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Photobiomodulation Directly Benefits Primary Neurons Functionally Inactivated by Toxins

ROLE OF CYTOCHROME *c* OXIDASE*

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Far red and near infrared (NIR) light promotes wound healing, but the mechanism is poorly understood. Our previous studies using 670 nm light-emitting diode (LED) arrays suggest that cytochrome *c* oxidase, a photoacceptor in the NIR range, plays an important role in therapeutic photobiomodulation. If this is true, then an irreversible inhibitor of cytochrome *c* oxidase, potassium cyanide (KCN), should compete with LED and reduce its beneficial effects. This hypothesis was tested on primary cultured neurons. LED treatment partially restored enzyme activity blocked by 10–100 μM KCN. It significantly reduced neuronal cell death induced by 300 μM KCN from 83.6 to 43.5%. However, at 1–100 mM KCN, the protective effects of LED decreased, and neuronal deaths increased. LED significantly restored neuronal ATP content only at 10 μM KCN but not at higher concentrations of KCN tested. Pretreatment with LED enhanced efficacy of LED during exposure to 10 or 100 μM KCN but did not restore enzyme activity to control levels. In contrast, LED was able to completely reverse the detrimental effect of tetrodotoxin, which only *indirectly* down-regulated enzyme levels. Among the wavelengths tested (670, 728, 770, 830, and 880 nm), the most effective ones (830 nm, 670 nm) paralleled the NIR absorption spectrum of oxidized cytochrome *c* oxidase, whereas the least effective wavelength, 728 nm, did not. The results are consistent with our hypothesis that the mechanism of photobiomodulation involves the up-regulation of cytochrome *c* oxidase, leading to increased energy metabolism in neurons functionally inactivated by toxins.

Near infrared (NIR)[1] light has been used in therapeutic devices for the treatment of a variety of injuries, especially infected, ischemic, and hypoxic wounds (1–4). NIR light penetrates more deeply than UV or visible light and is benign to living tissue. This presents clear clinical advantages to treatment within a tissue transparency window of 650–1000 nm. Most of the devices utilize lasers as the light source. Recently, however, light-emitting diodes (LEDs) have been found to be more beneficial than lasers in several respects (3, 5). LEDs can be constructed to form relatively large arrays to treat wounds that commonly involve areas much larger than the size of a laser beam. Unlike lasers, there is virtually no heat generated by the LED array and therefore no potential thermal injury to individuals being treated. LED is well tolerated by biological tissues and has no known detrimental effect. As a therapeutic device, LED has achieved FDA non-significant risk status. Moreover, LED units are more compact, portable, and affordable than lasers.

The cellular mechanisms of action of NIR in wound healing are not well understood. The basic premise is that long wavelength lights stimulate cellular energy metabolism and energy production. Three major photoacceptor molecules in mammalian tissues are known to absorb light in the NIR range: hemoglobin, myoglobin, and cytochrome *c* oxidase. Of the three, only cytochrome *c* oxidase (EC 1.9.3.1) has been associated with energy production. In our recent study, the first two candidates were excluded in our primary neuronal cultures in which more than 95% of the cells were neurons, and there were no blood elements or muscle cells (6). In the presence of a voltage-dependent sodium channel blocker, tetrodotoxin (TTX), which impedes neuronal impulse activity, decreases ATP demand, and down-regulates cytochrome *c* oxidase activity, LED at 670 nm was able to reverse the detrimental effect of TTX by bringing cytochrome *c* oxidase back to control levels. Moreover, LED treatment up-regulated enzyme activity of normal neurons above control levels (6).

The beneficial effect of NIR was further confirmed in our recent *in vivo* study. Rats intoxicated with methanol developed retinal dysfunction attributable to the inhibition of cytochrome *c* oxidase by formic acid, the toxic metabolite in methanol intoxication. Three brief LED treatments significantly improved retinal function as measured by the electroretinographic response and protected the retina from histopathological changes induced by methanol-derived formate (7). Thus, photobiomodulation is therapeutic in restoring visual functions after reversible inhibition of cytochrome *c* oxidase by formate.

Our *in vitro* and *in vivo* studies thus far suggest that cytochrome *c* oxidase plays an important role in the therapeutic process of photobiomodulation. A comparison of the action spec-

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[1] The abbreviations used are: NIR, near infrared; LED, light-emitting diode; TTX, tetrodotoxin; KCN, potassium cyanide; NaN_3, sodium azide.

trum for cellular proliferation following laser photoirradiation with the absorption spectra of potential photoacceptors led Karu (8) to suggest that cytochrome *c* oxidase is a primary photoacceptor of light in the far red to near infrared region. Britton Chance's group postulated that ~50% of near infrared light is absorbed by mitochondrial chromophores such as cytochrome *c* oxidase (9). To further investigate this question, the present study tested the following hypotheses: 1) cytochrome *c* oxidase is directly involved in the photobiomodulation reaction; therefore, a potent and irreversible inhibitor of cytochrome *c* oxidase such as potassium cyanide (KCN) will compete with and lessen the beneficial effect of LED. 2) LED treatment stimulates cytochrome *c* oxidase activity in neurons; therefore, LED pretreatment will further enhance the partial protective action of LED during exposure to KCN. 3) By increasing cytochrome *c* oxidase activity and cellular energetics, LED will reduce neuronal cell death caused by the cytotoxin KCN. Finally, 4) the effective action spectrum of LED in reversing the detrimental effect of the impulse blocker tetrodotoxin on cytochrome *c* oxidase activity should correspond to the NIR absorption spectrum of cytochrome *c* oxidase. Various wavelengths of LED were tested to see if specific ones were more beneficial to functionally inactivated neurons. Wavelengths were administered either singly or in combination to determine the optimal conditions for treatment. Primary neuronal cultures were again our model system because (*a*) primary neurons are more physiological than cell lines, (*b*) neurons are highly oxidative, and (*c*) experimental conditions can be manipulated to affect neurons without complications from other cell types.

EXPERIMENTAL PROCEDURES

Materials

LED arrays (25 × 10 cm) with peak wavelengths at 670, 728, 770, 830, or 880 nm were obtained from Quantum Devices, Inc. (Barnaveld, WI). ATP releasing reagent, bioluminescent somatic cell assay kit, 3,3′-diaminobenzidine tetrahydrochloride, equine heart cytochrome *c*, luciferase ATP assay mix, and tetrodotoxin were from Sigma. Potassium cyanide was from Fisher. All other chemicals were reagent grade or better.

Primary Neuronal Cultures

The general protocol for culturing neurons from postnatal rat visual cortex was as described previously (10, 11). Briefly, a glial feeder layer from postnatal 1–2-day-old rat cortex was cultured in 60-mm dishes. After 2 weeks, when this layer was subconfluent visual cortical neurons from postnatal 1–3-day-old rats were cultured on coverslips coated with poly-L-lysine. Cytosine arabinoside was added 1 day after plating of neurons to inhibit the replication of non-neuronal cells. Neurons were co-cultured with glial cells but were physically separated from them by wax spheres.

KCN Inactivation of Cytochrome c Oxidase

Sister cultures at 5 days of age were exposed to 10 μM, 100 μM, 1 mM, 10 mM, or 100 mM KCN for 5 days. All cell studies were done in covered Petri dishes to avoid evaporation of hydrogen cyanide. As a corollary to the KCN experiments, another set of sister cultures were exposed to 10 μM, 100 μM, and 1 mM of sodium azide (NaN_3) for 5 days.

Impulse Blockade with Tetrodotoxin

TTX, a blocker of voltage-dependent sodium channels, was added to sister cultures at 5 days of age at a final concentration of 0.4 μM, and cultures were maintained in such media for 6 days. TTX was replenished twice a week. Controls had no TTX.

Light-emitting Diode Treatment

GaAlAs light emitting diode arrays of five different wavelengths were used: 670, 728, 770, 830, and 880 nm, each with a bandwidth of 25–30 nm at 50% power. The power intensity chosen was 50 milliwatt/cm^2 (unless otherwise indicated), and energy density was 4 joules/cm^2 when applied for 1 min and 20 s (6).

Experimental Design

KCN Experiments—Five sets of sister cultures, each exposed to one of five KCN concentrations, were treated with LED at 670 nm and energy density of 4 joules/cm^2 twice a day on each of the 5 days of exposure to KCN. One set of normal controls had neither KCN nor LED. A second set of controls had KCN but no LED.

Pretreatment Experiments—Four sets of sister cultures were pretreated with LED at 670 nm and energy density of 4 joules/cm^2 (= 1 min and 20 s exposure) once a day for 3 days or 5 days *before* being exposed to either 10 or 100 μM of KCN for 5 days, during which they received LED treatment as described above. Ten groups were tested: (i) normal controls, (ii) pretreatment with LED for 3 days followed by 10 μM of KCN plus LED, (iii) 10 μM of KCN plus LED without pretreatment, (iv) pretreatment with LED for 3 days followed by 100 μM of KCN plus LED, and (v) 100 μM of KCN plus LED without pretreatment. Groups (vi) to (x) were the same as the above, except that groups (vii) and (ix) each had 5 days of pretreatment.

Varying Single Wavelength Experiments—Five sets of sister cultures, each exposed to TTX for 6 days, were treated with one of the five wavelengths (670, 728, 770, 830, and 880 nm) at a power intensity of 50 milliwatt/cm^2 and energy density of 4 joules/cm^2 for 1 min and 20 s per day for the last 5 of the 6 days grown in the presence of TTX. One set of normal controls had neither TTX nor LED. A second set of controls had TTX but no LED.

Varying Multiple Wavelengths Experiments—To determine whether the sequence of wavelengths, power intensities, or number of treatments affect cytochrome *c* oxidase activity in TTX-intoxicated neurons as compared with single wavelength treatment, eight sets of sister cultures (*a*–*h*) were each exposed to TTX for 6 days and were treated with LED of specified sequences of wavelengths as follows:

(*a*), 670 nm → 728 nm → 830 nm; (*b*), 670 nm → 830 nm → 728 nm; (*c*), 728 nm → 670 nm → 830 nm; (*d*), 728 nm → 830 nm → 670 nm; (*e*), 830 nm → 670 nm → 728 nm; (*f*) 830 nm → 728 nm → 670 nm.

For groups (*a*)–(*f*), each wavelength had a power intensity of 50 milliwatt/cm^2 and energy density of 4 joules/cm^2. Each group was exposed to a sequence of three wavelengths as indicated once a day for the last five of the 6 days of exposure to TTX.

(*g*), 670 nm (50 milliwatt/cm^2) → 728 nm (50 milliwatt/cm^2) → 830 nm (50 milliwatt/cm^2) →; 670 nm (10 milliwatt/cm^2) → 728 nm (10 milliwatt/cm^2) → 830 nm (10 milliwatt/cm^2) →; 670 nm (10 milliwatt/cm^2) → 728 nm (10 milliwatt/cm^2)→ 830 nm (10 milliwatt/cm^2); (*h*), 670 nm (50 milliwatt/cm^2) → 728 nm (50 milliwatt/cm^2) → 880 nm (50 milliwatt/cm^2) →; 670 nm (10 milliwatt/cm^2) → 728 nm (10 milliwatt/cm^2) → 880 nm (10 milliwatt/cm^2) →; 670 nm (10 milliwatt/cm^2) → 728 nm (10 milliwatt/cm^2) → 880 nm (10 milliwatt/cm^2).

Groups *g* and *h* were each treated with a sequence of nine wavelengths at the indicated power intensities, each of which was exposed for a length of time corresponding to an energy density of 4 joules/cm^2. All nine treatments were performed in sequence on the same day, which was 5 days after TTX exposure, and cells were harvested 24 h after each of the last (9th) treatment. Control cultures were not exposed to TTX or to LED treatment.

Cytochrome c Oxidase Assays

Cytochrome *c* oxidase reactions were performed concurrently for control and experimental cultures in an incubation medium containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.03% cytochrome *c* in phosphate-buffered saline, pH 7.2 (6, 11, 12).

ATP Content Bioluminescence Assays

Sister cultures from all the test groups, except for the pretreatment groups, were analyzed for ATP content by means of the luciferin-luciferase luminometry, using a modification of the luminescence method of Strehler (13) and components of the bioluminescent somatic cell assay kit. Briefly, cultured cells were rinsed with cold phosphate-buffered saline, then incubated in cold somatic cell ATP releasing reagent for 5 min, and harvested from the coverslips by means of a cell scraper. They were then mixed with the luciferase ATP assay mix and assayed with a luminometer (LB 9506). Values were expressed as nanomolar ATP content per milligram of protein.

Propidium Iodide DNA Staining of Neurons Undergoing Cell Death

The ratio of normal to neurons undergoing cell death were assessed with propidium iodide staining in methanol-permeabilized cells according to the method of Cebers *et al.* (14). Experiments were repeated three times. The frequency of neurons with intensely fluorescent, condensed

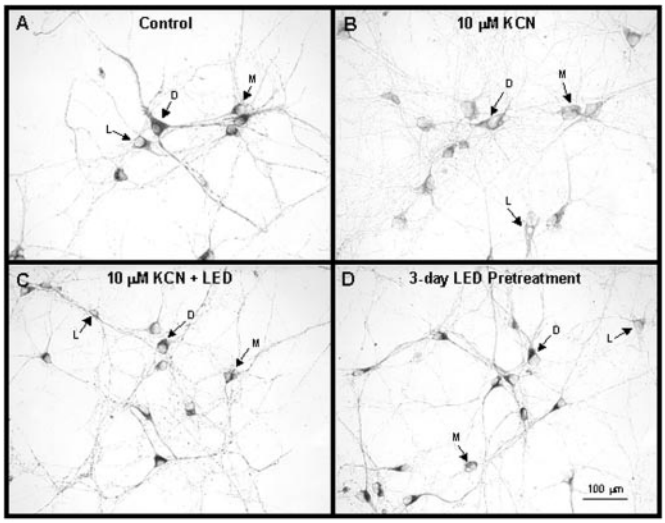


FIG. 1. **Primary visual cortical neurons in culture.** Neurons were broadly classified into darkly (*D*), moderately (*M*), and lightly (*L*) reactive categories for cytochrome *c* oxidase (*arrows*). *A*, normal control. *B*, neurons exposed to 10 μM KCN for 5 days. *C*, neurons were treated twice daily with 670 nm LED while being exposed to 10 μM of KCN for 5 days. *D*, neurons pretreated with 670 nm LED for 3 days before being exposed to 10 μM KCN for 5 days, during which LED treatment was continued twice daily.

chromatin (indicating cell death) was determined from the total number of cells counted in each of normal controls (n = 3,430), 100 μM KCN (n = 2,678), or 300 μM KCN (n = 3,825) exposure for 28 h and pretreatment with 670 nm LED (at 50 milliwatt/cm^2 and 30 joules/cm^2 for 10 min) before exposure to 28 h of 100 μM (n = 2,144) or 300 μM KCN (n = 2,572). A second inhibitor of cytochrome *c* oxidase, NaN_3, was also tested at concentrations of 100 μM, 300 μM, and 1 mM and with the same regimen of pretreatment.

Optical Densitometry and Statistical Analysis

To quantitatively analyze changes in cytochrome *c* oxidase activity following KCN or TTX exposure and LED treatment, optical densities of reaction product in individual cells from all of the above experiments were measured by means of a Zeiss Zonax MPM 03 photometer (Zeiss, Thornwood, NY) attached to a Zeiss compound microscope. Multiple, two micron spot size readings were taken from the cytoplasm of each cell. Between 150 and 300 cells were measured from each group (representing each bar of each histogram) using a ×25 objective lens. The background was subtracted by setting zero over a blank area (without cells) in each slide, and all lighting conditions, magnifications, and reference points were kept constant.

Two-tailed Student's t test for paired comparisons and analysis of variance for group comparisons was used to analyze differences between and among treated and untreated groups. Comparisons of each paradigm with every other paradigm within each group were also analyzed by the Tukey test. Values were expressed as mean ± S.E. A probability of 0.05 or less was considered significant.

RESULTS

KCN Experiments—As described previously (6, 11), cultured neurons were divided into three broad metabolic types: dark, moderate, and lightly reactive for cytochrome *c* oxidase. The goal was to determine whether different metabolic types would respond differently to toxins and LED. After KCN intoxication, the activity of cytochrome *c* oxidase was reduced in all cells, and reaction product in dark, moderate, and lightly reactive neurons all shifted toward lower optical densitometric values.

As shown in Figs. 1, *A* and *B*, and 2*A*, KCN treatment resulted in a significant reduction in cytochrome *c* oxidase activity of primary neurons even at the 10 μM concentration ($p < 0.001$). Increasing concentrations of KCN from 10 μM to 100 mM caused progressively greater decline in enzyme activity and neuronal viability.

LED treatment at 670 nm (energy density of 4 joules/cm^2 when exposed for 1 min and 20 s) twice a day for each of the 5 days of exposure to lower concentrations of KCN (10–100 μM) significantly reversed the detrimental effect of the toxin on cytochrome *c* oxidase activity ($p < 0.001$ for all three metabolic neuronal types). However, these reversals were complete only for the lightly reactive neurons but less than complete for the dark and moderately reactive neurons ($p < 0.05$ to $p < 0.001$, respectively, as compared with controls) (Figs. 1*C* and 2*A*). At higher concentrations of KCN (1–100 mM), increasing neuronal death and decreasing benefits from the LED were observed for all three metabolic cell types (Fig. 2*A*). Results of 100 mM KCN were not plotted because most of the cells did not survive at that concentration.

Sister cultures exposed to NaN_3 at various concentrations (10 μM, 100 μM, and 1 mM) resulted in progressively greater reduction of cytochrome *c* oxidase activity (data not shown). In comparison to KCN, a higher concentration of NaN_3 was needed to achieve the same level of inhibition. LED treatment partially reversed the inhibition but not to control levels (data not shown), again comparable to the findings with KCN.

Cellular ATP concentrations showed trends similar to those of cytochrome *c* oxidase in response to KCN or LED (Fig. 2*B*). However, LED treatment was able to reverse the effect of only the lowest concentration of KCN tested (10 μM) but not at concentrations of 100 μM or higher. The ATP content in cells subjected to 10 mM KCN was below the detection limit of the ATP assay, and LED treatment had little or no effect. Again, too few cells survived at 100 mM KCN for accurate determination of ATP content.

Pretreatment Experiments—Pretreatment with LED at 670 nm (energy density of 4 joules/cm^2, equivalent to 1 min and 20 s exposure), once a day for 3 days provided additional protection for visual cortical neurons that received LED treatment (twice a day) during their 5-day exposure to 10 or 100 μM of KCN (Figs. 1*D* and 3*A*). It significantly improved the level of cytochrome *c* oxidase activity in darkly and moderately reactive metabolic cell types above those provided by LED treatment *during* KCN exposure ($p < 0.01$ to $p < 0.001$). However, the values were still significantly below those of normal controls ($p < 0.001$ for all). Pretreatment did not appear to have additional benefit for the lightly reactive metabolic group. Higher concentrations of KCN were not tested.

Pretreatment with the same paradigm once a day for 5 days further increased cytochrome *c* oxidase activity in neurons that received LED *during* exposure to 10 μM or 100 μM of KCN ($p < 0.01$ to $p < 0.001$) (Fig. 3*B*). However, their values were still significantly lower than those of normal controls for each of the metabolic cell types ($p < 0.01$ to $p < 0.001$). Again, pretreatment did not provide further benefit for the lightly reactive cell group that received LED during exposure to 100 μM KCN.

Propidium Iodide DNA Staining of Neurons Undergoing Cell Death—The extent of cell death in various cultures and the effect of LED were evaluated with propidium iodide staining. Nuclei of intact cells had typical dispersed chromatin morphology, whereas neurons undergoing cell death had condensed, small, and intensely fluorescent nuclei with chromatin aggregates. As shown in Fig. 4*A*, control cultures had very few apoptotic nuclei (4.75% of total cell population). This number remained low at 100 μM KCN (9.3%) and was not different from that of pretreatment with 10 min of LED (9.28%) (data not shown). Higher concentrations of KCN resulted in increasing proportions of neurons with condensed chromatin. Cultures subjected to 300 μM KCN for 28 h showed a profusion of neurons with highly condensed chromatin (83.6%) (Fig. 4*B*). Significantly, 10 min of LED pretreatment at 670 nm (equivalent

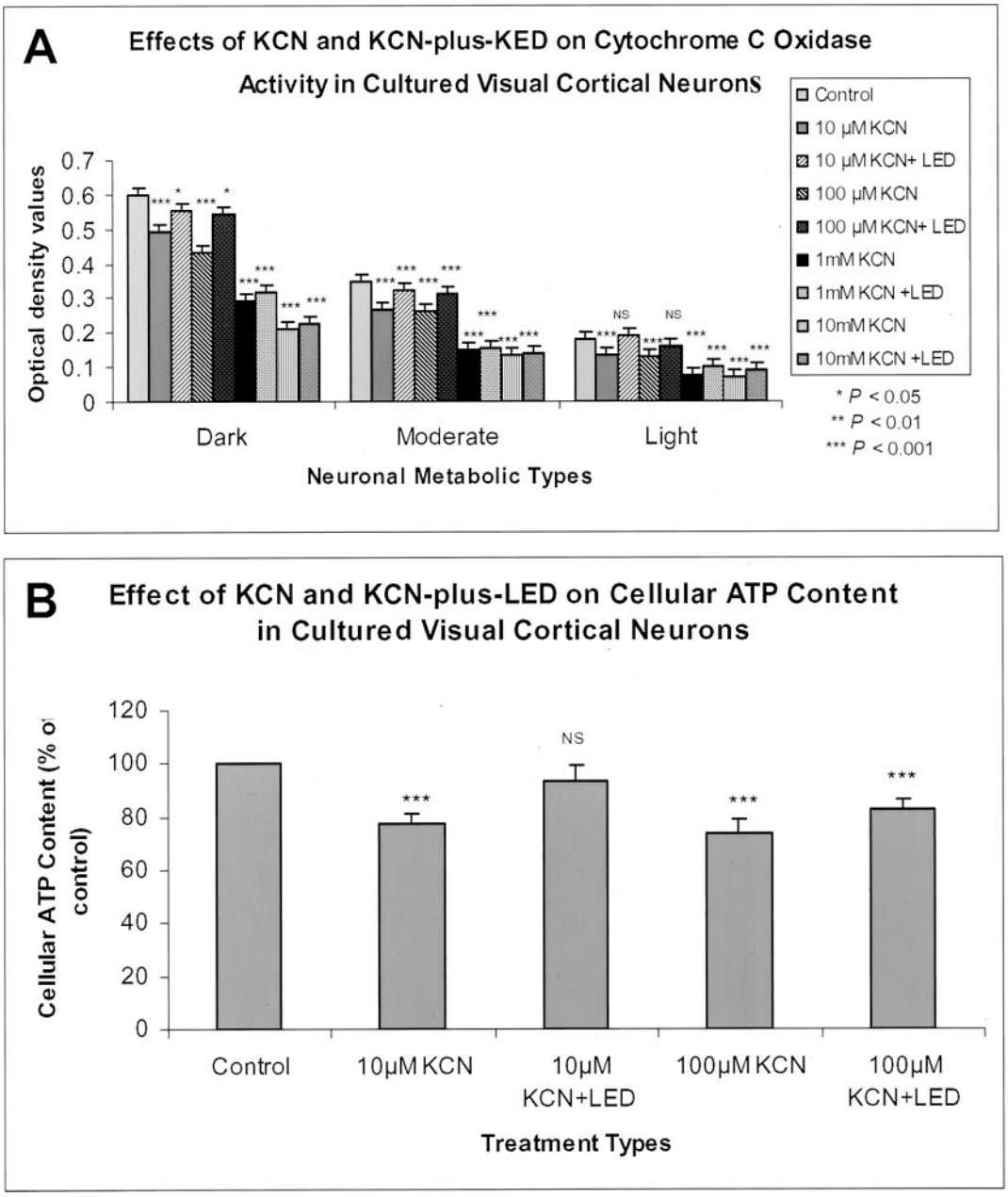
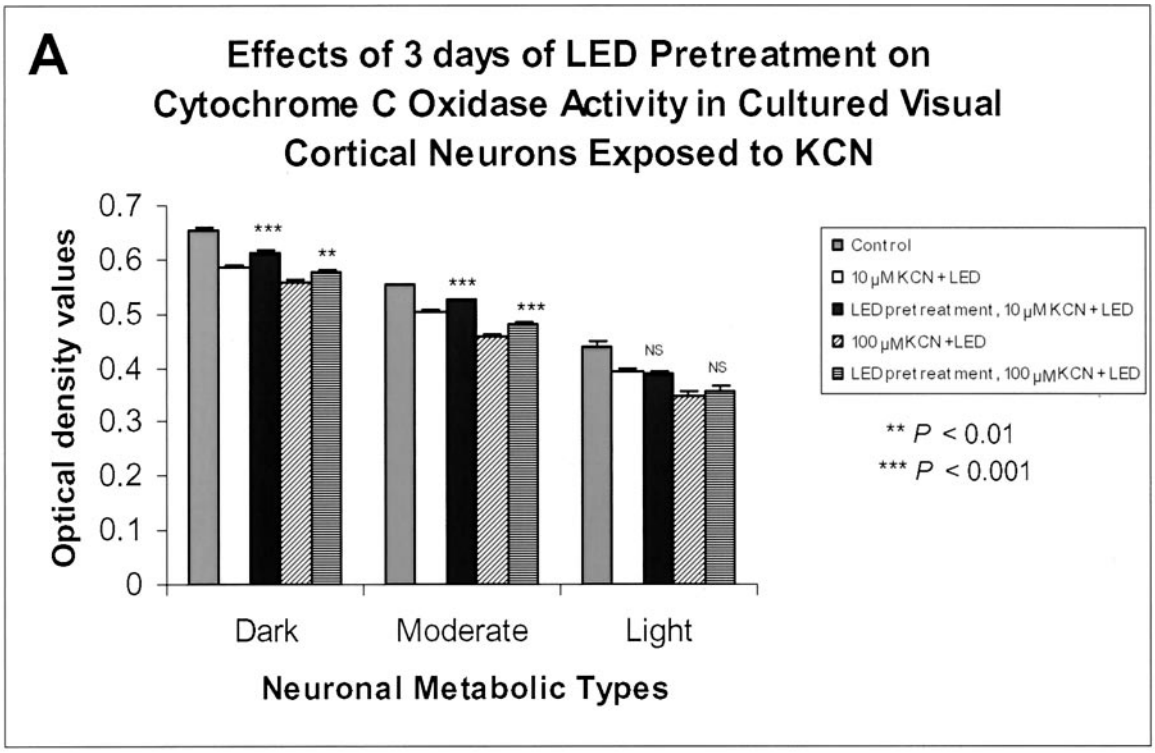


FIG. 2. *A*, effects of various concentrations of KCN and KCN plus LED on cytochrome *c* oxidase activity in cultured visual cortical neurons. Note that with increasing concentrations of KCN, enzyme levels progressively decreased in all three metabolic cell types. All *p* values indicate comparisons to controls in the respective metabolic categories. Reductions were highly significant even at the lowest concentration of KCN (10 μM). LED significantly reversed the reduction caused by 10 and 100 μM KCN, but the values reached control levels only in the lightly reactive metabolic cell type. Higher concentrations of KCN brought minimal relief from LED. *NS*, non-significant from controls. *B*, cellular ATP content was significantly reduced by KCN, and LED was able to reverse this process to control levels only in the presence of 10 μM but not 100 μM KCN. All *p* values were compared with the control.

to 30 joules/cm^2 of energy density) substantially reduced the number of neurons with condensed chromatin to 43.47% ($p < 0.001$) (Fig. 4*C*). Replacement of KCN with NaN_3 at 1 mM concentration generated 73.7% of neurons with condensed chromatin, and pretreatment with LED for 10 min significantly reduced the percentage of neurons undergoing cell death to 51.6% ($p < 0.001$). For comparison, the Hoechst stain showed comparable number of cell death with 300 μM KCN and comparable reduction in cell death with LED (data not shown).

Varying Single Wavelength Experiments—To determine the optimal wavelength for reversing the detrimental effect of TTX on neuronal cytochrome *c* oxidase activity, five wavelengths were tested: 670, 728, 770, 830, and 880 nm. Each was administered at a power intensity of 50 milliwatt/cm^2 and energy density of 4 joules/cm^2 equivalent to 1 min and 20 s per day for the last 5 of the 6 days of TTX exposure.

As shown in Fig. 5*A*, 670 and 830 nm were equally effective in reversing the deleterious effect of TTX on cytochrome *c* oxidase activity, with 830 nm being the most beneficial in completely restoring enzyme activity to slightly above (although not statistically different from) control levels (111.7%). 670 nm was equally effective in bringing about 100% recovery. Although 770 nm was as beneficial as 880 nm toward the darkly reactive metabolic cell type, overall it enabled 67.2% recovery of enzyme levels, whereas 880 nm brought about 98.4% recovery for all metabolic cell types. 728 nm, on the other hand, was the least effective and could only bring about 27.5% recovery of enzyme activity in TTX-intoxicated neurons.



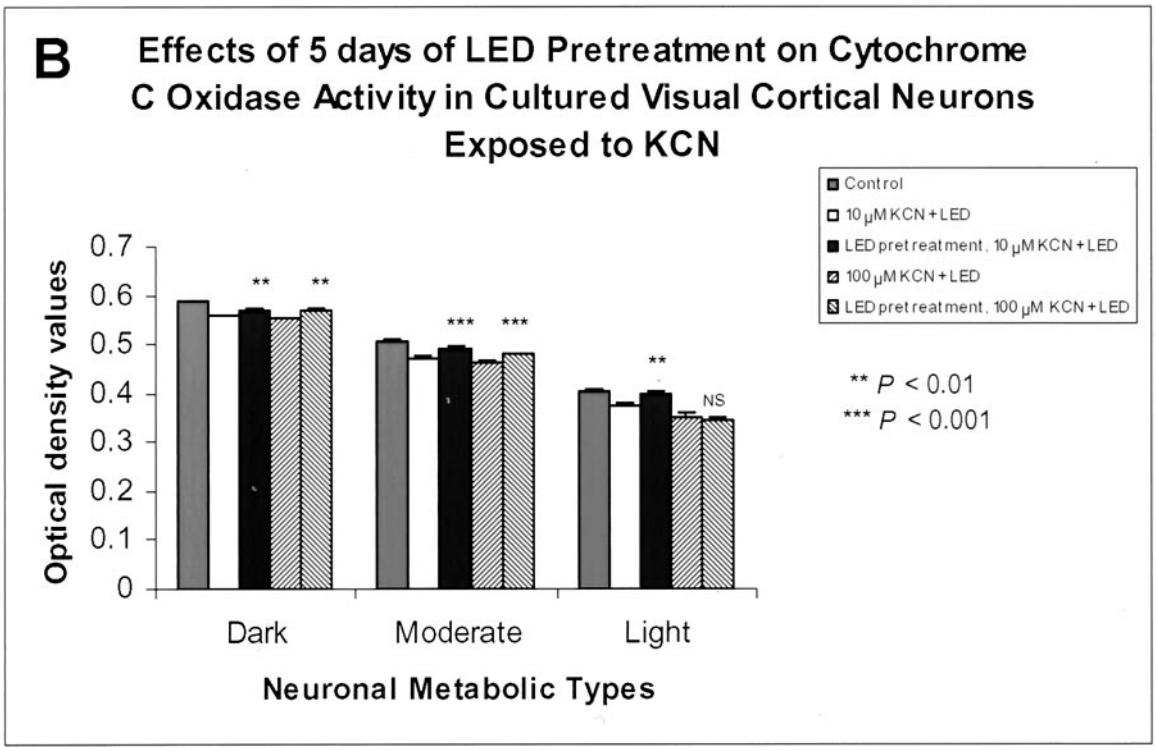


FIG. 3. **Pretreatment of neurons with 670 nm LED once a day for 3 days (*A*) or 5 days (*B*) further enhanced the beneficial effect of LED on cytochrome *c* oxidase activity *during* KCN exposure (10 or 100 μM KCN plus LED).** *p* values indicate comparisons between pretreatment and no pretreatment groups for each metabolic cell type at each concentration of KCN. However, pretreatment was able to rescue enzyme activity to control levels only in the lightly reactive cell group exposed to 10 μM KCN.

The ATP content of neurons was reduced to 82.5% of control levels by 6 days of exposure to 0.4 μM of TTX (Fig. 5*B*). All LED treatments except for 728 nm were able to completely reverse the detrimental trend. Again, 830 nm appeared to be very effective in restoring ATP content to slightly above (although not significantly different from) control levels (106.8%). 670 nm also restored the ATP content to a level above (but statistically comparable to) control levels (110%). Both 770 and 880 nm were effective in bringing ATP content to 98.4% and 91.5% of control levels, respectively. On the other hand, 728 nm was the least effective and showed minimal benefit in reversing the detrimental effect of TTX (to only 86.2% of controls, comparable to the value of TTX alone).

Varying Multiple Wavelengths Experiments—To determine whether certain sequences of wavelengths would be more beneficial than others, we tested six different sequences of three

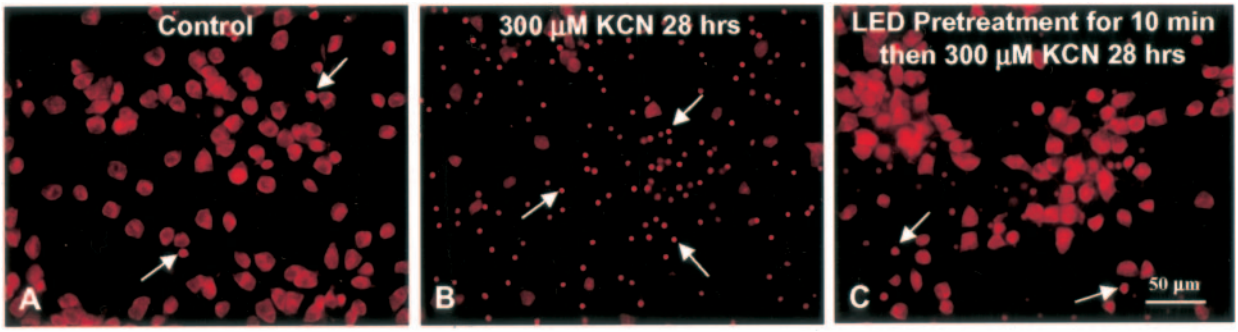
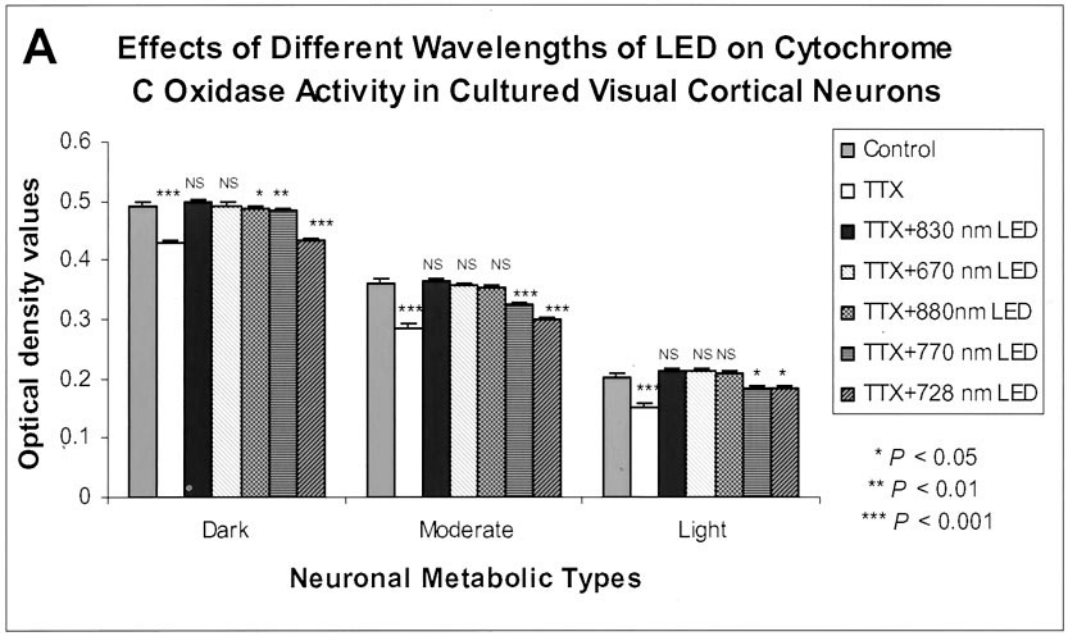


FIG. 4. **Cultures stained with propidium iodide for neurons undergoing cell death.** *A*, control cultures have relatively few apoptotic nuclei (*arrows*, 4.75% of total cell population). *B*, cultures subjected to 300 μM KCN for 28 h showed a profusion of neurons with highly condensed chromatin (*arrows*, 83.6%). *C*, 10 min of LED treatment at 670 nm substantially reduced the number of neurons exhibiting cell death to 43.5% (*arrows*).



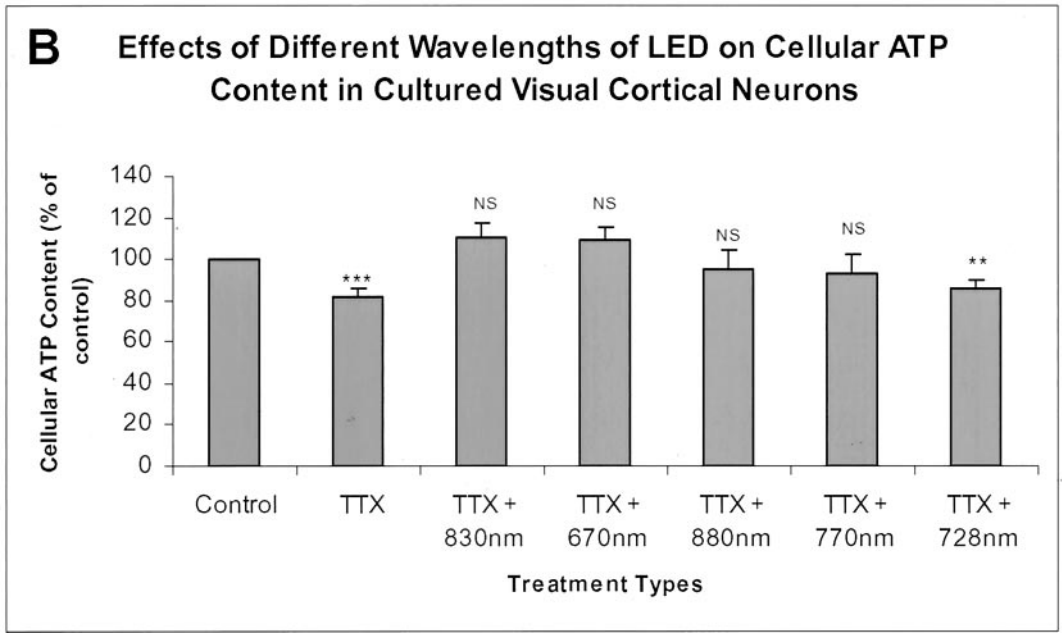


FIG. 5. **Effects of various wavelengths of LED on cytochrome *c* oxidase activity (*A*) and ATP content (*B*) in neurons exposed to TTX for 6 days.** All *p* values indicate comparisons with normal controls. Note that 670 and 830 nm were most effective in bringing enzyme activity and ATP content back to control levels; 880 and 770 nm were slightly less effective, whereas 728 nm was the least effective in reversing the detrimental effect of TTX.

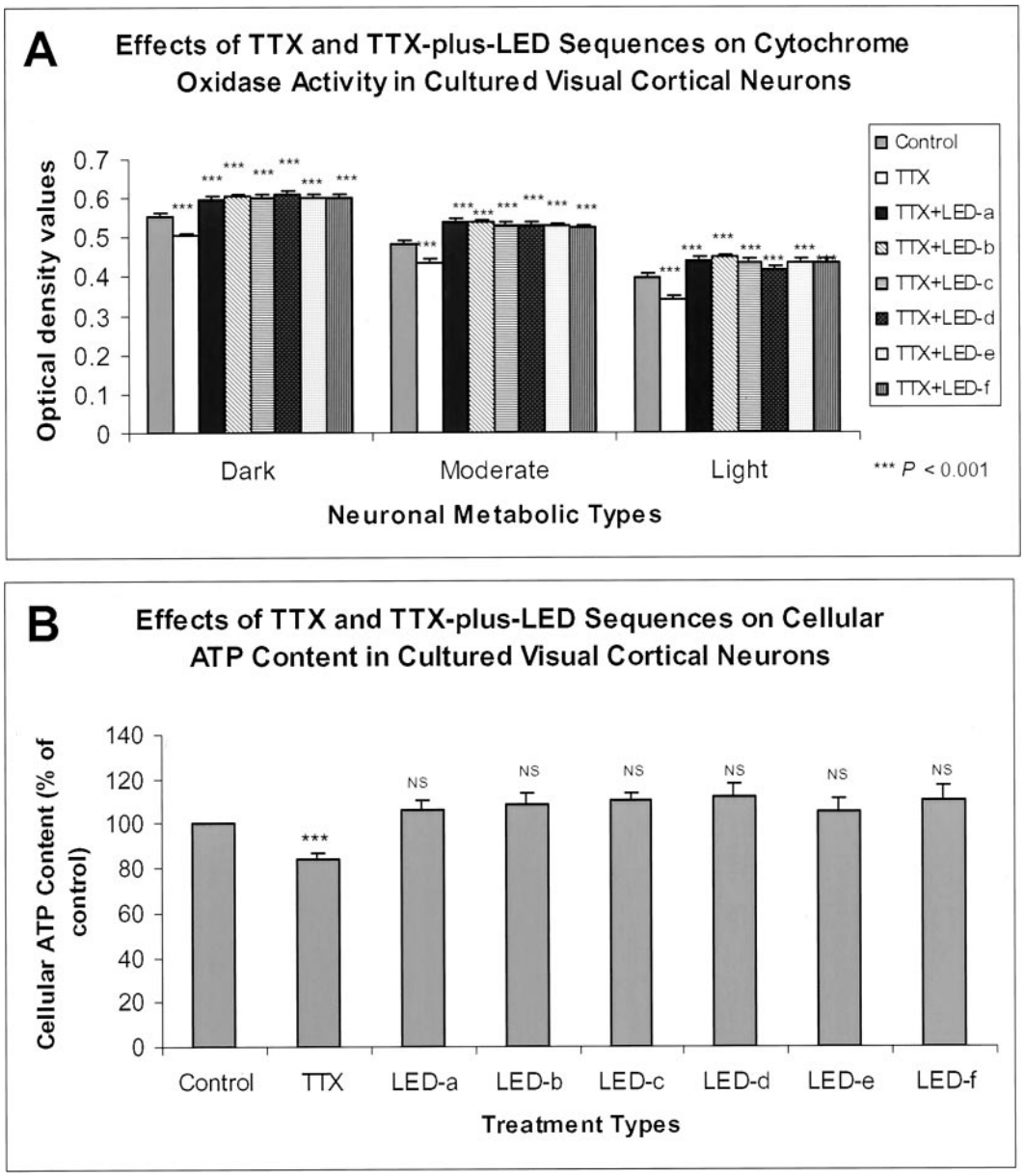


FIG. 6. **Effects of six different combinations of LED sequences (see "Experimental Procedures" for LED groups (*a*)–(*f*)) given once per day for 5 days on cytochrome *c* oxidase activity (*A*) and ATP content (*B*) in neurons exposed to 6 days of TTX.** All *p* values indicate comparisons with normal controls. Note that all combinations of LED not only reversed the detrimental effect of TTX but also brought enzyme and ATP values significantly above or at controls levels.

wavelengths, each given once a day for the last 5 of 6 days in TTX, all at the same energy density of 4 joules/cm^2 (see "Experimental Procedures" for groups (*a–f*)). Results indicated that all combinations tested resulted in cytochrome *c* oxidase levels above those of controls ($p < 0.001$ for all) (Fig. 6*A*). Cellular ATP content also showed the benefit of all combinations tested with values at or slightly above control levels (Fig. 6*B*).

The last two groups of neurons each had a sequence of nine LED treatments, all on the last day of a 6-day TTX exposure and were harvested 24 h later. Group (*g*) was treated sequentially with LEDs at 670, 728, and 830 nm at 50 milliwatt/cm^2 each for the first round and at 10 milliwatt/cm^2 each for the second and third rounds. Group (*h*) was similar to group (*g*) except that 830 nm was replaced by 880 nm LED (see "Experimental Procedures"). The energy density was kept constant at 4 J/cm^2 for each treatment to determine whether varying the power intensities would make a difference. Results indicated that both paradigms completely reversed the detrimental effect of TTX on cytochrome *c* oxidase activity, with group (*g*) reversing the darkly reactive cell type above control levels (Fig. 7*A*). The cellular ATP content showed the same trend with both paradigms reaching control levels (Fig. 7*B*).

Action Spectra versus Cytochrome c Oxidase Absorption Spectrum—The action spectra of cytochrome *c* oxidase activity and ATP content of neurons responding to various wavelengths were plotted against reported absorption spectrum of the enzyme (15). As shown in Fig. 8, the effective wavelengths, especially 670 and 830 nm, correlated positively with the known absorption spectrum of oxidized cytochrome *c* oxidase. On the other hand, the least effective wavelength, 728 nm, did not correspond to the absorption spectrum of the enzyme.

Equilibrium Constants of Cyanide and Azide with Cytochrome c Oxidase—Fig. 9 illustrates the equilibrium constants of cyanide (*A*) and azide (*B*) with cytochrome *c* oxidase with or without LED treatment. The cyanide data were for darkly reactive neurons as shown in Fig. 2*A*, and the azide data were

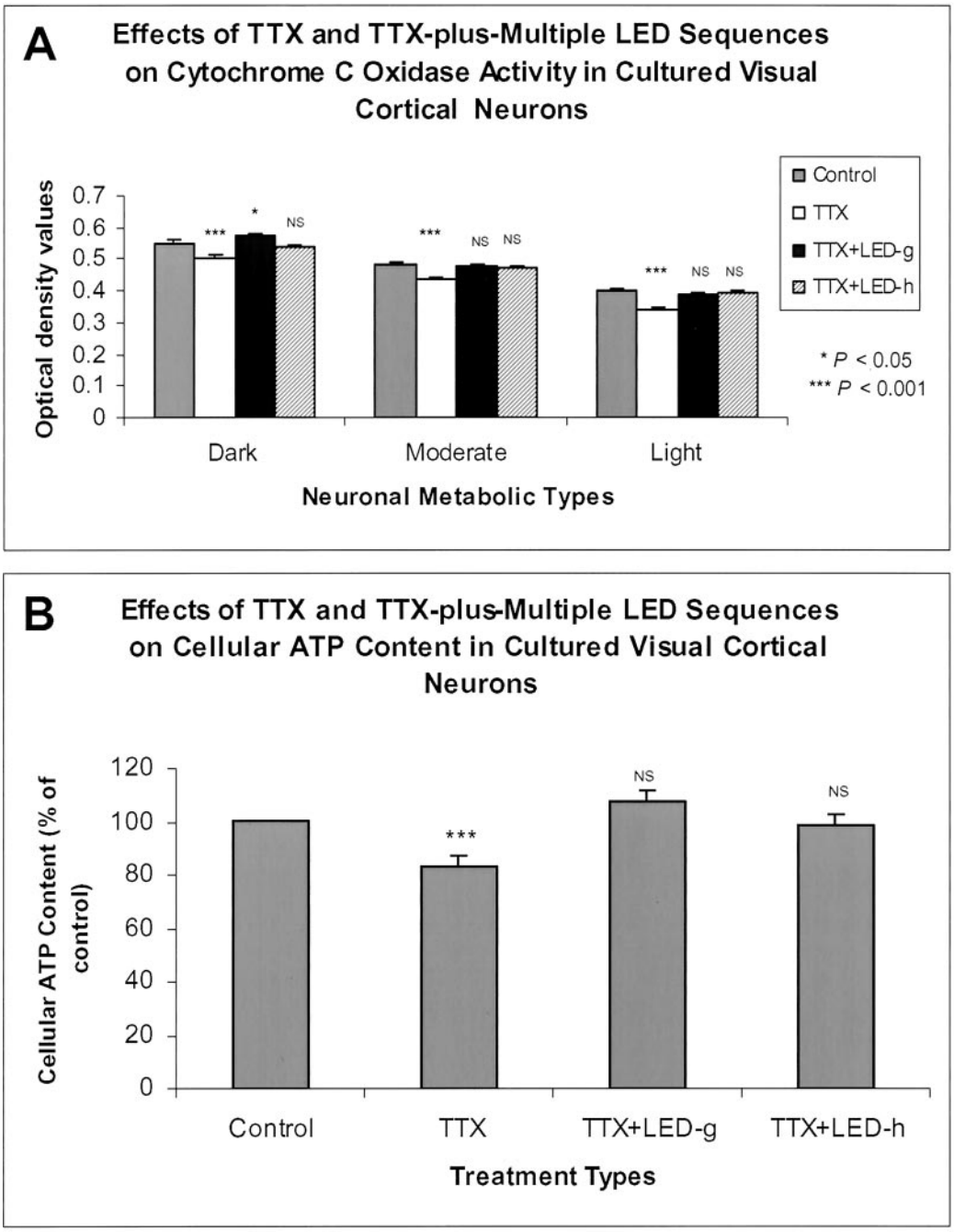


FIG. 7. **Effects of two different sequences of nine exposures each (see "Experimental Procedures" for LED groups (*g*) and (*h*)) administered on the last day of a 6-day TTX exposure on cytochrome *c* oxidase activity (*A*) and ATP content (*B*) in neurons.** All *p* values indicate comparisons with normal controls. Note that both paradigms completely reversed the down-regulation of cytochrome *c* oxidase and ATP content by TTX so that all values were either at or slightly above control levels.

for all three metabolic cell types from our unpublished observations. The cyanide concentrations plotted were 10 μM, 100 μM, 1 mM, and 10 mM, whereas those for azide were 10 μM, 100 μM, and 1 mM. In both cases, the "*a*" values of the constants were two to three times higher than the reported value of one (16). LED treatment reduced the "*a*" value of the constants for both KCN and NaN_3 (from 3.02 to 1.82 for KCN and from 2.56 to 1.65 for NaN_3).

DISCUSSION

The findings of the present study are consistent with our hypotheses that (*a*) cytochrome *c* oxidase is directly involved in the photobiomodulation of LED, (*b*) the beneficial actions of LED are attenuated by KCN, a known inhibitor of the oxidase, (*c*) LED pretreatment *before* exposure to KCN enhances the positive effect of LED during KCN intoxication, (*d*) the action spectra of effective wavelengths of LED correspond to the absorption spectrum of oxidized cytochrome *c* oxidase in the near infrared range, and (*e*) LED significantly reduces the percentage of cell death induced by KCN.

Both KCN and TTX significantly down-regulated cytochrome *c* oxidase activity in neurons, and LED reversed the detrimental effects of the toxins. However, the degree of reversal differed for the two toxins. Potassium cyanide is a potent, *direct*, and irreversible inhibitor of cytochrome *c* oxidase activity. At lower concentrations of KCN, LED up-regulated the enzyme against the inhibitory action of KCN, but the reversal never reached control levels. At higher concentrations of KCN, LED could no

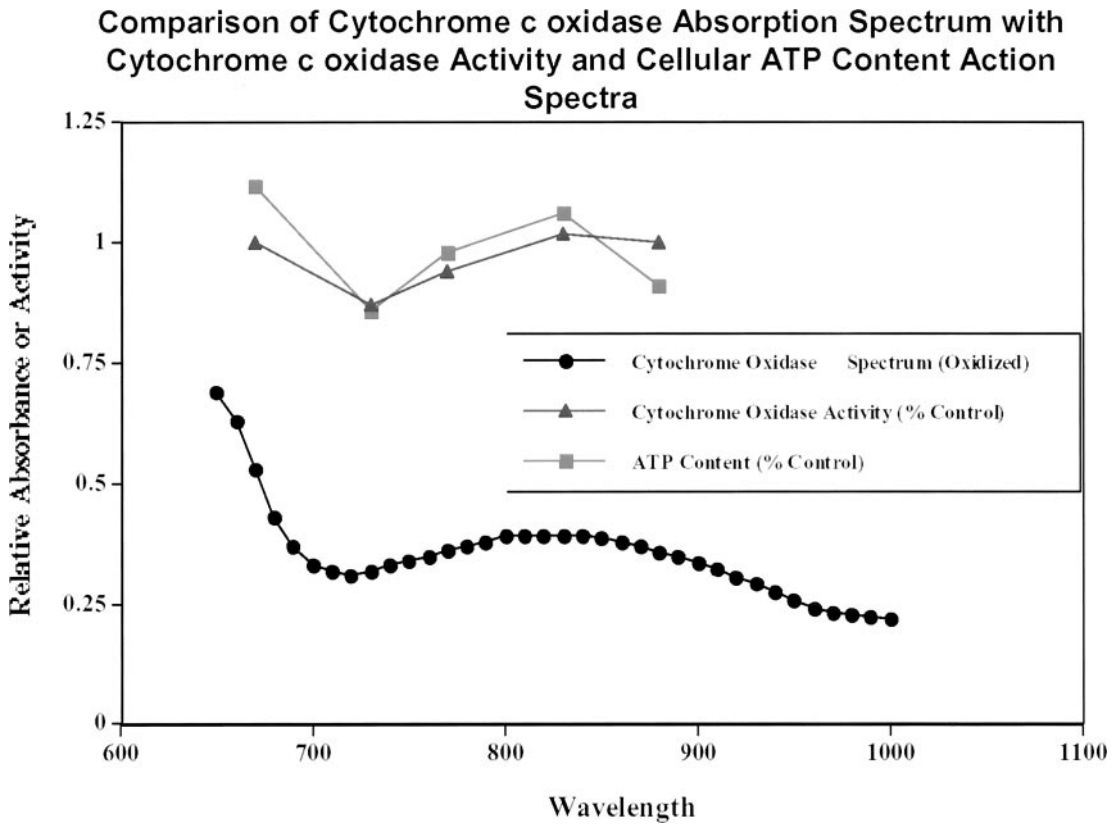


FIG. 8. **Action and absorption spectra in the far red and near infrared range for oxidized cytochrome *c* oxidase (15), relative cytochrome *c* oxidase activity, and relative ATP content in TTX-treated neurons exposed to LED treatments at different wavelengths expressed as percentages of controls (present study).** Note the correspondence of effective wavelengths (especially 670 and 830 nm) with the absorption spectrum of oxidized cytochrome *c* oxidase and the non-correspondence of the least effective wavelength (728 nm).

longer rescue enzyme activity effectively as shown by increasing cell death. Thus, LED and KCN have direct, opposing influences on the enzyme. On the other hand, TTX *indirectly* affects cytochrome *c* oxidase activity by blocking neuronal action potentials, thereby lessening energy requirement of these functionally quiescent cells. LED was able to completely reverse the detrimental effect of TTX by up-regulating the enzyme to its control levels. Previously, we found that a difference of 0.1 optical densitometric unit is equivalent to a change in cytochrome *c* oxidase specific activity of ~500 milliunits/mg protein, which represents about 29% of normal specific enzyme activity in highly active neuronal regions (~1700 milliunits/mg protein) (17). The present study is based on measurements in individual neurons, and the three broad metabolic neuronal types have different baseline levels of enzyme activity. Nevertheless, the down-regulation of cytochrome *c* oxidase specific activity by 10 μM KCN in darkly reactive neurons was ~500 milliunits/mg protein, whereas that incurred by 0.4 μM TTX, a dosage that effectively blocks all impulse activity in neurons (18), approached 350 milliunits/mg protein, or ~21% of the normal values for highly active neurons. Previously, we showed that the activity of this enzyme is regulated mainly at its protein level (19). Thus, the up-regulation of enzyme activity by LED is likely to involve synthesis of new proteins. In the presence of TTX, LED is able to stimulate synthesis of sufficient amounts of proteins to return the oxidase to its control level. In the case of KCN exposure, however, the population of newly synthesized proteins will be partially blocked by low doses of KCN and completely blocked by high doses of KCN. Thus, LED is prevented from reversing the enzyme to its control levels in the presence of KCN.

The cellular content of ATP was likewise reduced by KCN, and LED in the presence of 10 μM KCN was able to restore the ATP content to control levels. However, 100 μM or more of KCN abolished the beneficial effect of LED in ATP production, indicating that even though cytochrome *c* oxidase activity was partially enhanced by LED, it was not sufficient for a significant recovery of ATP content.

Remarkably, only 10 min of LED pretreatment at a total energy density of 30 joules/cm^2 effectively reduced by 48% the number of neurons exhibiting cell death in 300 μM KCN. Likewise, LED pretreatment significantly reduced the percentage of dying cells with condensed chromatin induced by another inhibitor of cytochrome *c* oxidase, sodium azide. The cause of the KCN-induced apoptosis is thought to be the generation of reactive oxygen species (20). The ability to arrest cell death strongly suggests that within the parameters tested LED is able to up-regulate cytochrome *c* oxidase and ATP production and to trigger a signaling cascade that prevents KCN- (or NaN_3)-poisoned neurons from undergoing cell death.

The use of various wavelengths provided insight into the relative benefits of different LEDs. Both 670 and 830 nm were equally effective in restoring cytochrome *c* oxidase to control levels in the presence of TTX, whereas 880 and 770 nm were slightly less effective, and 728 nm was the least effective. These results are strikingly comparable to those of Karu (8) in which the generalized action spectrum of HeLa cell DNA synthesis was analyzed after treatment with lasers of different wavelengths. In that study, Karu found four action peaks in the far red to near infrared range: 620, 680, 760, and 820 nm, which she suggested belong to absorption spectra of redox active copper centers of cytochrome *c* oxidase, reduced Cu_A, oxidized Cu_B, reduced Cu_B, and oxidized Cu_A, respectively. Our LEDs have an effective bandwidth of 25–30 nm, so our 670 and 830 nm treatments fall within the proposed absorption spectra of oxidized Cu_B and oxidized Cu_A, respectively. 830 nm is known

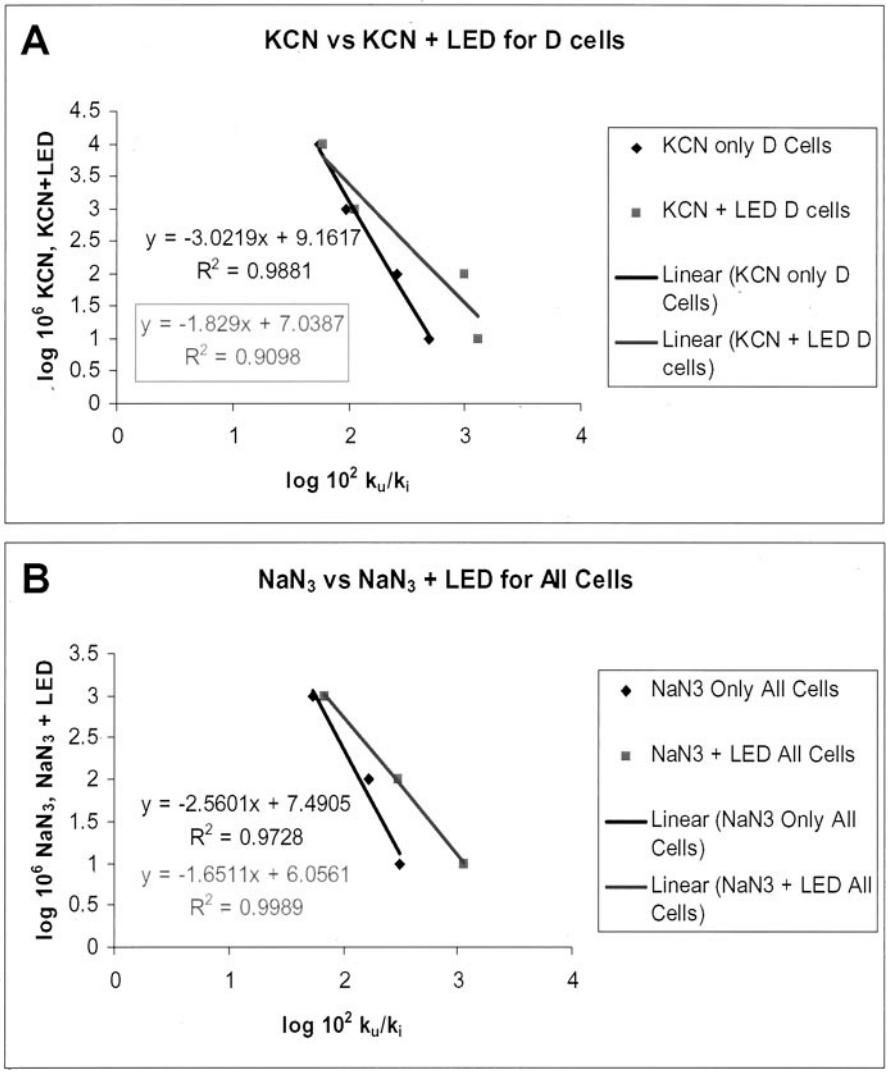


FIG. 9. **Equilibrium constants of cyanide (*A*) and azide (*B*) with cytochrome *c* oxidase based on the data for darkly reactive neurons in Fig. 2*A* and our unpublished data of azide for all cells.** The cyanide concentrations plotted were 10 μM, 100 μM, 1 mM, and 10 mM, whereas those for azide were 10 μM, 100 μM, and 1 mM. Note the reduction in the constants for both cyanide and azide with LED treatment.

to be the maximal absorption spectrum of oxidized cytochrome *c* oxidase because of the cupric ion of Cu_A (21–23) and is the most effective wavelength in the present study. 880 nm falls within the broad range of oxidized Cu_A, and 770 nm is close to the reported absorption spectrum of reduced Cu_B, whereas the reported spectrum of reduced Cu_A (620 nm) was not tested in the present study. 770 nm is also close to 784 nm, the suspected absorption band of ferroheme a3 in cytochrome *c* oxidase of beef heart (24). Another candidate to consider for 770 nm is the known absorption peak for deoxyhemoglobin at 760 nm (25). However, in the present study, there was no blood element that would contribute to the 770 nm response, so cytochrome *c* oxidase is the most likely candidate. On the other hand, 728 nm was the least effective in our study, and Karu's data also showed that lasers within this range did not up-regulate DNA synthesis. The effective wavelengths also compared positively with the known absorption spectrum of oxidized cytochrome *c* oxidase (26). Within the broad range of wavelengths between 770 and 900 nm, Cooper *et al.* (27) claimed that most of the *in vivo* signal can be attributed to cytochrome *c* oxidase.

The positive relationship between wavelength efficacy and cytochrome *c* oxidase absorption spectrum strengthens our hypothesis that cytochrome *c* oxidase plays an important role in therapeutic photobiomodulation by LED (5–7). As discussed above, the increase in enzyme activity is likely to be a reflection of an increase mainly in the amount of the enzyme (19) because values taken immediately after 80 s of LED treatment did not show a detectable change from the control (data not shown), whereas 1 day after the treatment, a significant increase in activity was present in the darkly reactive neurons (6).

Results from our ATP experiments corroborated the data obtained in the enzyme studies in that 670 and 830 nm were able to restore ATP content to or above control levels in the presence of TTX. 770 and 880 nm were also effective, although to a slightly less degree, whereas 728 nm was again the least effective. Thus, LED most likely up-regulates both the activity and synthesis of cytochrome *c* oxidase, resulting in increased energy production in neurons.

Multiple treatments for all wavelength sequences tested yielded comparable results in restoring cytochrome *c* oxidase activity above control levels. This suggests that the less effective wavelength at 728 nm was offset by the more beneficial ones at 670 and 830 nm when performed in succession. Furthermore, the marginally effective 728 nm did not compromise the positive actions of the other wavelengths. Nine treatments done on the last day were able to restore cytochrome *c* oxidase activity close to (but generally not above) control levels, indicating that treatments spread out over days is more beneficial than that concentrated to 1 day. The nine exposures were also slightly more effective for the sequence of 670/728/830 nm than that of 670/728/880 nm, confirming the slight advantage of 830 nm over 880 nm.

Heat production is a concern of laser therapy. In contrast, the LED arrays, originally developed for NASA plant growth experiments in space and used in our studies, produce virtually

no heat and have attained FDA approval for non-significant risk status. If heat were a major cause of cellular response in our studies, then responses would be predicted to be incremental or decremental with increasing wavelengths used. As shown by the present data, this is not the case. 728 nm was the least effective in comparison to either 670 or 830 nm. Thus, even if LED should prove to increase cellular temperature to a fraction of a degree higher than normal, and if it should elicit further cellular events, heat is not likely to be a major factor in the therapeutic treatment with LED. Further investigation into this issue is underway.

One aspect that deserves some consideration is that other cellular proteins may also bind to cyanide and azide and may participate in the light-activated response. When the equilibrium constants of cyanide and azide with cytochrome *c* oxidase were plotted, we found that in both cases the "*a*" value of the constants were two to three times higher than the reported value of one (16). This strongly implies that other protein or proteins were also binding to the toxins. This is not surprising because there are many varieties of heme proteins in cells. We can, however, rule out hemoglobin and myoglobin in our study, as there were no blood elements or muscle fibers in our cultures. Other heme proteins, such as cytochrome *b*, cytochrome *c*, catalase, and nitric-oxide synthase, as well as other metallic proteins, cannot be ruled out at this time.

The fact that LED treatment caused a reduction in the "*a*" value of the constants for both cyanide and azide strongly indicates that LED induced an increase in the synthesis of cytochrome *c* oxidase consistent with our deductions discussed above. Such synthesis undoubtedly involves a signaling molecule (or molecules) that directly or indirectly caused transcription and translation of cytochrome *c* oxidase subunit genes from both the mitochondrial and nuclear genomes. The prolonged cellular effect of brief LED treatments further suggests that there is an induction of a cascade of events leading to the activation and repression of a variety of genes (28, 29). Karu *et al.* (30) have proposed that intracellular signaling secondary to photon interaction with cytochrome *c* oxidase could be mediated by redox changes in the electron transport chain, by the generation of reactive oxygen species or nitric oxide signaling molecules, as well as by pathways involving the Na^+,K^+-ATPase and the Na^+/H^+ exchanger. Additional signaling molecules that may link light absorption with DNA induction such as phytochromes (in plants) (31) and cryptochromes (in plants and animals) (32) also warrant further investigation.

In sum, although other photoacceptor molecules remain to be investigated, the present study supports our hypothesis that the endogenous mitochondrial enzyme, cytochrome *c* oxidase, is an important biological photoacceptor that mediates photobiomodulation in the far red and near infrared range. It further shows that the ATP content of primary neurons rises or falls in concert with the activity of cytochrome *c* oxidase, a known energy-generating enzyme. The therapeutic effects of photobiomodulation in promoting wound healing (1–5) and in overcoming intoxication by various toxins that inactivate cytochrome *c* oxidase both *in vitro* (Ref. 6, present study) and *in vivo* (7) are documented. There are clear clinical advantages to treatment using wavelengths within the tissue transparency window of 650–1000 nm. Future applications of this approach, especially with the low risk LED arrays, can and should be considered for various biological ailments and diseases such as those that involve cellular metabolism and energy production.

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