Internalization of Near-Infrared Fluorescent Dyes within Isolated Leukocyte Populations

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Abstract: Scientists have expressed continued interest in the development of microsensor technology that can indicate toxicity that occurs within a cell after a chemical challenge. One of the more useful approaches of microsensor technology is the application of fluorescent spectroscopy to indicate early-stage injury with optimal specificity and decreased background interference. If the toxicity is detected during the early onset period of injury, then the probability for therapeutic recovery is promising. There has been increasing interest in the use of infrared (IR) and near infrared (NIR) dyes as biological microsensors due to their fluorescent spectral characteristics. Three of the most essential characteristics are the ability to minimize background interference by extraneous biological matrices, to exhibit optimal molar absorptivity and quantum yields, to maintain chronic cellular homeostasis. Therefore, the present study was developed to determine if selected NIR dyes would distribute within isolated neutrophil populations without altering normal cellular homeostasis using fluorescent wavelength analysis. The results demonstrate that selected NIR dyes undergo internalization within target cell populations while maintaining cell viability and homeostasis. In addition, these dyes demonstrate changes in absorbance and fluorescence analysis after the immune cells were challenged with a stimulant. Moreover, critical cellular functions, such as degranulation and superoxide production were not compromised by the internalization of the NIR dyes. These data suggest that selected NIR dyes can undergo intracellular uptake within neutrophil cultures without altering the normal functional capabilities of the targeted cell population.

Keywords: Fluorescent, Dyes, Microsensor, Neutrophils, Near-Infrared, Cellular uptake

Introduction

The degree of environmental contamination has increased in recent years. Exposure to these contaminants could result in severe changes in normal cellular activity. Accordingly, there is a need for innovative approaches that will indicate the occurrence of these cellular changes. One such approach is the development of fluorescent microsensors that can detect intracellular changes after internalization. Fluorescence is an energy phenomenon that involves the photoemission from excited molecules as their excited electrons return to their ground states [1, 2]. When light is absorbed by a photoluminescent molecule, electrons in the molecule are excited from the lowest energy level, or ground state, to one of the excited energy levels. When the electron returns to the ground state, the absorbed energy is either emitted through a nonradiative process or a radiative one. If the process is nonradiative, the energy is either transferred to another molecule or it is released as heat. If the process is radiative, energy is released as a photon with a wavelength characteristic of the emitted energy [1, 3]. Because of the nature of its photon emission, fluorescence is highly sensitive to protein structures and energy transfer to proteins. Thus the present study designed to determine if the selected NIR dyes can undergo intracellular uptake within neutrophil cultures without altering the normal functional capabilities of the cells. Neutrophils were chosen as the *in vitro* cell model because they are an essential component of the body's innate immune response. They are a part of the first line of defense against antigenic invaders and they

interact with many toxic substances or chemicals that may be distributed by the circulatory system to various tissues. If neutrophil activity is altered by any foreign invader, it would be beneficial to have the capability to monitor/indicate the development of cellular toxicity so that corrective therapy could be initiated.

Materials and Methods

Isolation of Neutrophils

Neutrophils were isolated from male, Sprague-Dawley, retired breeder rats (Harlan Laboratories, Fredrick, MD) by glycogen elicitation [5, 6]. Thirty mL of 1% glycogen (sterile saline vehicle) were injected into the peritoneum of anesthetized rats. After a 4-hour period, the rats were anesthetized with diethyl ether and decapitated. Neutrophils were isolated via a thirty-mL rinse of the peritoneum with phosphate-buffered saline (PBS) containing 1 unit heparin/mL. The neutrophil-containing solution was then filtered through gauze and spun at room temperature for 7 minutes at 500 x g. Red blood cells contaminating the cell preparation were lysed with NH4Cl solution [5]. Next, the isolated neutrophils were washed twice with PBS, resuspended in Williams' Medium-E (SigmaAldrich, St. Louis, MO) containing gentamycin and cultured in six-well plates at a density of 1×10^7 cells/well. Purity of neutrophil preparations was greater than 95%, and neutrophil viability was greater than 98% (lactate dehydrogenase release and trypan blue exclusion) [5]. The isolated neutrophils were used as the cell population for analyzing dye uptake.

Preparation of Fluorescent Dyes

Calcein, fluorscein, Fluorescent dye 780 phosphonate and 786 phosphonate are commercial dyes that were purchased from (SigmaAldrich, St. Louis, MO). Calcein and fluorscein were used as control dyes for cellular uptake, while dye 780 and 786 were the test dyes for cellular uptake. The crystalline form of the dye was dissolved in .05% DMSO and 99.95% William's Medium E. The dyes were subjected to several centrifugal elutriations in which the supernatant was retained and the pelleted debris was removed. The remaining supernatant was filtered through a Biopore filter sieve (SigmaAldrich, St. Louis, MO). The filtrate was collected, incubated at 40^oC for 1 hour and then refrigerated at 4^oC.

Administration of Fluorescent Dyes and Stimulant to Isolated Neutrophils

Neutrophils were isolated and cultured (Williams' Medium-E containing gentamycin and supplements) in six-well plates at a density of 1×10^7 cells/well. Calcein, fluoroscene, dye 780 or dye 786 was added to cultures at a final concentration of 20 μg/1×10^7 cells and incubated for 24 hours. Previous temporal/response studies conducted in the laboratory indicated that optimal internalization of the dyes required a 24 hours. After this 24 hour incubation, the culture medium was collected, sonicated and centrifuged at room temperature for 15 minutes at 120 x g to remove cellular debris, then

refrigerated at -20^{0}C. This medium was then analyzed for fluorescent dye content. Next, neutrophils were removed from the wells by .02% trypsin, washed and re-suspended in PBS, then analyzed for fluorescent dye content and neutrophil function. This was the initial phase of the isolated neutrophil treatments. The second phase of the treatments involved the administration of a stimulant to the fluorescent dye-treated cultures. For the second phase, neutrophils were cultured (1×10^7 cells/well) in Falcon 3046, six-well culture plates. The stimulant (FMLP; f-Met-Leu-Phe; 1 μM/well) was administered to the neutrophil cultures 12 hours after treatment with the fluorescent dyes and incubated for the remaining 12 hours. After this incubation time, the medium was collected, centrifuged at room temperature for 15 minutes at 120 x g to remove cellular debris, and then refrigerated at -20°C. The conditioned medium was analyzed for lactate dehydrogenase (LDH) activity as a marker of cell toxicity. This medium was also analyzed for fluorescent dye content as a marker of non-internalization. In addition, treated neutrophils were collected from the wells by .02% trypsin, washed and re-suspended in PBS, then analyzed for fluorescent dye content and neutrophil function. Trypan blue exclusion/eosin Y uptakes were used as indicators of cellular toxicity and viability, respectively. Treatment of cells with triton detergent was used as a positive lethality control for trypan blue/eosin y analysis

Measurement of Intracellular Absorptivity

The concentration detection limits for the dyes were determined using a Cary Eclipse Fluorometer (Varian Instruments, Palo Alto, CA) equipped with a red sensitive R928S photomultiplier tube (PMT). From the detection limit concentrations, an optimal concentration range centered near 7.5×10^{-8} mol was adopted for this study. The cells were inoculated in 10^{-7} molar dye solution (< 2% EtOH, DMSO). In addition, readings were performed using a Cary 100 Spectrophotometer (Varian Instruments, Palo Alto, CA). Samples were transferred by Pasteur pipette to standardized 3.4 mL disposable polystyrene fluorescence cuvets (Fisher Scientific, Suwanee, GA).

Measurement of Intracellular Fluorescence

After the treatment and incubation period, separated cell solutions and medium solutions, were analyzed (triplicate) for absorbance and fluorescence. Absorbance scans (600-900 nm) were performed on both cell and medium solutions to quantitatively identify the presence of dye and possible interferents. Dyes were identified by their absorbance maximum wavelengths and their concentration in each sample was determined by their molar absorptivities. The absorbance maximum wavelengths were used as the excitation wavelengths in the subsequent fluorescence analysis. Maximum emission wavelengths were determined from the emission spectra obtained from scans performed on the initial sample in a triplicate set of samples. The fluorescence intensity for the remaining samples was obtained as single reads from the previously determined maximum emission wavelengths.

Measurement of Neutrophil Degranulation

Neutrophil degranulation was measured according to the extent of release of the granule-derived enzyme, myeloperoxidase (MPO). Neutrophils were incubated at a concentration of 10^7/ml in Hanks' balanced salt solution (HBSS) for 10 min at 37°C. The stimulant, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; 1 μM), a synthetic chemotactic tripeptide, in combination with a submaximal concentration of cytochalasin B (CB; 1 μM) was then added to the cells, which were incubated for 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at $400 \times g$ for 5 min to pellet the cells [5]. The neutrophil-free supernatants were then decanted and assayed for MPO activity by micromodifications of standard colorimetric procedures [5]. Briefly, in the case of MPO, neutrophil supernatants (20 μl) were added to guaiacol and H_2O_2 (final concentrations of 10 and 5 mM, respectively) in a final reaction volume of 200 μl, and enzyme activity was monitored spectrophotometrically at 450 nm.

Measurement of Superoxide Release by Neutrophils

Neutrophil-derived superoxide production after treatment with fluorescent dyes was measured by reduction of cytochrome c (100, μM; horse heart, type III, SigmaAldrich, St. Louis, Mo), followed by monitoring the change spectrophotometrically (absorbance - 550 nm). The rapid reduction of cytochrome C by neutrophil-derived superoxide was calculated as nmol of superoxide formed via extinction coefficient of 21×10^3 M-1 cm-1 but expressed as percentage positive control (FMLP; f-Met-Leu-Phe). The production of superoxide in response to FMLP, with/without the internalization of the fluorescent dyes, was completed after a 25 minute incubation and ended via superoxide dismutase (30 units/ml; SigmaAldrich, St. Louis, Mo) administration [10].

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Data were analyzed by analysis of variance (ANOVA), and individual means were compared using Dunnett's test. Appropriate transformations were performed on data for which variances were not homogeneous. For each treatment group, cell populations were prepared from different animals and different cell isolations, respectively; *N* = 8 for all studies unless otherwise indicated. The criterion for significance was $p \leq 0.05$ [5].

Results

Internalization of VIS Microsensor Dyes by Neutrophils

Applying the Beers-Lambert law, the molar absorptivity of the VIS microsensor dyes can be used to determine the concentration of dyes associated with neutrophil uptake. The relationship between the absorbance and concentration is given by (1), where a is the path length in centimeters, c is the concentration

expressed as mols/mL, ε is the molar absorptivity, and abs is the absorbance.

$$ac\varepsilon = abs \tag{1}$$

Equation 1 can be rearranged and the concentration of dyes can be calculated using the measured absorbance values associated with dye uptake by the neutrophil population. The determined concentration of the dyes indicated no significant difference in the uptake of calcein and fluorescein in non-stimulated cells. For the stimulated cells, a moderate increase was observed for fluorescein uptake when compared to calcein uptake (Figure 1).
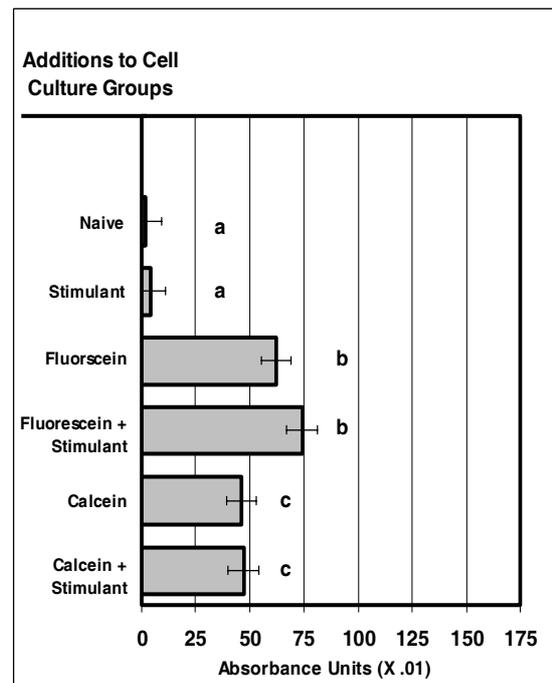
Figure 1: VIS micro-sensor dyes are internalized neutrophils. Neutrophil cultures were treated with either vehicle or visible-spectra micro-sensor dyes (fluorescein or calcein; 20 μg/10^6 cells) and incubated for 24 hours. After this incubation period, neutrophil cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, neutrophil cultures were analyzed for VIS micro-sensor dye uptake. Spectral absorptivity was used as the endpoint for indicating cellular uptake. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a), (b), and (c) were expressed as $P \leq 0.05$ in comparison to each other.

Internalization of NIR Microsensor Dyes by Neutrophils

When comparing the neutrophil uptake of near-infrared dyes to that of the visible dyes, the uptake of near-infrared dyes was comparable or greater to that of the visible control dyes, fluorescein and calcein (Figure 2). The uptake of NIR-spectra microsensor dyes was as much as 3-fold greater than that of visible-spectra microsensor dyes. While the uptake of dye 780 and dye 786 were of similar magnitude for non-stimulated neutrophils, dye 786 demonstrated significantly more uptake than dye 780 for the stimulated group of neutrophils.
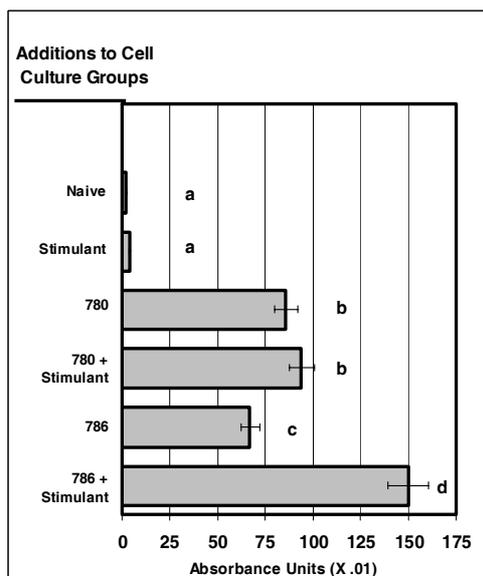
Figure 2: NIR micro-sensor dyes are internalized neutrophils. Neutrophil cultures were treated with either vehicle or NIR-spectra micro-sensor dyes (NIR-786 or NIR-780; 20 µg/10^6 cells, respectively) and incubated for 24 hours. After this incubation period, neutrophil cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, neutrophil cultures were analyzed for micro-sensor dye uptake. Spectral absorptivity was used as the endpoint for indicating cellular uptake. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a), (b), (c), and (d) were expressed as P $\leq$ 0.05 in comparison to each other.

IntracellularFfluorescence of VIS Microsensor Dyes after Internalization by Neutrophils
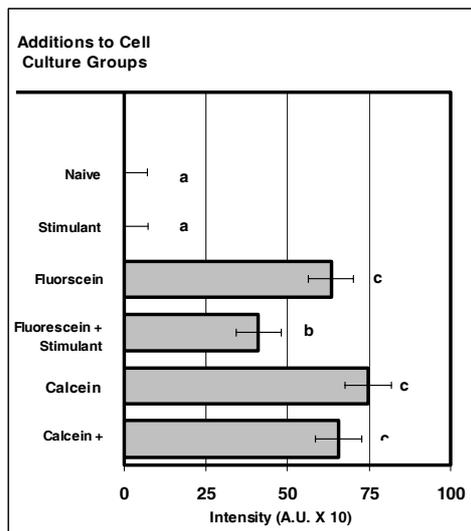
Figure 3: VIS micro-sensor dyes exhibit intracellular fluorescence after internalization by neutrophils. Neutrophil cultures were treated with either vehicle or VIS micro-sensor dyes (fluorescein or calcein; 20 µg/10^6 cells), and incubated for 24 hours. After this incubation period, the cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, intracellular fluorescence of the VIS micro-sensor dyes was analyzed. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a), (b), and (c) were expressed as P $\leq$ 0.05 in comparison to each other.

The fluorescence exhibited by calcein after its internalization by neutrophils was notable (Figure 3). There was no significant difference between the fluorescence associated with calcein for non-stimulated and stimulated neutrophils (Figure 3). The fluorescence intensity of fluorescein demonstrated significant difference between non-stimulated and stimulated neutrophils (Figure 3).

Intracellular Fluorescence of NIR Microsensor Dyes after Internalization by Neutrophils

In comparison to control groups, a significant degree of fluorescence was generated by visible and NIR microsensor dyes. The fluorescence intensity displayed by the two microsensor dye groups was comparable (Figure 4). No significant difference between stimulated and non-stimulated cells was observed for the fluorescence intensity of either IR780 or IR786. The fluorescence intensity of IR786 was greater than the fluorescence intensity determined for IR780 in both cell populations.
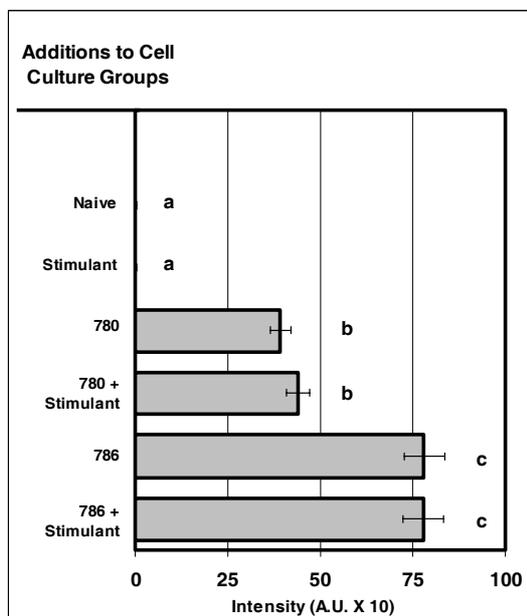
Figure 4: NIR micro-sensor dyes exhibit intracellular fluorescence after internalization by neutrophils. Neutrophil cultures were treated with either vehicle or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 µg/10^6 cells) and incubated for 24 hours. After this incubation period, the cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, intracellular fluorescence of the micro-sensor dyes was analyzed. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a), (b), and (c) were expressed as P $\leq$ 0.05 in comparison to each other.

NEUTROPHIL Degranulation after Internalization NIR Microsensor Dyes

Internalization of NIR microsensor dyes did not alter neutrophil degranulation in response to stimuli (Figures 5 and 6). The neutrophils containing the microsensor dyes demonstrated a very moderate degree of degranulation similar to that of neutrophils void of the microsensor-dyes.
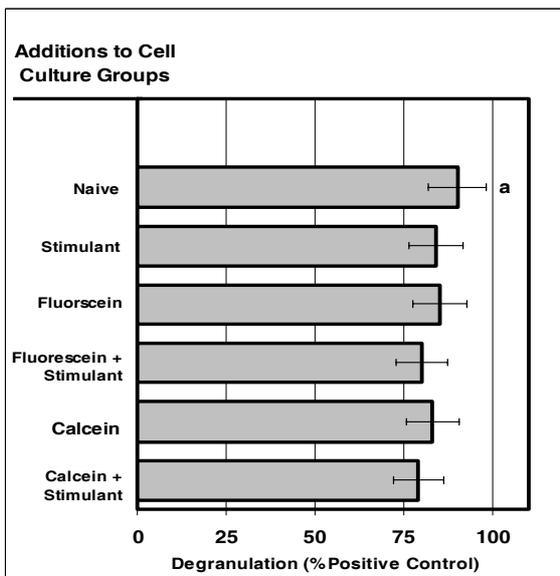
Figure 5: Internalization of VIS micro-sensor dyes did not alter neutrophil degranulation in response to stimuli. Neutrophil cultures were treated with either vehicle or visible micro-sensor dyes (fluorescein or calcein; 20 $\mu g/10^6$ cells) and incubated for 24 hours. After this incubation period, neutrophil cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, neutrophil degranulation was analyzed using myeloperoxidase assays. Degranulation was used as a marker of normal NEUTROPHIL function. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a) was expressed as P $\leq$ 0.05.
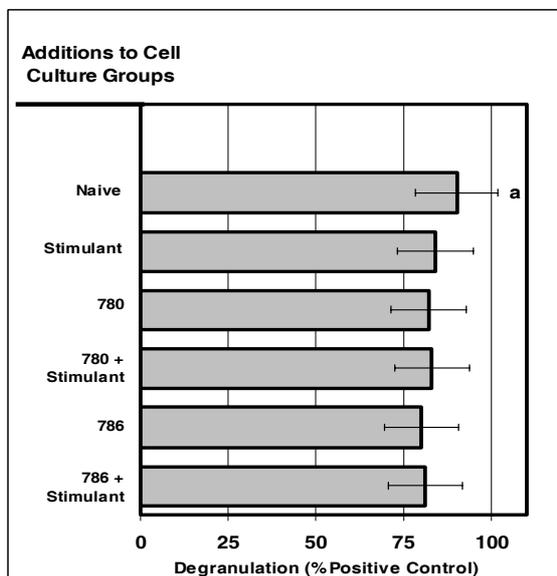
Figure 6: Internalization of NIR micro-sensor dyes did not alter neutrophil degranulation in response to stimuli. Neutrophil cultures were treated with either vehicle or NIR-spectra micro-sensor dyes (NIR-786 or NIR-780; 20 $\mu g/10^6$ cells) and incubated for 24 hours. After this incubation period, the cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, neutrophil degranulation was analyzed using myeloperoxidase assays. Degranulation was used as a marker of normal neutrophil function. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a) was expressed as P $\leq$ 0.05.

The Effect of VIS and NIR Dyes on Neutrophil Viability

The internalization of VIS or NIR microsensor dyes did not alter normal cell viability (Figure 7). The neutrophils containing the microsensor dyes did not exhibit any degree of cytotoxicity as measured through the release of LDH (marker of cytotoxicity) nor did cellular lethality occur as indicated by trypan blue exclusion and eosin y uptake (markers of cell death).
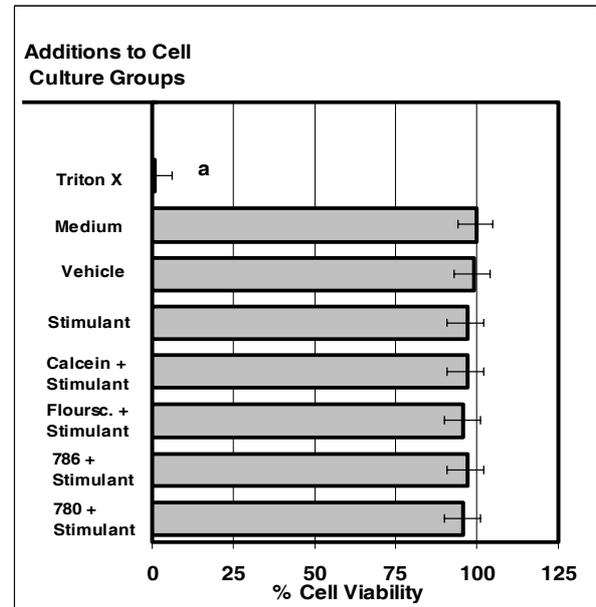
Figure 7: The internalization of VIS or NIR micro-sensor dyes did not alter normal cell viability. Neutrophil cultures were treated with either vehicle, visible micro-sensor dyes (fluorescein or calcein; 20 $\mu g/10^6$ cells), or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 $\mu g/10^6$ cells) and incubated for 24 hours. After this incubation period, neutrophil cultures were stimulated with LPS for 24 hours. After this second incubation period, neutrophil cultures were analyzed for viability. Trypan blue exclusion and eosin Y uptake were used as endpoints for the determination of lethality. Percent values are expressed as the mean $\pm$ SEM, N = 8. Statistical significance (a) was expressed as P $\leq$ 0.05 in comparison to other groups.

The Effect of Internalized Dyes on Neutrophil Super Oxide Production

The internalization of VIS or NIR microsensor dyes did not alter neutrophil-induced super oxide production in response to stimuli (Figure 8). Neutrophils that had internalized the microsensor dyes produced super oxide anion at levels similar to those of control neutrophils.

Discussion

The current study has demonstrated that the internalization of NIR micro-sensor dyes (NIR micro-sensor dyes 780 and 786) by isolated neutrophils did not result in a cellular lethality. In addition, this internalization did not alter the normal responses of the neutrophils to stimuli. Moreover, once intracellular distribution of the microsensor dyes has occurred, the spectral property of fluorescence was easily measured.

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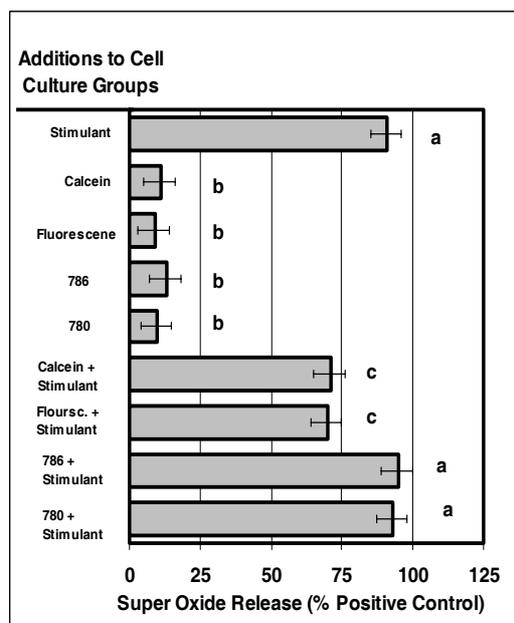
Figure 8: The internalization of VIS or NIR micro-sensor dyes did not alter neutrophil super oxide production in response to stimuli. Neutrophil cultures were treated with either vehicle, VIS micro-sensor dyes (fluorescein or calcein; 20 µg/10^6 cells), or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 µg/10^6 cells) and incubated for 24 hours. After this incubation period, the cultures were stimulated with LPS for 24 hours. After the 24-hour stimulation period, the ability of neutrophils to produce super oxide was analyzed. Super oxide production was also used as a marker of normal neutrophil function. Percent values are expressed as the mean + SEM, N = 8. Statistical significance (a), (b), and (c) were expressed as P < 0.05 in comparison to each other.

These observations have demonstrated that the NIR micro-sensor dyes are not harmful once internalization within neutrophil populations has occurred. Moreover, it is conceivable that the aforementioned NIR micro-sensor dyes could be modified to detect or monitor changes/alterations of intracellular activity without adversely effecting cellular homeostasis. The use of fluorescence and fluorescent constructs as indicators of intra- and extra-cellular activity is a widely used application [7, 8, 11, 12]. The parameter of concern regarding these applications is the ability of the constructs to distribute into the intracellular environment without compromising normal cellular homeostasis. Studies conducted by Fehr [7] have demonstrated the use of fluorescent nanosensors to monitor transport of maltose inside yeast cell populations. Another study conducted by Sato [8] used fluorescent sensors constructs (phocuses) to indicate the degree of phosphorylation within living cell populations. These studies have provided very intriguing insights into the development and use of fluorescent sensors to monitor intracellular events with minimal cytotoxicity. One concern, regarding the construction and application of many fluorescent nanosensors is the difficulty in constructing sensors that demonstrate fluorescence or increased fluorescent intensity for specific intracellular events only, and are not committed to a constant/continuous fluorescent state [12, 13]. In an attempt to address this concern, a study conducted by Heo [9] was able to construct a fluorescent dye (BCECF-AM) that exhibits extra-cellular non-fluorescence, until translocation across the cellular membrane. Once this intracellular translocation occurs, the BCECF-AM is converted into a fluorescent form (BCECF) for the analysis of intracellular activity. These developments demonstrate promise for the continued development and application of micro sensors technology to detect intracellular changes after exposures to toxic agents.

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