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Activity-Dependent Neural Tissue Oxidation Emits Intrinsic Ultraweak Photons

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Living organisms have been known to spontaneously emit ultraweak photons *in vivo* and *in vitro*. Origin of the photon emission remains unclear, especially in the nervous system. The spontaneous ultraweak photon emission was detected here from cultured rat cerebellar granule neurons using a photomultiplier tube which was highly sensitive to visible light. The photon emission was facilitated by the membrane depolarization of neurons by a high concentration of K^+ and was attenuated by application of tetrodotoxin or removal of extracellular Ca^{2+}, indicating the photon emission depending on the neuronal activity and likely on the cellular metabolism. Furthermore, almost all the photon emission was arrested by 2,4-dinitrophenylhydrazine, indicating that the photon emission would be derived from oxidized molecules. Detection of the spontaneous ultraweak photon emission will realize noninvasive and real-time monitoring of the redox state of neural tissue corresponding to the neuronal activity and metabolism. © 2001 Academic Press

Key Words: **ultraweak photon emission; cerebellar granule cell; rat; photomultiplier tube; neuronal activity; oxidation; photo-dynamic tissue oxidation; 2,4-dinitrophenylhydrazine; carbonyl.**

Almost all living organisms intrinsically and spontaneously emit ultraweak photons. Intensity of the ultraweak photon emission is less than 10^{-16} W/cm^2 or 10^3 photons/s · cm^2 estimated at around 600 nm wavelength at the sample surface (1, 2), and is known to vary under physiological or pathological states; i.e., germinating soybean seedling (2, 3), isolated hepatocytes (4), and human skin *in vivo* (5) under oxidative stress. Spectrum of the photon emission includes various ranges of wavelength including ultraviolet, visible, and infrared ranges (4, 6, 7). Origin of the photon emission are thought to be cellular metabolism, especially respiratory activity or oxidative stress (4, 7), although the exact chemical reactions or related molecules are remained to be clarified.

In the central nervous system, Isojima *et al.* (8) reported the neural activity-dependent ultraweak photon emission from hippocampal slices of rat brain, detected by a Si avalanche photodiode (9), while the origin of the photon emission was not studied. In the present report, we detected the neuronal activity-dependent ultraweak photon emission from primary monolayer culture of rat cerebellar granule neurons which develop the neural networks, and demonstrated suppressive effect of 2,4-dinitrophenylhydrazine (DNPH) on the photon emission; DNPH is a delivertizing agent of carbonyl groups, which arise under oxidative stress, to 2,4-dinitrophenylhydrazone.

MATERIALS AND METHODS

Primary culture of cerebellar granule neurons. After dissecting the cerebellum from 8-day-old male rats deeply anesthetized with ethyl ether, the cerebellar granule neurons was cultured for 4–7 days as previously described (10). In brief, the cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml; Worthington) at 37°C. The cells were then seeded onto polylysine (Sigma, St. Louis, MO)-coated quartz glass sheets (4 cm diameter) in culture medium consisting of 5% (vol/vol) precolostrum newborn calf serum, 5% (vol/vol) heat-inactivated horse serum, 1% (vol/vol) rat serum, and 89% 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium containing 15 mM Hepes buffer, pH 7.4, 30 nM selenium, and 1.9 mg/ml sodium bicarbonate. The cells

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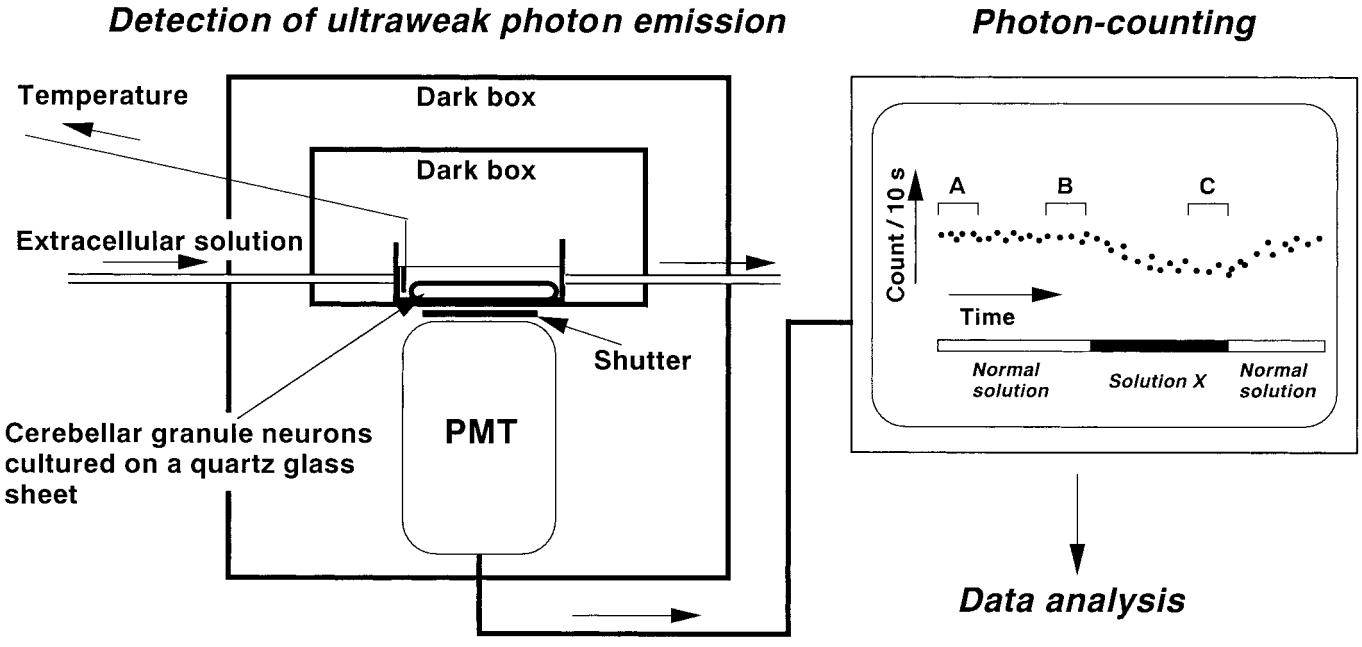

FIG. 1. Schematic drawing of the detecting system of ultraweak photon emission. Emitted photons were detected by a photomultiplier tube (PMT) and the number of detected photons in every 10 s was plotted against time in a computer display (photon-counting) by the photon processing system. The mean values for 500 s at the start of each experiment (duration A), immediately before the timing for exchange of extracellular condition (duration B), and immediately before washout of the experimental solution (Solution X in the figure, duration C) were used as data for calculation of the count ratios.

were seeded at a density of 5×10^5 cells/cm^2. After 1 day of culture in a humidified CO_2 (5%) incubator, the medium was changed to high-K^+ minimum essential medium (MEM) supplemented with 5% heat-inactivated horse serum and 3 μM cytosine arabinoside. MEM was supplemented with 2.2 mg/ml glucose and 2.2 mg/ml sodium bicarbonate. High K^+ MEM was prepared by increasing the $KHCO_3$ concentration from the normal value of 5.4 to 26 mM, with the omission of the corresponding concentration of $NaHCO_3$.

Detection of ultraweak photon emission. Ultraweak photon emission from cultured rat cerebellar granule neurons at 35°C in a completely dark box (*Detection of ultraweak photon emission* in Fig. 1) was detected using a photomultiplier tube [PMT in Fig. 1; R331-09, Hamamatsu Photonics K. K., Hamamatsu, Japan] which was highly sensitive to visible light (>50 mA/W in the wavelength of 310–520 nm). The composition of the control extracellular solution (normal solution) was as follows (in mM): NaCl, 113; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1; glucose, 11; $NaHCO_3$, 25. The solution was equilibrated with 95% O_2/5% CO_2. The composition of the Ca^{2+}-free extracellular solution was as follows (in mM): NaCl, 113; KCl, 3; $MgCl_2$, 5; NaH_2PO_4, 1; glucose, 11; $NaHCO_3$, 25. Tetrodotoxin (TTX) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2,4-Dinitrophenylhydrazine solution at 10 mM was made as previously reported (11). The solution was diluted to 5 μM DNPH with extracellular solution.

Photon-counting and data analysis. The photon detection system (Fig. 1) showed the back ground noise of about 28 cps (counts/s), including dark noise in PMT (about 12 cps) and background fluorescence from a poly-lysine-coated quartz glass sheet without cells in the extracellular solution at 35°C. Changing the quartz glass sheet without cells for the sheet on which neurons were cultured showed increase of 2–5 cps in photon emission which was brought about by cultured neurons. Total counts (back ground noise + photon emission from the cells) for 10 s was plotted against time (*Photon-counting* in Fig. 1), and the mean values for 500 s at the start of each experiment (indicated as duration A in Fig. 1) and immediately before each timing for exchange of extracellular condition (indicated as durations B and C in Fig. 1) were used as data for calculation of the count ratios in the normal solution (Normal) and in exchanged solution (Solution X) as follows: (Count ratio in Normal) = [(Count in B)/(Count in A)] $\times$ 100 (Count ratio in Solution X) = [(Count in C)/(Count in B)] $\times$ 100

Photo-dynamic tissue oxidation of brain slices. Coronal slices (400 μm thick) of the whole brain containing the cerebral cortex, hippocampus, and thalamus were obtained from deeply anesthetized rats (3–5-week-old) with ethyl ether by a previously published method (12), following incubation in the normal solution for an hour at 37°C. Then each brain slice was photo-irradiated with a xenon metal halide lamp (10 W/cm^2; 1 min) in the 3 μM Rose Bengal-containing Hepes-buffered solution (Photo-dynamic tissue oxidation technique, 13). The composition of the Hepes-buffered solution was as follows: NaCl, 153 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1 mM; glucose, 17 mM; Hepes, 10 mM (buffered to pH 7.4 with KOH). Rose Bengal was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Each photo-oxidized slice was placed in an experimental chamber filled with the Hepes-buffered solution without Rose Bengal in the dark room for detection of ultraweak photon emission (Fig. 1), immediately after the photo-oxidation; detection of the photon emission was started 1 min after withdrawal of the photo-irradiation. The Rose Bengal-containing solution without brain slices did not induce photon emission 1 min after the photo-irradiation.

RESULTS

The photon detection system showed the back ground noise of 279 $\pm$ 3 counts/10 s (mean $\pm$ SE, n = 5 experiments), including dark noise in PMT [121 $\pm$ 4 counts/10 s (mean $\pm$ SE, n = 5 experiments)] and back ground fluorescence from a poly-L-lysine-coated quartz

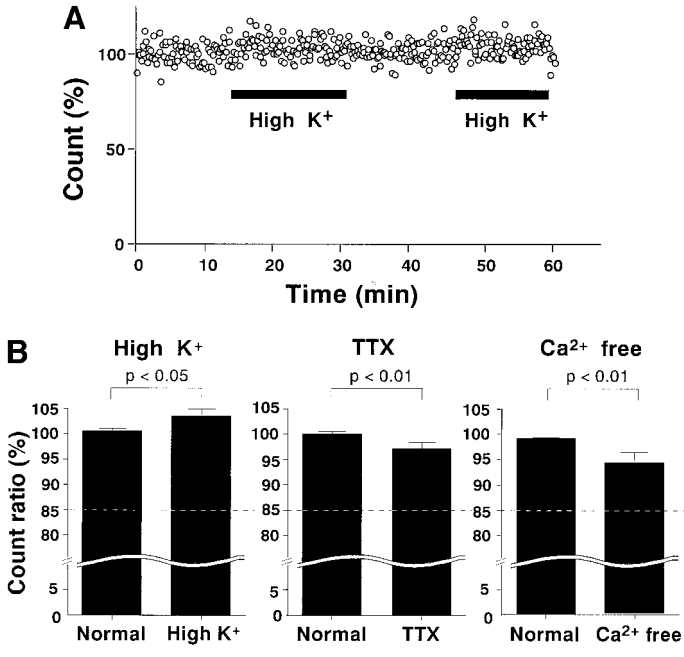

FIG. 2. Neuronal activity affected the spontaneous ultraweak photon emission from cultured neurons. (A) A typical example of high concentration of K^+-induced facilitation of the photon emission. Data were plotted as relative values against mean value of the data during the first 500 s (100%). Experimental solution including K^+ at a high concentration was applied to cultured neurons at timings indicated by bars (High K^+). (B) Effects of various kinds of experimental solutions on the photon emission are plotted as the count ratios (mean ± SE) with the ratio in the normal solution (Normal); solution containing K^+ at a high concentration (High K^+; $n = 25$ cultured materials), 1 μM TTX-containing solution (TTX; 27 cultured materials), and Ca^{2+} free solution (Ca^{2+} free; $n = 9$ cultured materials). Broken lines indicate the estimated minimum value of back ground noise in the present experimental system. Statistical significance is shown in each graph (*t*-test).

glass sheet without cells in the extracellular solution at 35°C. The background noise was very stable against time. Changing the quartz glass sheet without cells for the sheet on which neurons were cultured showed increase of 20–50 counts/10 s in photon emission.

After confirming the stable photon emission from cultured neurons in the normal extracellular solution (Normal in Fig. 2B), the normal solution was changed with the experimental solution including K^+ at a high concentration (High K^+ in Fig. 2B) for depolarizing the plasma membrane of cultured neurons (the estimated EK, −30 mV). The high concentration of K^+ slightly but significantly facilitated the photon emission after completing the solution exchange (Figs. 2A and 2B), and withdrawal of the high K^+ solution restored the emission to the basal level observed in the normal solution. On the contrary, inactivation of the axonal conduction of action potential in cultured neurons by application of 1 μM tetrodotoxin (TTX) to the extracellular solution significantly reduced the photon emission (TTX in Fig. 2B). Furthermore, removal of Ca^{2+} from the extracellular solution significantly attenuated the photon emission from cultured neurons (Ca^{2+} free in Fig. 2B). Those experimental solutions (High K^+, TTX, and Ca^{2+} free) did not affect the back ground noise which was observed without cells (data not shown). In the present experimental system, more than 85% of total counts was estimated to consist of the back ground noise (broken lines in Fig. 2B), indicating that high concentration of K^+ increased photon emission from cultured neurons as more than 20% of that in the normal solution, and that TTX application and Ca^{2+} removal decreased the photon emission as more than 18 and 30%, respectively.

Application of 2,4-dinitrophenylhydrazine (DNPH) at 5 μM in the extracellular solution immediately and remarkably attenuated the photon emission (Figs. 3A and 3B). DNPH at such a low concentration did not affect the background noise nor additional weak white noise light passage, indicating that DNPH attenuated the cell-derived photon emission likely by the effects on biochemical reactions in the cells. This was supported by assessing amplitude of the DNPH-induced reduction of photon emission under a condition of high concentration of K^+ in extracellular solution or that of TTX applied in the solution; amount of DNPH-induced reduction in count ratio was larger in the high K^+ solution which facilitated the cell-derived photon emission, and smaller in the TTX-applied solution which attenuated the photon emission from the cells (Fig. 3C). Almost all cell-derived photon emission could be attenuated by DNPH application, since more than 85% of count ratio consisted of the back ground noise (broken line in Fig. 3B). DNPH at such a low concentration did not induce any histological abnormalities in cultured neurons in observation with a phase-contrast microscope, i.e., cell shrinkage, cell swelling, or neurite disruption (data not shown).

Photon emission from oxidized brain slices were demonstrated to be attenuated by DNPH (5 μM). Each coronal section (400 μm thick) of rat brains was oxidized by photo-dynamic tissue oxidation technique (13) (Fig. 3D) or by application of chemicals (Fenton reaction; data not shown). Immediately after the photo-oxidation, a brain slice was placed in the dark box for detection of ultraweak photon emission (Fig. 1). Photon emission declining against time could be detected from oxidized brain tissue, and that was remarkably attenuated by DNPH applied into the extracellular solution ($n = 5$ brain slices; one typical example is shown in Fig. 3D), indicating that DNPH attenuated photon emission which was likely derived from oxidized tissue.

DISCUSSION

In the present photon detection system, less than 15% of the total counts was estimated to originate in the cultured neurons, and more than 85% of that was

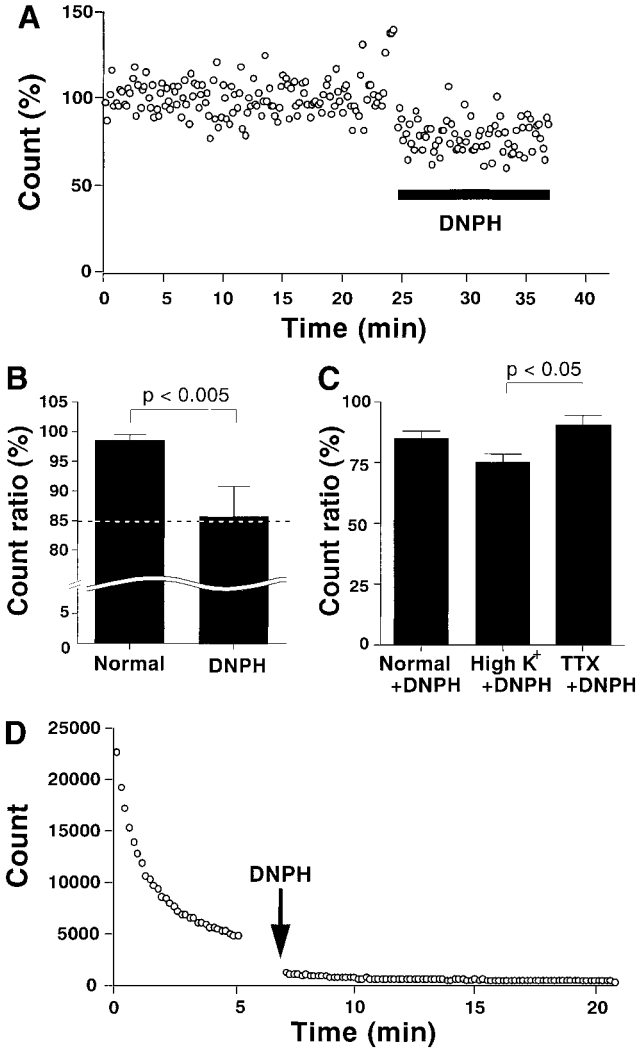

FIG. 3. 2,4-Dinitrophenylhydrazine attenuated spontaneous photon emission from cultured neurons and that from an extrinsically-oxidized brain slice. (A) A typical example of DNPH-induced attenuation of photon emission. Data were plotted as relative values against mean value of the data during the first 500 s (100%). Experimental solution including DNPH at 5 μM was applied to cultured neurons at the timing indicated by the bar (DNPH). (B) Effects of DNPH application on the photon emission are plotted as count ratio with that in the control solution (Normal). Mean values of count ratio (n = 4 cultured materials) were plotted with SE. Broken line indicates the estimated minimum value of back ground noise in the experimental system. Statistical significance is shown in the graph (t-test). Note that extracellular application of DNPH arrested almost all the photon emission from the neurons. (C) Effects of DNPH application on the photon emission in various extracellular conditions are plotted as count ratios (mean ± SE); in the normal solution (Normal+DNPH; n = 4 cultured materials), in the solution containing K^+ at a high concentration (High K^++DNPH; n = 6 cultured materials), and in the solution containing TTX (TTX+DNPH; n = 10 cultured materials). Statistical significance is shown in the graph (t-test). (D) Amount of photon emission (count/10 s) from a photo-oxidized brain slice was plotted against time. DNPH (5 μM) was applied to the extracellular solution at the timing indicated by an arrow (DNPH). Data were not obtained for 2 min immediately before application of DNPH, since a window of the dark room of photon detection system was opened for one shot injection of DNPH into the incubation fluid of the slice.

back ground noise (broken lines in Fig. 2B). In spite of such an amount of back ground noise, the system allowed statistical analysis of the photon emission originating from the cells because of stable monitoring of the emission for more than a few hours. High concentration of K^+ increased photon emission from the cultured tissue as more than 20% of that in the normal solution, and TTX application and Ca^{2+} removal decreased the photon emission as more than 18% and 30%, respectively (Fig. 2). It indicates that spontaneous ultraweak photon emission from neural tissue depends on the neuronal activity, i.e., neuronal membrane depolarization and Ca^{2+} entry into the cells. Photomultiplier tube in the present photon detecting system (Fig. 1) is sensitive to visible light (>50 mA/W in the wavelength of 310–520 nm). Photons with such a range of wavelength are known to be detected from yeast cells (14) or oxidized hepatocytes (4). In the present study, we could not identify the wavelengths more precisely because of extremely low intensity of the emission.

Most part of ultraweak photon emission from the cultured neurons was attenuated by application of DNPH which is a chemical agent to derivatize carbonyl groups (ketone and aldehyde) in protein side chains or lipids to 2,4-dinitrophenylhydrazone (Fig. 3B). DNPH applied at such a low concentration (5 μM) did not induce any histological abnormalities in the cultured neurons, i.e., neither neuronal degeneration nor neurite disruption. It indicates that the attenuation of emission by DNPH was not brought about by decrease in number of living cells in the culture, but extinction of carbonyl groups, especially excited triplets of them which are known to emit photons of the spectral range of 400–580 nm (4) including the wavelength detected in the present experiment. Such carbonyl groups are believed to be produced under oxidative stress including generation of reactive oxygen species in the cells; therefore it is likely that the activity-dependent ultraweak photon emission from neural tissue originated from intrinsically oxidized molecules. It is supported by the DNPH-induced attenuation of photon emission from triplet states of carbonyl groups which were produced in extrinsically photo-oxidized brain slices (Fig. 3D), and also by some other reports which have assumed oxidized molecules or radical oxidation reactions as origin of emission in isolated hepatocytes (4) or human skin (5) under oxidative stress, formaldehyde-stimulated yeast cells (14), or apoptotic neuroblastoma cells (7). The present report is the first one demonstrating neuronal activity-dependent intrinsic photon emission via tissue oxidation.

Recently, newly developed experimental techniques realizing detection of neuronal and/or brain activity has brought about great progresses in neuroscience, i.e., monitoring membrane potential using voltage sensitive dye or neural activity by change of light scatter-

ing in the tissue. Noninvasive detection of spontaneous ultraweak photon emission, however, has not been employed for that, because of unknown mechanism of the emission. The present study demonstrating activity-dependent photon emission from neural tissue via the activity-dependent tissue oxidation, could bring about the noninvasive monitoring of the neural activity in various experimental studies. Furthermore, the present technique could also realize noninvasive estimation of oxidative stress or diagnosis of neurological disorders involving tissue oxidation. Especially in combination with photo-dynamic tissue oxidation technique (13), detection of the ultraweak photon emission is very useful for noninvasive and real-time estimation of tissue oxidation level, since photo-oxidized tissue induces much more intensive photon emission exactly corresponding to the oxidation level (Fig. 3D). In photodynamic therapy, also, the present technique will be applied for estimation of oxidation-induced lethal effect on the therapeutically targeted tissue.

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REFERENCES

1. Usa, M., Devaraj, B., Kobayashi, M., Takeda, M., Ito, H., Jin, M., and Inaba, H. (1994) Detection and characterization of ultraweak biophotons from life processes *in* Optical Methods in Biomedical and Environmental Sciences (Ohzu, H., and Komatsu, S., Eds.), pp. 3–6, Elsevier, Amsterdam.
2. Kobayashi, M., Devaraj, B., Usa, M., Tanno, Y., Takeda, M., and Inaba, H. (1997) Two-dimensional imaging of ultraweak photon emission from germinating soybean seedlings with a highly sensitive CCD camera. *Photochem. Photobiol.* **65,** 535–537.
3. Scott, R. Q., Usa, M., and Inaba, H. (1989) Ultraweak emission imagery of mitosing soybeans. *Appl. Physiol. B* **48,** 183–185.
4. Cadenas, E., Wefers, H., and Sies, H. (1981) Low-level chemiluminescence of isolated hepatocytes. *Eur. J. Biochem.* **119,** 531–536.
5. Sauermann, G., Mei, W. P., Hoppe, U., and Stäb, F. (1999) Ultraweak photon emission of human skin *in vivo:* Influence of topically applied antioxidants on human skin. *Methods Enzymol.* **300,** 419–428.
6. Boveris, A., Varsavsky, A. I., Goncalves da Silva, S., and Sanchez, R. A. (1983) Chemiluminescence of soybean seeds: Spectral analysis, temperature dependence and effect of inhibitors. *Photochem. Photobiol.* **38,** 99–104.
7. Maccarrone, M., Salucci, M. L., Melino, G., Rosato, N., and Finazzi-Agro, A. (1999) The early phase of apoptosis in human neuroblastoma CHP100 cells is characterized by lipoxygenase-dependent ultraweak light emission. *Biochem. Biophys. Res. Commun.* **265,** 758–762, doi:10.1006/bbrc.1999.1744.
8. Isojima, Y., Isoshima, T., Nagai, K., Kikuchi, K., and Nakagawa, H. (1995) Ultraweak biochemiluminescence detected from rat hippocampal slices. *NeuroReport* **6,** 658–660.
9. Isoshima, T., Isojima, Y., Hakomori, K., Kikuchi, K., Nagai, K., and Nakagawa, H. (1995) Ultrahigh sensitivity single-photon detector using a Si avalanche photodiode for the measurement of ultraweak biochemiluminescence. *Rev. Sci. Instrum.* **66,** 2922–2926.
10. Kudo, T., Nonomura, T., Enokido, Y., and Hatanaka, H. (1995) Brain-derived neurotrophic factor (BDNF) can prevent apoptosis of rat cerebellar granule neurons in culture. *Dev. Brain Res.* **85,** 249–258.
11. Levine, R. L., Williams, J. A., Stadtman, E. R., and Shacter, E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233,** 346–357.
12. Nicoll, R. A., and Alger, B. E. (1981) A simple chamber for recording from submerged brain slices. *J. Neurosci. Methods* **4,** 153–156.
13. Kataoka, Y., Morii, H., Imamura, K., Cui, Y., Kobayashi, M., and Watanabe, Y. (2000) Control of neurotransmission, behaviour, and development, by photo-dynamic manipulation of tissue redox state of brain targets. *Eur. J. Neurosci.* **12,** 4417–4423.
14. Godlewski, M., Rajfur, Z., Slawinski, J., Kobayashi, M., Usa, M., and Inaba, H. (1993) Spectra of the formaldehyde-induced ultraweak luminescence from yeast cells. *J. Photochem. Photobiol. B: Biol.* **21,** 29–35.