Selective delivery of laser energy to ester bonds of triacylglycerol in lipid droplets of adipocyte using a quantum cascade laser

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Abstract: The recent development of quantum cascade lasers (QCLs) has facilitated the irradiation of a mid-infrared laser beam that is specifically absorbed by a target molecular bond. Aiming for a selective delivery of laser energy to a specific absorption at 1,738 cm$^{-1}$ by the ester bonds of triacylglycerol (TAG), a QCL beam with a wavenumber of 1,710 cm$^{-1}$ was irradiated to 3T3–L1 adipocytes and preadipocytes. Neutral red staining, and FITC-labeled annexin V and ethidium homodimer-III assays revealed the occurrence of adipocyte-specific cell death 24 h after QCL irradiation. The selective delivery of laser energy to endogenous molecules can affect biological processes in a living organism.

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1. Introduction

Laser beams in the mid-infrared (MIR) (2–13 µm) region, referred to as the molecular fingerprint region, can be absorbed by molecular bonds [1]. The MIR absorption spectrum can non-destructively provide useful information about molecular composition, even for living organisms. In order to characterize endogenous biomolecules, which exhibit a three-dimensional arrangement at the subcellular level (even at the intra-nuclear level) [2], a selective excitation of vibrational states is employed for measurements using coherent anti-Stokes Raman spectroscopy [3–5] and vibrational sum frequency generation spectroscopy [6, 7]. The effects of molecular vibrations, excited by an infrared laser energy, on biological objects, are not yet fully understood.

In general, the reactivity of the molecules [8], re-radiation at a longer wavelength, and radiation as heat can be observed as effects of the excited vibrational states. Heat causes a change in reactivity and radiation of IR light [9] by the fluctuating molecular vibrations, regardless of the chemical bonds’ type. In contrast to the selective effects of the MIR laser beams, the heat affects almost all molecules; therefore, it has been challenging to distinguish the effects of MIR lasers in biological objects. MIR lasers are considered harmless, while the absorbance by the water molecules can affect as heat.

The recent development of quantum cascade lasers (QCLs) [10, 11] has facilitated the irradiation of a pure MIR laser line. The energy of the QCL can be effectively delivered to target molecules, without absorption by water molecules. QCLs exhibit a high sensitivity, sufficient even for ultra-microanalysis. However, the highly effective delivery of the QCL energy could also create concerns of a potential damage in the human body by the MIR laser irradiation.

Abundance and specificity in the absorption are required to investigate the selective delivery of MIR laser energy. Lipids satisfy both requirements, as the ester bonds exhibit a specific absorption in the range of 1,700–1,750 cm$^{-1}$ [12, 13], and the lipids are the second most abundant molecular species in an animal body, e.g., 15–40% in the mouse body [14]. In fact, influence of MIR radiation on fat cells has been investigated [15, 16]. In addition, the functions of lipids are of interest in various fields including medical sciences [17, 18], biology [19, 20], food science [21, 22], etc. The abundant lipids are challenging to be manipulated, while the molecular biological manipulation of nucleic acids and proteins has been developed and widely used. Therefore, the development of methods for manipulation of lipids in living organisms is of key importance.

In this study, we demonstrated a selective delivery of MIR laser energy to triacylglycerol (TAG), leading to adipocyte-specific cell death. Using a QCL (1,710 cm$^{-1}$), energy was delivered to the specific absorption of TAG at 1,738 cm$^{-1}$. In order to confirm that the QCL...
caused damage to TAG, an adipocyte, differentiated from a cell line of a mouse fibroblast, 3T3–L1, was compared with an undifferentiated pre-adipocyte, as TAG is almost specific in lipid droplets of adipocyte [23]. Finally, the damage to adipocytes by QCL was characterized using the typical features of apoptosis and necrosis.

2. Materials and methods

2.1 Cell culture and differentiation

A cell line derived from a mouse, 3T3–L1 (DS Pharma Biomedical, Osaka, Japan), was cultured and differentiated to adipocyte, according to the manufacturer's protocol. A preadipocyte was cultured in a Dulbecco’s Modified Eagle Medium containing a high glucose level (Wako Pure Chemical Industries, Osaka, Japan) and 10% heat-inactivated fetal bovine serum (Corning, NY, USA) in a film bottom dish, µ-Dish35 mm, high ibiTreat (ibidi, Martinsried, Germany). The medium was exchanged every second day until confluent conditions were obtained. The differentiation to adipocyte was induced using the 3T3–L1 differentiation medium (DS Pharma Biomedical) after two days of culture under a confluent condition. The differentiation medium was exchanged to a fresh differentiation medium after two days. After four days of stimulation, the differentiation medium was exchanged with the 3T3–L1 adipocyte medium (DS Pharma Biomedical). The adipocyte medium was exchanged every three days during the experiment. It was used for experiments within 7–14 days from the induction of differentiation; the preadipocyte was used as control within 2–4 days after reaching the confluent condition.

2.2 Irradiation with an infrared laser

A custom-made QCL (HAMAMATSU, Shizuoka, Japan) operated with a C10338 HAMAMATSU pulse driver was employed to irradiate a laser beam with a wavenumber of 1,710 cm\(^{-1}\) in a quasi-continuous wave mode with duty cycle 25% (500 nsec, 500kHz). The irradiated area was expanded using a CaF\(_2\) plano-concave lens (Thorlabs, Tokyo, Japan); the laser spot was determined using an MIR liquid-crystal detector card VRC6S (Thorlabs). The irradiation was limited at 30 s using an SH–05 (Thorlabs) shutter with an SC–10 controller (Thorlabs). In order to reduce the diffraction and absorption of the laser beam, the MIR beam was irradiated from the bottom of the sample dish at room temperature. The laser intensity was checked using a FieldMaxII (Coherent, CA, USA) power meter before and after the irradiation. Prior to the experiments, the cells were washed and submerged in a warmed Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher, Waltham, MA, USA). After the irradiation, DPBS was exchanged to a warmed 3T3–L1 adipocyte maintenance medium and incubated. As increase of temperature during QCL irradiation was less than 1 °C by observation with a thermography FLIR ONE (FLIR Systems, Wilsonville, OR, USA) temperature of the sample dish was not controlled. A near-infrared (NIR) laser beam, 7300 Laser Diode Module (Spectra-Physics, Santa Clara, CA, USA), with a wavenumber of 12,345 cm\(^{-1}\) (810 nm), was employed as a negative control laser beam, which is absorbed neither by biomolecules nor by water molecules.

2.3 Cell stain and image acquisition

In order to evaluate the differentiation of 3T3–L1 to adipocyte, an oil red O staining was employed. The adipocyte fixed with a 10% paraformaldehyde solution was stained for 20 min with a 1.8-mg/ml oil red O (Wako Pure Chemical Industries) in 60% isopropyl alcohol [24]. The cell viability was tested with a neutral red uptake assay [25]. The viable-cell staining was performed with 33-µg/ml neutral red (Wako Pure Chemical Industries) dissolved in the culture medium for 2 h after the 24 h of laser irradiation. The stained cells were imaged using a color MC120HD complementary metal–oxide–semiconductor (CMOS) (Leica Microsystems, Wetzlar, Germany) camera through a 10 × objective using a DMi1 (Leica
microsystems) inverted microscope. An apoptotic/necrotic cells detection kit (PromoCell, Heidelberg, Germany) was employed to determine the damage characteristic according to the type of cell death [26]. The fluorescences of fluorescein isothiocyanate (FITC) and ethidium homodimer III were observed through a 20 × objective using an inverted microscope (IX81, Olympus). Fluorescence images were acquired using a CMOS monochrome camera (WRAYCAM SR130M, WRAYMER, Osaka, Japan). The acquired images were analyzed with the Fiji program [27]. The statistics were described as average ± standard deviation. The significance in a difference was analyzed with the one-tailed $t$-test using the R program (Version 3.2.4) [28].

2.4 Fourier-transform infrared (FTIR) measurements

Both preadipocyte and adipocyte were washed twice with a warmed DPBS. DPBS was then removed and dried in a laminar-flow culture hood. The dried cells were scraped and put on a polyethylene IR sample card (International Crystal Laboratories, Granfield, NJ, USA). The absorption spectrum (500–4,000 cm$^{-1}$) was obtained using an FTIR spectrometer (Spectrum Two, Perkin Elmer, Waltham, MA, USA). Glyceryl trioleate (Sigma-Aldrich, St. Louis, MO, USA) was analyzed as a TAG standard. The obtained spectra were processed using the Igor Pro 6.34 (Wave Metrics, Portland, OR, USA) program.

3. Results

3.1 Selective absorption by the ester bonds of TAG in adipocyte

![Image of specific absorption by the ester bonds of TAG in adipocyte]

Fig. 1. Specific absorption by the ester bonds of TAG in adipocytes. Oil red O staining of: (a) adipocytes and (b) preadipocytes. The scale-bar corresponds to 200 µm. (c) FTIR absorption spectra of adipocytes and preadipocytes. The absorption characteristic that is specific to the adipocytes at 1,738 cm$^{-1}$ is indicated by the arrow. (d) FTIR absorption spectrum of the standard sample of glyceryl trioleate.

After differentiation to adipocyte, the mouse fibroblast 3T3–L1 has lipid droplets filled with neutral lipids; most of them are TAG. The 3T3–L1 adipocytes and preadipocytes were stained with oil red O, as shown in Figs. 1(a) and 1(b). All of the droplets in adipocytes were stained
with oil red O, as shown in Fig. 1(a); however, almost no oil red O stain was observed in the preadipocytes, as shown in Fig. 1(b). The MIR absorbance characteristic of the adipocytes was evaluated by comparison with that of the preadipocytes using FTIR spectroscopy. The absorption spectra of the adipocytes and preadipocytes are shown in Fig. 1(c). The adipocyte sample exhibited a specific absorbance at 1,738 cm$^{-1}$ (indicated by the arrow), attributed to the absorbance by the ester bonds (C = O stretching) [12, 13]. Consistently, the standard TAG sample of glyceryl trioleate also exhibited an intense absorption at 1,738 cm$^{-1}$, as shown in Fig. 1(d). Therefore, the MIR beam at 1,738 cm$^{-1}$ was selectively absorbed by the ester bonds of TAG in the lipid droplets.

3.2 Cell death induced by the selective delivery of laser energy

A QCL beam with a wavenumber of 1,710 cm$^{-1}$ was irradiated at average power of 180 mW for 30 s onto the adipocytes in the DPBS at room temperature. The adipocytes, 24 h after a MIR laser irradiation, died and were negative for neutral red staining, as shown in Fig. 2(a), while the adipocytes that were not exposed to the MIR laser beam were alive and stained with neutral red (Fig. 2(b)). Area of neutral red negative region increased depending on delivered laser energy. The neutral red level, quantified as the average intensity of the red channel in the neutral red negative regions ($n = 5$), was calculated to be 83.5±3.6, significantly low by the t-test ($p = 0.046 < 0.05$), compared with that of the neutral red positive regions ($n = 5$) of 87.4±1.6. On the other hand, the preadipocytes exposed to the MIR laser beam (78.9 ± 2.4, $n = 5$) were alive and positive for neutral red stain, regardless of the exposure to the MIR laser beam, as shown in Figs. 2(c) and 2(d). The neutral red level of the unexposed preadipocytes was 77.6 ± 4.1, and the difference was not significant ($p = 0.36$). Therefore, the laser energy was selectively delivered to adipocytes, and caused cell death. Consistently, both adipocytes and preadipocytes were not affected by a 30-s-long NIR laser irradiation (data not shown), although its power was 440 mW, almost 2.5 times larger than that of the QCL.

![Fig. 2. Adipocytes and preadipocytes after the MIR laser irradiation. Viable adipocytes and preadipocytes were stained with neutral red 24 h after a MIR laser irradiation; (a) adipocytes and (c) preadipocytes exposed to the MIR laser beam; (b) adipocytes and (d) preadipocytes those were not exposed to the MIR laser irradiation. The neutral red negative cells are outlined by the dashed circle. The scale-bar corresponds to 200 µm.](image)
3.3 Apoptosis and necrosis assays

In order to characterize the damage in the adipocytes as a biological feature in the cell death process, the adipocytes, 24 h after the MIR laser irradiation, were stained with FITC-labeled annexin V and ethidium homodimer III (EthD-III), representative markers for two major processes leading to cell death. Annexin V specifically connects to phosphatidylserine (PS) translocated from the inner to the outer surface of the cell during the apoptosis [29, 30]. EthD-III is impermeant to live or apoptotic cells, and is a highly positively charged nucleic acid probe [31]. The fluorescence results of EthD-III showed a cluster of adipocytes, consistent with the neutral red stain result, as shown in Fig. 3(b). The fluorescence of FITC was also concentrated at the same region, as shown in Figs. 3(c) and 3(d). Therefore, the adipocytes 24 h after the MIR laser irradiation could be characterized as either necrotic or late apoptotic cells [32].

Fig. 3. Characterization of the MIR laser damage in adipocytes. The adipocytes were stained with an apoptotic/necrotic detection kit 24 h after the MIR laser irradiation. (a) Phase contrast image. Fluorescence of (b) EthD-III and (c) FITC-labeled annexin V. (d) Merged fluorescence image. The scale-bar corresponds to 200 µm.

4. Discussion

4.1 Estimation of the energy delivered by the MIR laser

In this study, the QCL laser was irradiated at average power of 180 mW for 30 s. It was challenging to determine the exact QCL energy delivered to adipocytes under the irradiation conditions, confluent in a monolayer, as the sensitivity of the spectrometer was not sufficiently high. As the FTIR measurement of the scraped adipocytes, corresponding to approximately 2–3 layers, showed an absorption of 13.7% at 1,738 cm⁻¹ (Fig. 1(c)), 5–7% of the QCL energy was considered to be absorbed. Therefore, the estimated QCL energy delivered to the adipocytes was approximately 0.3 J, which corresponds to a heat quantity that
can increase the temperature of 1 µl TAG oil by approximately 150 °C [33]; the volume of the irradiated adipocytes was estimated by multiplying the laser spot size of 1 cm² by the adipocytes height of 10 µm. Similarly, the unexpected absorption of QCL energy (most likely by the water molecules) by the preadipocytes was calculated to be 0.2 J, from the absorption of 11.4% at 1,738 cm⁻¹ in the FTIR spectrum of the scraped preadipocytes (Fig. 1(c)). This energy corresponds to a heat quantity that can increase the temperature of 1 µl water by approximately 50 °C. As a 1-min-long exposure at 45–50 °C is lethal for adipocytes [34], the above estimates seem reasonable.

It is worth noting that the QCL irradiation at a power smaller than 150 mW did not affect the 3T3–L1 adipocytes while occurrence of neutral red negative region was stochastic at a power range of 160–170 mW. Therefore, the effect of the QCL energy to adipocytes cannot be only attributed to thermal damage. The region with dead 3T3–L1 adipocytes (Fig. 2(a)) was significantly smaller than the QCL spot size of approximately 1 cm², observed using the MIR liquid-crystal detector card. The neutral red negative area, calculated from the images, was 0.19 ± 0.03 mm² (n = 5). As the photon density in the laser spot was considered to be varied such as Gaussian fluctuation, energy density could be higher in the neutral red negative area, e.g. × 2.3 by rough estimation assuming Gaussian spot with 1/e² width. As a temperature increase by more than 300 °C is sufficient for a non-catalytic thermal crack of TAG [35], the activation of biological processes by the increase of diacylglycerol (DAG) or free fatty acids contents [18, 36] was also presumable.

4.2 Biological features of the effect by the QCL

The effects of the QCL exposure on the adipocytes were characterized using FITC-labeled annexin V and EthD-III. The FITC-labeled adipocytes and EthD-III-positive adipocytes were not exclusive but overlapped. Therefore, the adipocytes 24 h after the QCL irradiation were either late apoptotic or necrotic cells. Although a late apoptosis 24 h after the irradiation is reasonable, according to a previous study with a terahertz laser [37], necrosis is also likely to appear. The temperature increase can cause necrosis by deforming or disordering the cellular membrane through denaturation or change of the phase of the lipid membrane. It also causes apoptosis through upregulation of leptin in a 3T3–L1 adipocyte [38, 39].

The non-catalytic increase of the DAG or fatty acids contents [40], which are signal molecules associated with various biological processes, can cause necrosis or apoptosis. The increase of the free fatty acids content could trigger necrosis through mitochondrial dysfunction [41, 42], and it is also attributed to apoptosis [43, 44]. The production of reactive oxygen species, such as hydrogen peroxide [32] or singlet oxygen [45], should also be considered. In addition, it is of interest to investigate whether the excited molecular vibrations are related to the functions of lipids, recently revealed in other types of programmed cell death [19].

5. Conclusion

The aim of this study was to selectively deliver MIR laser energy to TAG. We demonstrated an adipocyte-specific cell death caused by QCL irradiation at 1,710 cm⁻¹, which was absorbed specifically by the ester bonds of TAG at 1,738 cm⁻¹ in lipid droplets of adipocytes. The QCL exposure caused cell death specific to adipocytes, and the damage was characterized through FITC-labeled annexin V and EthD-III. This study revealed an optical manipulation technique to stimulate biomolecules directly by choosing the optimal wavenumber for a target bond. These findings reveal that the stimulation of endogenous molecules could be performed directly, transiently, and locally, according to the spatiotemporal limit of the MIR laser.
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Disclosures
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