of CFP decreased (Fig. 4A), thus the FRET ratio increased (Fig. 4B). The FRET ratio of the health cells after the specific PKC inhibitor Gö 6983 treatment, a negative control, also increased (Fig. 4C). The baseline FRET ratio decreased a little, implying that a portion of PKCs in health cells were activated (Fig. 4C). These results showed that the PKCs activity decreased when the cells were treated with high fluence LPLI, which was opposite to that of LPLI treatment. CKAR was successfully designed to be a specific, reversible reporter of phosphorylation by PKCs and it could monitor the ongoing balance between PKCs and phosphatases (Violin et al., 2003). Therefore, the increase of FRET ratio of CKAR may be due to the decrease of PKCs activity and/or the increase of phosphatase activities in living cells.

Effects of He-Ne laser irradiation at different doses on serum-starved ASTC-a-1 cells proliferation

To further confirm the effects of laser irradiation on the ASTC-a-1 cells, we used CCK-8 to observe the cell viability after the cells were treated with different doses of laser irradiation. In these experiments, cells were irradiated with He-Ne laser (632.8 nm, 5 mw) at fluence of 0, 0.5, 0.65, 0.8 J/cm^2, respectively. After irradiation, cells were cultured for 2 days and 5 days at 37°C, 5% CO_2 before using CCK-8 assay. Irradiated and control non-irradiated cells were held in 1% serum-containing

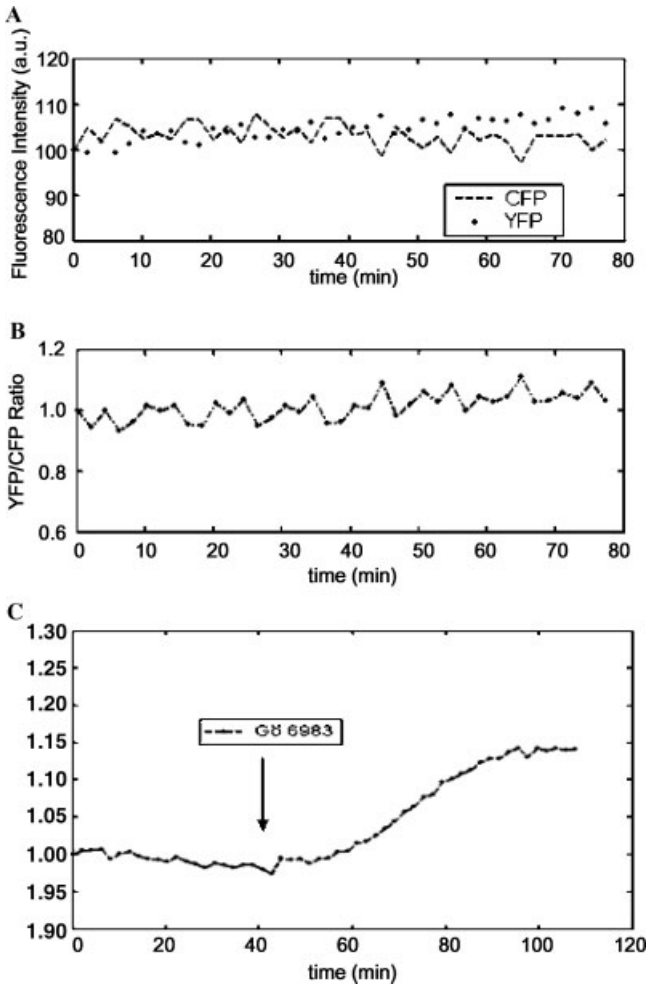

Fig. 4. Dynamics of PKCs activity induced by high fluence LPLI in single living cell. **A**: Dynamics of YFP emission intensities and CFP emission intensities. ASTC-a-1 cells stably expressing CKAR reporter were cultured in DMEM-15% serum and then irradiated with He-Ne laser (632.8 nm, 40 mw) at fluence of 60 J/cm^2. Quantification of YFP and CFP emission intensities excited by Ar-Ion laser (458 nm) were recorded by performing on LSM microscope. **B**: Dynamic FRET changes of CKAR representing the PKCs activity according to the data in A. **C**: Dynamic FRET changes after Gö 6983 treatment. The cells were cultured in DMEM-15% serum and treated with the specific PKC inhibitor Gö 6983 after the baseline fluorescence of CFP and YFP emissions with LSM was recorded. The FRET ratio was processed with the same method as B. Results represent one of three replicates.

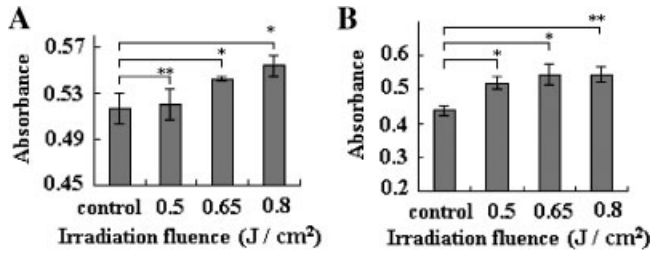

Fig. 5. Proliferation of ASTC-a-1 cells induced by LPLI. ASTC-a-1 cells were seeded on 96-well microplates for 24 h in 1% serum-containing medium and then irradiated with He-Ne laser (632.8 nm, 5 mw, spot diameter 0.635 cm) at fluence of 0, 0.5, 0.65, and 0.8 J/cm^2, respectively. Cell viability was assessed by the CCK-8 assay 2 days (**A**) and 5 days (**B**) after the laser irradiation, respectively. Results represent one of three replicates. $^*P < 0.05$; $^{**}P < 0.01$.

medium throughout the whole experiment. The states of proliferation of the irradiated and control non-irradiated cells were analyzed 2 days (Fig. 5A) and 5 days (Fig. 5B) after LPLI treatment, respectively. OD_{450} absorbance value represented the number of live cells and the cell proliferation. The results showed that the effects of laser irradiation on proliferation of ASTC-a-1 cells were dose-dependent. Compared with non-irradiated controls, significant differences in cell proliferation were observed for different doses (Fig. 5). Both the two results showed that LPLI promoted proliferation of ASTC-a-1 cells, and that the fluence of 0.8 J/cm^2 significantly stimulated ASTC-a-1 cells proliferation, which were consistent with the above results that LPLI (0.8 J/cm^2) could activate PKCs.

DISCUSSION

Laser irradiation is regarded to have a significant role in triggering cellular proliferation, differentiation, and apoptosis in various cell types (Bibikova and Oron, 1993). However, the regulatory mechanisms have not been clearly understood. PKCs are involved in various functions, including the cellular proliferation and apoptosis of many kinds of cells (Musashi et al., 2000; Braun et al., 2005). To elucidate the contribution of laser irradiation to the signal regulatory pathways of cell proliferation and apoptosis, we studied the effects of laser irradiation on Ca^{2+}-dependent PKCs activity. In this study, we monitor for the first time the dynamics of PKCs activation in living cells during cell proliferation and apoptosis induced by laser irradiation. Our results show that laser irradiation affects the ASTC-a-1 cell proliferation or apoptosis by selectively activating or inactivating PKCs, and that the effects of laser irradiation on cellular physiology and PKCs activity dependent on the dosage of laser irradiation. When the cells are treated with LPLI (0.8 J/cm^2), a typical dosage of low fluence LPLI to induce the proliferation of ASTC-a-1 cells in our conditions (Fig. 5), both FRET imaging and spectrofluorometric analysis show an increase in the CFP emission and a corresponding decrease in the YFP emission in living cell in real-time, consistent with a decrease in FRET (Figs. 1 and 3). The results imply that PKCs are activated by LPLI, and PKCs are involved in the regulation of cell proliferation in these processes. On the other hand, when the cells are treated with PMA, the CKAR reporter behaves similarly (Fig. 2). Previous studies have demonstrated that LPLI can transiently increase the intracellular Ca^{2+} concentration by affecting the Ca^{2+} transport of the plasmalemma (Breitbart et al., 1996; Alexandratou et al., 2002). The increases of Ca^{2+} concentration and DG in the cytoplasm in the process of respiratory burst of neutrophils induced by LPLI have been observed (Duan et al., 2001). Furthermore, PKCs could be activated by DG and/or regulated by Ca^{2+} concentration (Braun et al., 2004). Taken together, our data suggest that the effects of LPLI on cell proliferation are partially dependent on the PKCs activation.

LPLI activates PKCs, but high fluence LPLI inactivates PKCs.

High fluence LPLI significantly inhibits cell viability and induces its apoptosis (Gross and Jelkmann, 1990; Lubart et al., 1992). With the SCAT-3 probe and FRET technique, our previous study showed that caspase-3 was activated and involved in the subsequent apoptosis induced by high fluence LPLI (Wang et al., 2005). The caspase-3 is an important molecule in executing apoptosis (Thornberry and Lazebnik, 1998). CKAR has been successfully designed to be a specific, reversible reporter

of phosphorylation by PKCs and it could monitor the ongoing balance between PKCs and phosphatases in living cells (Violin et al., 2003). In our experiments, when ASTC-a-1 cells stably expressing CKAR reporter are treated with high fluence LPLI (60 J/cm^2), a typical dosage of high fluence LPLI to induce the activation of caspase-3 and the apoptosis of ASTC-a-1 cells in our previous study, the FRET imaging shows that the emission intensity in CFP channel decreases and the emission intensity in YFP channel increases (Fig. 4), which are opposite to the FRET changes of LPLI treatment (Fig. 1). The results imply that high fluence LPLI inactivates PKCs activity. What will happen when the irradiation fluence is between 0.8 J/cm^2 and 60 J/cm^2? We suppose that the cells would proliferate if the irradiation fluence is between 0.8 and 15 J/cm^2. When the dosage of laser irradiation exceeds 15 J/cm^2, the apoptosis has been observed (Wang et al., 2005; Fig. 1). PKCs are involved in apoptosis of many types of cells (Musashi et al., 2000; Braun et al., 2005). CKAR reporter is sensitive to all isoforms of PKC family (Violin et al., 2003). Therefore, based on our results, we speculate that high fluence LPLI-mediated PKCs inactivation may be due to the increase of phosphatases activities or other mechanisms.

What is the possible mechanism by which LPLI activates PKCs? One possibility is that tyrosine protein kinases (TPKs)/phospholipase C (PLC)-gamma/PKCs pathway mediate PKCs activation. The activation of TPKs is related to the releases of NGF (nerve growth factor) (Schwartz et al., 2002), PDGF (platelet-derived growth factor) (Yu et al., 1994), and VEGF (vascular endothelial growth factor) (Kipshidze et al., 2001) from various cell types after LPLI treatment. It is known that the acceptors of these cytokines belong to TPKs family. It is possible that LPLI induces ligand-free dimerization and activation of TPKs that are in the "right energetic state" to accept the laser energy, leading to their autophosphorylation and downstream effects (Karu, 1999; Shefer et al., 2001). Activated TPKs can activate the extracellular signal regulated protein kinase ERK1/2 (Shefer et al., 2001), promoting cell proliferation (Seko et al., 1997; Khanna et al., 1999; Maruyama et al., 1999). The catalytic activity of PLC-gamma could be stimulated by TPKs (Palmier et al., 1999; Duan et al., 2001). The activated PLC could catalyze the hydrolysis of some phospholipids, and then increase the concentration of DG and Ca^{2+} in the cytoplasm, which could function to activate PKCs (Rhee and Choi, 1992; Rhee and Bae, 1997; Braun et al., 2005). Our speculation is that LPLI activates PKCs through the pathway: TPKs/PLC-gamma/PKCs. Nevertheless, one cannot rule out the possibility that other mechanisms are associated with the activation of PKCs. For instance, LPLI has been demonstrated to increase the production of ROS and sequential elevation of intracellular Ca^{2+} concentration (Grossman et al., 1998; Lavi et al., 2003), which have been shown to stimulate signal transduction pathways and cell growth (reviewed in Behrend et al., 2003). Recent studies have shown that high glucose increases cellular ROS by activating PKCs, and then ROS may activate PKCs thus provide signal amplification in cells (Lee et al., 2004).

How are PKCs inhibited by high fluence LPLI? It has been postulated that high fluence LPLI produces large amounts of singlet oxygen (Lubart et al., 1990). Recent studies have demonstrated that the elevation of intracellular Ca^{2+} concentration following light irradiating or addition of H_2O_2 is found to be dose-dependent in the cardiac myocytes (Lavi et al., 2003). Other studies indicate that the oxidative stress produced by singlet oxygen rapidly disrupts epidermal growth factor receptor (EGFR)-mediated signaling by activating of caspase-3 and phosphatases (Zhuang et al., 2003). It is possible that the mitogenic tyrosine kinase signal could be converted into an apoptotic response by recruiting apical components of the caspase pathway to interact with TPKs (Howard et al., 2003), and that the decreased TPKs activity could inhibit PLC-gamma activation, and then inactivate PKCs.

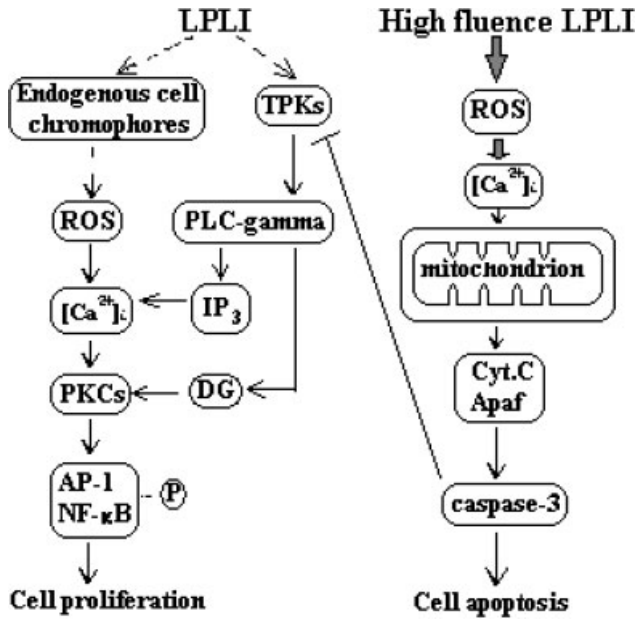

Fig. 6. A model of the signal pathways of PKCs activation and inactivation induced by laser irradiation. Broken lines mean that the downstream changes produced by the upstream don't be proved clearly. Real lines mean that the downstream effects induced by the upstream have been proved already. The thick arrowheads mean the corresponding effects are much stronger than the thin ones.

According to the above experimental data and the related analysis, we summarize the signaling pathways related to PKCs activation induced by laser irradiation (Fig. 6).

In conclusion, this study demonstrates that PKCs are involved in the regulation of cell proliferation and apoptosis induced by laser irradiation. The importance of these findings needs to be explored further. How PKCs are activated and inhibited and how the activated PKCs interact with other signal pathways need to be further studied. Further experiments are in progress to verify whether caspase-3 is related to the inactivity of PKCs by high fluence LPLI as well as how ROS regulates the PKCs activity.

ACKNOWLEDGMENTS

The authors thank Professor Alexandra C. Newton at Department of Pharmacology, University of California for providing C kinase activity reporter plasmid.

LITERATURE CITED

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.

Barushka O, Yaakobi T, Oron U. 1995. Effect of low-energy laser (He-Ne) irradiation on the process of bone repair in the rat tibia. Bone 16:47–55.

Behrend L, Henderson G, Zwacka RM. 2003. Reactive oxygen species in oncogenic transformation. Biochem Soc Trans 31:1441–1444.

Belkin M, Zaturunsky B, Schwartz M. 1988. A critical review of low energy laser bioeffects. Lasers Light Ophthalmol 2:63–71.

Ben-Dov N, Shefer G, Irintchev A, Wernig A, Oron U, Halevy O, Irinitchev A. 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.

Boulton M, Marshall J. 1986. He-Ne laser stimulation of human fibroblast proliferation and attachment in vitro. Lasers Life Sci 1:125–134.

Braun DC, Garfield SH, Blumberg PM. 2005. Analysis by fluorescence resonance energy transfer of the interaction between ligands and protein kinase C in the intact cell. J Biol Chem 280:8164–8171.

Breitbart H, Levinshal T, Cohen N, Friedmann H, Lubart R. 1996. Changes in calcium transport in mammalian sperm mitochondria and plasma membrane irradiated at 633 nm (HeNe laser). J Photochem Photobiol B 34:117–121.

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

Duan R, Liu TC, Li Y, Guo H, Yao LB. 2001. Signal transduction pathways involved in low intensity He-Ne laser-induced respiratory burst in bovine neutrophils: A potential mechanism of low intensity laser biostimulation. Lasers Surg Med 29:174–178.

Gaits F, Hahn K. 2003. Shedding light on cell signaling: Interpretation of FRET biosensors. Sci STKE 165:PE3.

Gross AJ, Jelkmann W. 1990. Helium-neon laser irradiation inhibits the growth of kidney epithelial cells in culture. Lasers Surg Med 10:40–44.

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. Lasers Surg Med 22:212–218.

Heim R, Tsien RY. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr Biol 6:178–182.

Hilf R, Murant RS, Narayanan U, Gibson SL. 1986. Relationship of mitochondrial function and cellular adenosine triphosphate levels to hematoporphyrin derivative-induced photosensitization in R3230AC mammary tumors. Cancer Res 46:211–217.

Howard PL, Chia MC, Rizzo SD, Liu FF, Pawson T. 2003. Redirecting tyrosine kinase signaling to an apoptotic caspase pathway through chimeric adaptor proteins. Proc Natl Acad Sci USA 100:11267–11272.

Jaken S, Parker PJ. 2000. Protein kinase C binding partners. Bioessays 22:245–254.

Johnson KR, Becker KP, Facchinetti MM, Hannun YA, Obeid LM. 2002. PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). J Biol Chem 277:35257–35262.

Karu T. 1999. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B 49:1–17.

Karu T, Pyatibrat L, Kalendo G. 1995. Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro. J Photochem Photobiol B 27:219–223.

Khanna A, Shankar LR, Keelan MH, Kornowski R, Leon M, Moses J, Kipshidze N. 1999. Augmentation of the expression of proangiogenic genes in cardiomyocytes with low dose laser irradiation in vitro. Cardiovasc Radiat Med 1:265–269.

Kipshidze N, Nikolaychik V, Keelan MH, Shankar LR, Khanna A, Kornowski R, Leon M, Moses J. 2001. Low-power helium: Neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells in vitro. Lasers Surg Med 28:355–364.

Kiwamu T, Takeharu N, Atsushi M, Masayuki M. 2003. Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. J Cell Biol 160:235–243.

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.

Lee HB, Yu MR, Song JS, Ha H. 2004. Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by human peritoneal mesothelial cells. Kidney Int 65:1170–1179.

Lubart R, Malik Z, Rochkind S, Fisher T. 1990. A possible mechanism of low lever laser-living cell interaction. Laser Ther 2:65–68.

Lubart R, Wollman Y, Friedmann H, Rochkind S, Lanlicht I. 1992. Effects of visible and near-infrared lasers on cell cultures. J Photochem Photobiol B 12:305–310.

Maruyama K, Mori Y, Murasawa S, Masaki H, Takahashi N, Tsutusmi Y, Moriguchi Y, Shibazaki Y, Tanaka Y, Shibuya M, Inada M, Matsubara H, Iwasaka T. 1999. Interleukin-1 beta upregulates cardiac expression of vascular endothelial growth factor and its receptor KDR/flk-1 via activation of protein tyrosine kinases. J Mol Cell Cardiol 31:607–617.

Mitra RD, Silva CM, Youvan DC. 1996. Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. Gene 173:13–17.

Mizuno H, Sawano A, Eli P, Hama H, Miyawaki A. 2001. Red fluorescent protein from Discosoma as a fusion tag and a partner for fluorescence resonance energy transfer. Biochemistry 40:2502–2510.

Musashi M, Ota S, Shiroshita N. 2000. The role of protein kinase C isoforms in cell proliferation and apoptosis. Int J Hematol 72:12–19.

Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. 2004. Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. Proc Natl Acad Sci USA 101:10554–10559.

Newton AC. 2001. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101:2353–2364.

Nishizuka Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.

Ocana-Quero JM, Perez de la Lastral J, Gomez-Villamandos R, Moreno-Milla'n M. 1998. Biological effect of helium-neon (He-Ne) laser irradiation on mouse myeloma (Sp2-Ag14) cell line in vitro. Lasers Med Sci 13:214–218.

Palmier B, Vacher M, Harbon S, Leiber D. 1999. A tyrosine kinase signaling pathway, regulated by calcium entry and dissociated from tyrosine phosphorylation of phospholipase Cγ-1, is involved in inositol phosphate production by activated G protein-coupled receptors in myometrium. J Pharmacol Exp Ther 289:1022–1030.

Rhee SG, Bae YS. 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 272:15045–15048.

Rhee SG, Choi KD. 1992. Regulation of inositol phospholipid-specific phospholipase C isozymes. J Biol Chem 267:12393–12396.

Schaffer M, Sroka R, Fuchs C, Schrader-Reichardt U, Schaffer PM, Busch M, Duhmke E. 1997. Biomodulative effects induced by 805 nm laser light irradiation of normal and tumour cells. J Photochem Photobiol B 40:253–257.

Schwartz F, Brodie C, Appel E, Kazimirsky G, Shainberg A. 2002. Effect of helium/neon laser irradiation on nerve growth factor synthesis and secretion in skeletal muscle cultures. J Photochem Photobiol B 66:195–200.

Sekar RB, Periasamy A. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J Cell Biol 160:629–633.

Seko Y, Imai Y, Suzui S, Kamijukkoku S, Hayasaki K, Sakomura Y, Tobe K, Kadowaki T, Maekawa H, Takahashi N, Yazaki Y. 1997. Serum levels of vascular endothelial growth factor in patients with acute myocardial infarction undergoing reperfusion therapy. Clin Sci 92:453–454.

Shefer G, Oron U, Irintchev A, Wernig A, Halevy 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, Barash I, Oron U, Halevy O. 2003. Low-energy laser irradiation enhances de novo protein synthesis via its effects on translation-regulatory proteins in skeletal muscle myoblasts. Biochim Biophys Acta 1593:131–139.

Spivak JM, Grande DA, Ben-Yishay A, Menche DS, Pitman MI. 1992. The effect of low level Nd:YAG laser energy on adult articular cartilage in vitro. Arthroscopy 8:36–43.

Takai Y, Kishimoto A, Inoue M, Nishizuka Y. 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. J Biol Chem 252:7603–7609.

Thornberry NA, Lazebnik Y. 1998. Caspases: Enemies within. Science 281:1312–1316.

Ting AY, Kain KH, Klemke RL, Tsien RY. 2001. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. Proc Natl Acad Sci USA 98:15003–15008.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. 1996. Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. Nature 380:75–79.

Violin JD, Zhang J, Tsien RY, Newton AC. 2003. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J Cell Biol 161:899–909.

Wang F, Chen TS, Xing D, Wang JJ, Wu YX. 2005. Measuring dynamics of caspase-3 activity in living cells using FRET technique during apoptosis induced by high fluence low-power laser irradiation. Lasers Surg Med 36:2–7.

Yu W, Naim JO, Lanzafame RJ. 1994. The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. Photochem Photobiol 59:167–170.

Zhang J, Campbell RE, Ting AY, Tsien RY. 2002. Creating new fluorescent probes for cell biology. Nat Rev Mol Cell Biol 3:906–918.

Zhuang S, Ouedraogo GD, Kochevar IE. 2003. Downregulation of epidermal growth factor receptor signaling by singlet oxygen through activation of caspase-3 and protein phosphatases. Oncogene 22:4413–4424.