Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death

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Knight, Martin M., Susan R. Roberts, David A. Lee, and Dan L. Bader. Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death. Am J Physiol Cell Physiol 284: C1083–C1089, 2003; 10.1152/ajpcell.00276.2002.—Isolated chondrocytes stained with fluo 4-AM and visualized using standard confocal microscopy techniques exhibited Ca\(^{2+}\) transients and oscillations. Decreasing the power of the laser light decreased the percentage of cells exhibiting these Ca\(^{2+}\) signals. Treatment with the antioxidant ascorbate reduced the Ca\(^{2+}\) response, suggesting that it was mediated by light-induced release of reactive oxygen species (ROS). Cell viability 24 h after the 1-h confocal imaging period was ~90% for cells that were neither fluorescently stained nor subjected to laser excitation. By contrast, fluorescently stained cells imaged for 1 h exhibited greatly reduced viability. Treatment with ascorbate reduced the level of cell death, suggesting that the effect was mediated by release of exogenous ROS associated with the interaction of light and the fluorochrome. Ca\(^{2+}\) oscillations were not always associated with cell death, suggesting that separate light-sensitive pathways mediate the two processes. Light-activated Ca\(^{2+}\) signaling may trigger alterations in numerous cell processes and thereby represent an important and hitherto overlooked artifact in fluorescent microscopy of viable cells.

photoactivation; free radicals; reactive oxygen species; oxygen; chondrocyte

THERE IS INCREASING USE of fluorescence-based microscopy techniques for the study of physiology within viable cells. This is supported by the expanding array of fluorescent probes and the development of confocal laser scanning microscopes designed to provide quantitative, real-time imaging of viable cells. Fundamental to this approach is the assumption that the imaging process does not significantly influence normal cell physiology. However, light is known to stimulate the production of oxygen free radicals or reactive oxygen species (ROS) either via damage to biological molecules or degradation of photosensitive agents (7, 8, 14) or fluorochromes (6, 10). ROS, which include singlet oxygen, superoxide anions, and hydrogen peroxide, function as intracellular second messengers in healthy tissue (13, 16, 28). These ROS are also involved in various disease conditions, including ischemic reperfusion injury, atherosclerosis, Alzheimer’s disease, osteoarthritis, and cancer (1, 16, 17, 22, 31, 34). Numerous previous studies using specialized photosensitive agents have reported light-induced release of exogenous ROS leading to activation of intracellular Ca\(^{2+}\) signaling and cell death (2, 7, 8, 19, 24). Indeed, the latter phenomenon is the basis of photodynamic therapy (PDT), which is used clinically for the treatment of some forms of cancer (23). The present study tests the hypothesis that Ca\(^{2+}\) signaling and phototoxicity also occur due to release of ROS from standard fluorochromes used for fluorescent microscopy, thereby representing a significant artifact associated with these methodologies.

MATERIALS AND METHODS

Preparation of chondrocyte/agarose constructs. This study employs a well-characterized experimental model consisting of isolated bovine articular chondrocytes seeded in agarose gel (20, 21). Culture medium consisted of Dulbecco’s minimal essential medium supplemented with penicillin/streptomycin (5 mg/ml), L-glutamine (2 mM), HEPES (20 mM), L-ascorbic acid (0.85 mM), and 20% (vol/vol) fetal calf serum (FCS) (DMEM + 20% FCS; all from Sigma, Poole, UK). Full-depth slices of articular cartilage were removed from the proximal surfaces of metacarpophalangeal joints from steers aged between 18 and 30 mo. The cartilage was diced finely and incubated at 37°C on rollers for 1 h in DMEM + 20% FCS + 700 units/ml pronase (VWR International, Lutterworth, UK). The tissue was subsequently incubated at 37°C in DMEM + 20% FCS + 100 units/ml collagenase type XI (Sigma) for a further 16 h on rollers. The supernatant, containing released chondrocytes, was passed through a 70-μm pore-size sieve (Falcon, Oxford, UK), washed twice in DMEM + 20% FCS, and resuspended at 2 × 10^7 cells/ml in 20% FCS at 37°C and 5% CO\(_2\). The chondrocyte suspension was added to an equal volume of molten 8% (wt/vol) agarose (type IX-A; Sigma) in Earl’s balanced salt solution (EBSS; Sigma) to give a final concentration of 1 × 10^7 cells/ml in 4% agarose. The chondrocyte/agarose suspension was plated in specially designed stainless steel moulds and allowed to gel at 4°C for 20 min to produce cylindrical constructs, 5 mm in diameter and 5 mm high. The constructs were maintained in DMEM + 20% FCS at 37°C and 5% CO\(_2\).

Fluorescent staining. After 24 h in culture, chondrocyte/agarose constructs were incubated for 60 min at 37°C in a...
5-μM solution of either fluo-4 AM or calcein-AM (both from Cambridge Bioscience, Cambridge, UK) prepared in EBSS containing 1.8 mM Ca\(^{2+}\) and supplemented with 20 mM HEPES at pH 7.4. These staining protocols were optimized for cells seeded within three-dimensional agarose gel, which, unlike two-dimensional model systems such as monolayer cultures, inhibits the diffusion of the fluorescent probe and thereby necessitates an extended incubation period. Control constructs remained unstained in EBSS. All constructs were then washed twice in EBSS and incubated in EBSS for a further 15 min at room temperature to allow complete esterification of the stains.

**Confocal laser scanning microscopy.** Individual constructs were placed on a sterile coverslip, bathed in EBSS, and mounted on the stage of an inverted microscope (Eclipse, Nikon, Kingston-upon-Thames, UK) associated with a confocal laser scanning microscope (UltraView, Perkin Elmer, Cambridge, UK). A plan apo ×20/0.75 N.A. objective lens (Nikon) was used to visualize cells within a field of view of 265 × 217 μm and at a depth of ~50 μm into the agarose construct. Laser excitation light was provided at a wavelength of 488 nm, and fluorescent emissions were collected at wavelengths above 515 nm. For image acquisition, an exposure time of 0.8 s was adopted with a binning of 2 × 2 on the charge-coupled device camera, yielding a pixel size of 0.68 μm. Pixel intensities were recorded within the range 0 to 4,095, the latter value representing saturation. Individual cells within a single field of view were imaged over a 60-min period, with a 10-s shuttered interval between each image.

The argon-ion laser (Coherent, Ely, UK) associated with the confocal microscope system was operated at two different laser power settings, the normal power setting recommended by the manufacturer and a reduced laser power. With the use of a light meter (Coherent), the corresponding power at the objective aperture was 30 and 15 μW at a wavelength of 488 nm. All imaging was conducted at room temperature of 25°C ± 4°C to minimize dye leakage. A transmitted light reference image was captured after completion of the 1-h imaging protocol. The construct was subsequently placed in an individual 7-ml bijou tube and cultured for a further 24 h in DMEM + 20% FCS at 37°C and 5% CO₂.

The **influence of confocal laser power on Ca\(^{2+}\) signaling.** Cells stained with fluo-4 AM were imaged for the 1-h period at either 30 or 15 μW laser power as described above, and the resulting images were used for analysis of Ca\(^{2+}\) signaling as indicated in the flow chart in Fig. 1. Approximately 300 cells were imaged in 8 separate constructs at 30 μW laser power and a similar number at 15 μW laser power.

With the use of the software associated with the confocal microscope (Temporal Mode, UltraView Software, Perkin Elmer, Cambridge, UK), a circular region of interest (ROI) was drawn around each cell within a field of view. For each ROI, the mean intensity was calculated for each image in the 1-h sequence. These intensity values were plotted against time to indicate the temporal changes in intracellular Ca\(^{2+}\) concentration as shown in Fig. 2. A-C. A Ca\(^{2+}\) transient was defined as a rise in intracellular Ca\(^{2+}\) followed by a return to basal levels (Fig. 2B). For each cell, the number of Ca\(^{2+}\) transients in the 1-h period and the time at which the peak of each transient occurred were recorded. The periodicity of Ca\(^{2+}\) oscillations, defined as the mean time between successive transients, was calculated for cells exhibiting four or more transients within 1 h (Fig. 2C).

The **influence of confocal laser power on cell viability.** Unstained cells and cells stained with either fluo-4 AM or calcein-AM were exposed to the 1-h imaging period at either 30 or 15 μW laser power or no laser power and then returned to culture conditions for a further 24 h before analysis of cell viability. The different test conditions are illustrated in the flow chart in Fig. 1. Viability was assessed as follows using an established fluorescent live/dead assay.

Cell/agarose constructs were incubated in EBSS supplemented with 5 μM calcein-AM and 5 μM ethidium homodimer-1 (Cambridge Bioscience) for 1 h at 37°C. The transmitted light reference image was used to relocate the same groups of cells previously subjected to the 1-h confocal imaging protocol. Live cells stained with calcein-AM and the nuclei of dead cells stained with ethidium homodimer-1 were visualized at excitation wavelengths of 488 and 568 nm, respectively. Cell viability was calculated as the percentage of live cells.

The **effect of antioxidant ascorbate.** To determine whether any Ca\(^{2+}\) signaling response or change in cell viability was mediated by free radicals, a further group of cell-agarose constructs was treated with the antioxidant, ascorbate (16, 26, 29, 34). Constructs were incubated in EBSS supplemented with 1 mM ascorbate (Sigma) for 10 min before and throughout the 1-h confocal imaging protocol as indicated in Fig. 1. Ca\(^{2+}\) signaling characteristics and cell viability were then assessed as previously described.

**Statistical analysis.** The χ² statistical analysis was used to compare the proportion of cells exhibiting different modes of Ca\(^{2+}\) response. Unpaired Student t-tests were used to compare the percentage viability values calculated for the constructs in each experiment group. In all cases, \( P < 0.05 \) was taken to indicate a statistically significant difference.
RESULTS

The influence of confocal laser power on Ca\(^{2+}\) signaling. During the 1-h imaging period, a subpopulation of cells stained with fluo 4-AM exhibited Ca\(^{2+}\) transients, whereas the remainder of cells maintained a constant level of intracellular Ca\(^{2+}\) (Fig. 2A). Cells exhibiting Ca\(^{2+}\) transients were subdivided into those cells that exhibited one to three spontaneous transients in the 1-h period (Fig. 2B) and those that exhibited four or more regular oscillatory transients (Fig. 2C). The percentage of cells showing these different modes of Ca\(^{2+}\) response at 30 and 15 \(\mu\)W laser power, with and without ascorbate treatment, is illustrated in Fig. 3. During the 1-h imaging period, 58% of cell exhibited 1–3 transients at 30 \(\mu\)W laser power compared with 45% at 15 \(\mu\)W laser power, the difference being statistically significant. There was a similar statistically significant difference in the percentages of cells exhibiting four or more transients with values of 28 and 14% at 30 and 15 \(\mu\)W laser power, respectively. At each laser power setting, treatment with ascorbate significantly reduced both the percentage of cells exhibiting one to three transients and the percentage of cells exhibiting four or more transients (Fig. 3). Indeed, ascorbate treatment reduced the total percentage of cells exhibiting Ca\(^{2+}\) transients at 30 \(\mu\)W laser power to a value similar to that with 15 \(\mu\)W laser power in the absence of ascorbate.

For those cells showing Ca\(^{2+}\) oscillations, the mean periodicity of oscillations was 308 s. For all cells, there was minimal photobleaching of the fluo 4.

The influence of confocal laser power on cell viability. The representative confocal images in Fig. 4 indicate how the same groups of cells exposed to the 1-h imaging protocol (Fig. 4, A, C, and E) were assayed for viability after a further 24 h in culture (Fig. 4, B, D, and F). The mean cell viability values for each experimental condition are presented in Fig. 5. Cell viability was \(\sim\)90% for unstained control cells not exposed to laser excitation during the 1-h imaging protocol. Fluorescent staining with either fluo 4-AM or calcein-AM, without exposure to laser light, produced no statistically significant change in cell viability compared with unstained controls. There was also no change in viability when unstained cells were exposed to either 30 or 15 \(\mu\)W laser power during the 1-h imaging protocol. However, the combination of fluorescent staining with either fluo 4-AM or calcein-AM and exposure to laser light resulted in substantial cell death. The loss of
viability was greater for cells exposed to the higher laser power and for cells stained with calcein-AM compared with fluo 4-AM. In both cases, the differences in cell viability were statistically significant.

Treatment with ascorbate significantly reduced the level of cell death caused by the synergistic effects of fluorescent staining and laser excitation during the 1-h imaging protocol (Fig. 5). At 15 μW laser power, the ascorbate treatment in the presence of fluo 4-AM resulted in viability levels indistinguishable from those obtained in unstained controls. Ascorbate had no effect on the viability of cells stained with fluo 4-AM and not exposed to laser excitation.

**DISCUSSION**

Intracellular Ca^{2+} is a ubiquitous second messenger controlling numerous aspects of cell function, including cell division, apoptosis, cytoskeletal organization, transport, and movement (3). It is widely reported that Ca^{2+} signaling can be triggered by ROS released during the exposure of specific photosensitive agents to light (2, 7, 8, 19, 24). Further studies have reported that exposure of the mitochondrial membrane potential probe, TRTM, to high-powered fluorescent light causes the release of ROS, resulting in mitochondrial depolarization (6, 10). However, it remains unclear as to whether Ca^{2+} signaling can be initiated by ROS released during fluorescent microscopy of viable cells.

Previous studies by the authors have investigated the role of Ca^{2+} signaling in chondrocyte mechanotransduction using a well-characterized experimental model consisting of isolated chondrocytes seeded in agarose gel (25). In the present study, the same chondrocyte-agarose model was employed with chondrocytes exposed to a 1-h imaging protocol using a confocal laser scanning microscope (UltraView, Perkin Elmer, UK). The Ca^{2+} signaling characteristics during this imaging period were examined by incubating cells with the intracellular Ca^{2+} probe fluo 4-AM. Laser light, at a wavelength of 488 nm, was used at either 30 or 15 μW. It should be noted that these power levels are considerably lower than that generated by a standard fluorescent mercury lamp (Nikon) for which the power at the objective ranged from 400 to 12.8 mW, depending on the number of neutral density filters within the light path.
siss, whereas lower levels induce cell death through a slower apoptotic pathway (14). In the present study, at the end of the 1-h imaging protocol at both laser power settings, cells stained with fluo 4-AM were still able to exhibit characteristic rapid Ca\(^{2+}\) oscillations in response to stimulation by ATP (data not shown). These findings indicate a maintenance of cell viability at this time point, suggesting that the subsequent cell death occurred by apoptosis rather than necrosis.

It is unclear whether the Ca\(^{2+}\) signaling response is mediated by exogenous or endogenous ROS because it was not possible to measure intracellular Ca\(^{2+}\) signaling in the absence of a fluorescent probe. Although electrophysiology techniques would overcome this problem, such techniques are unsuitable for cells seeded within a 3-D agarose construct. In previous studies, Cui et al. (7, 8) reported that exogenous ROS released from photosensitive agents induced Ca\(^{2+}\) oscillations with a periodicity similar to that observed in the present study. This effect appears to be mediated by hydrogen peroxide (H\(_2\)O\(_2\)) because the Ca\(^{2+}\) response could be eliminated by treatment with catalase, which selectively removes this species (11, 32). It has been shown that H\(_2\)O\(_2\) activates phostidylinositol-specific phospholipase C (15, 32). This causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5 trisphosphate (IP3), which binds to receptors to induce the release of Ca\(^{2+}\) from intracellular stores (3). In this way, light-activated Ca\(^{2+}\) signaling may modulate existing intracellular signaling

During the 1-h imaging protocol at the 30 \(\mu\)W laser power setting, 86% of cells stained with fluo 4-AM exhibited at least one Ca\(^{2+}\) transient, with 33% of these cells showing Ca\(^{2+}\) oscillations of 4 or more regular transients (Figs. 2C and 3). This Ca\(^{2+}\) signaling behavior was followed by a substantial loss of cell viability measured after a further 24 h in culture (Fig. 5). Reducing the laser power to 15 \(\mu\)W decreased the percentage of cells showing Ca\(^{2+}\) transients and oscillations (Fig. 3) and reduced the level of cell death (Figs. 4 and 5). At both laser power settings, treatment with the antioxidant ascorbate also reduced both the percentage of cells showing a Ca\(^{2+}\) response and also the level of cell death. Consequently, confocal laser excitation light induces an immediate Ca\(^{2+}\) signaling response followed by cell death, both of which appear to be mediated by the release of ROS. These effects are similar to those reported using specialized photosensitive agents (2, 7, 8, 19, 24) but have not previously been reported in association with fluorescent microscopy of standard fluorochromes.

The ROS that trigger cell death are exogenous free radicals generated by action of light on the fluorochrome, because there was no loss of viability in unstained cells subjected to the 1-h imaging protocol at either 30 or 15 \(\mu\)W laser power (Fig. 5). Light-induced cell death was not specific to the use of fluo 4-AM because cell death also occurred in cells stained with calcine-AM. For these cells, there was a greater loss of viability, reflecting the fact that the calcine-AM had a greater emission intensity and, hence, a greater propensity to release ROS than fluo 4-AM. It is widely accepted that high levels of ROS initiate rapid necro-

![Fig. 5. The mean percentage viability of cells 24 h after the 1-h imaging protocol at either 30 \(\mu\)W laser power, 15 \(\mu\)W laser power, or no laser. Error bars indicate SE for between 4 and 10 specimens.](http://ajpcell.physiology.org/)

![Fig. 6. The relationship between the mode of Ca\(^{2+}\) signaling exhibited during the 1-h imaging protocol at either 30 or 15 \(\mu\)W laser power and the subsequent cell viability. The percentage cell viability is calculated separately for cells exhibiting no Ca\(^{2+}\) transients, 1–3 Ca\(^{2+}\) transients, and Ca\(^{2+}\) oscillations. Values in parentheses represent the percentage of cells exhibiting each mode of Ca\(^{2+}\) response as presented graphically in Fig. 3.](http://ajpcell.physiology.org/)
pathways (5). In addition to the phospholipase C pathway, other possible signaling mechanisms may be involved, such as ROS-induced changes in the conformation of membrane ion channel proteins (4, 27). Thus different signaling mechanisms may be activated in a dose-dependent manner, resulting in various modes of Ca$^{2+}$ response, as observed in the present study, with different downstream effects. Articular chondrocytes are known to produce ROS such as H$_2$O$_2$ in the presence of inflammatory cytokines (18, 30). The present study demonstrates that in addition to the well-known stimulation of nitric oxide synthesis, ROS may also trigger alterations in Ca$^{2+}$ signaling in chondrocytes.

Although treatment with ascorbate reduced the Ca$^{2+}$ signaling response, it failed to completely eliminate it, even at the lower laser power setting (Fig. 3). This may be due to the inability of the ascorbate to sufficiently scavenge all the ROS. Alternatively, the residual Ca$^{2+}$ signaling may represent a resting basal level of response.

The present study examined whether light-activated Ca$^{2+}$ signaling was associated with subsequent cell death. The percentage viability of cells exhibiting different modes of Ca$^{2+}$ response during the 1-h imaging protocol is plotted in Fig. 6. At 15 µW laser power, 14% of cells exhibited Ca$^{2+}$ oscillations and of these, 100% remained viable after a further 24 h in culture (Fig. 6). Thus ROS-induced Ca$^{2+}$ oscillations are not a prerequisite to cell death, and therefore there is no evidence to suggest that they form part of the same signaling cascade. This agrees with previous studies in which low levels of H$_2$O$_2$ generated by the interaction of glucose oxidase and glucose triggered similar subtoxic Ca$^{2+}$ oscillation, whereas higher levels of H$_2$O$_2$ induced cell death (32). In addition, it has been suggested that changes in intracellular Ca$^{2+}$ may be cytoprotective and thereby reduce cellular toxicity (2, 19, 24). Figure 6 further indicates that cells exhibiting between 1 and 3 Ca$^{2+}$ transients during the 1-h imaging protocol had a significantly lower viability than cells that either exhibited Ca$^{2+}$ oscillations or maintained a constant basal level of intracellular Ca$^{2+}$. This trend was observed at both laser power settings.

Spontaneous single Ca$^{2+}$ transients have previously been reported in chondrocytes both in monolayer culture (9, 33) and in three-dimensional agarose constructs, as used in the present study (12, 25). However, because these previous studies also used fluorescent microscopy techniques to quantify Ca$^{2+}$ signaling, it is unclear the extent to which light may have influenced the results. Ca$^{2+}$ oscillations, such as those observed in the present study with a mean periodicity of ~300 s, have not been reported in unstimulated chondrocytes. Although preliminary studies have also found similar light-induced Ca$^{2+}$ signaling using chondrocytes in intact tissue or cultured in monolayer (data not shown), it remains to be demonstrated whether this response is exhibited in other cell types. The response of different cell types is likely to be influenced by their ability to scavenge or enzymatically degrade ROS, as well as their inherent ROS sensitivity. Because the Ca$^{2+}$ response appears to be mediated by low levels of H$_2$O$_2$, it is possible that the effect will not be triggered by microscope configurations in which cells are exposed to greater light intensities. However, in these systems, there is likely to be greater loss of cell viability.

The present study has demonstrated that confocal laser scanning microscopy using standard fluorochromes incorporated in isolated chondrocytes initiates two important cellular events. First, there is a rapid onset of intracellular Ca$^{2+}$ signaling in the form of spontaneous Ca$^{2+}$ transients and oscillations. Second, there is a significant loss of cell viability observed 24 h after the initial confocal imaging period. Both these events are mediated by light-induced release of oxygen free radicals or ROS. This study therefore demonstrates for the first time that light-induced Ca$^{2+}$ signaling may represent a significant and hitherto overlooked artifact in long-term fluorescent microscopy studies of viable cells. Consequently, it may be necessary to develop a new generation of fluorochromes and microscopy techniques that limit the release of specific ROS.

REFERENCES