Oxidative Stress-Induced Increase in Intracellular Ca\(^{2+}\) and Ca\(^{2+}\)-Induced Increase in Oxidative Stress: An Experimental Model Using Dissociated Rat Brain Neurons

Yasuo Oyama\(^1\), Eisuke Okazaki\(^1\), Lumi Chikahisa\(^1\), Takayuki Nagano\(^1\) and Chiharu Sadakata\(^1\)

\(^1\)Laboratory of Cellular Signaling, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770, Japan
\(^2\)Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., Hanno 357, Japan

Received August 19, 1996 Accepted October 21, 1996

ABSTRACT—In order to study the oxidative stress-induced change in intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and Ca\(^{2+}\)-induced oxidative stress, effects of hydrogen peroxide and ionomycin, a calcium ionophore, on rat cerebellar neurons were examined using a flow cytometer and fluorescent dyes: fluo-3 for monitoring [Ca\(^{2+}\)]\(_i\); 2',7'-dichlorofluorescin, for reactive oxygen species; and 5-chloromethylfluorescein, for cellular nonprotein thiols. Oxidative stress induced by hydrogen peroxide dose-dependently increased [Ca\(^{2+}\)]\(_i\), and decreased the content of nonprotein thiols. Ionomycin increased oxidative metabolism and decreased the content of nonprotein thiols. Results suggest that oxidative stress induces an increase in [Ca\(^{2+}\)]\(_i\) while an increase in [Ca\(^{2+}\)]\(_i\) increases oxidative stress in neurons.

Keywords: Intracellular Ca\(^{2+}\), Oxidative stress, Neuron

We have shown that the calcium ionophore ionomycin increases the oxidation of intracellular 2',7'-dichlorofluorescin which is considered to be oxidized by cellular peroxidants including hydrogen peroxide, in mammalian brain neurons; this is associated with an increase in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) (1). Thus, excessive influx of Ca\(^{2+}\) into brain neurons may result in an increased formation of reactive oxygen species that are involved in ischemic brain damages (2-4). The oxidative stress to mammalian cells was shown to induce an increase in [Ca\(^{2+}\)]\(_i\), a process which is proposed to be largely responsible for subsequent cell death or injury (3-5). In this study, to characterize the oxidative stress-induced increase in [Ca\(^{2+}\)]\(_i\), we have examined the effects of hydrogen peroxide as a biological peroxide and ionomycin for increasing [Ca\(^{2+}\)]\(_i\) on the fluo-3,2',7'-dichlorofluorescin and 3-chloromethylfluorescein fluorescences of individual cells using a flow cytometer and a combination of the respective dye with ethidium bromide, which identifies cells that are dead or have compromised membranes so that they can be neglected (6, 7).

Experimental methods for dissociation of rat cerebellar neurons were similar to those described in previous studies (6). In brief, slices of the cerebellum dissected from 10- to 14-day-old Wistar strain rats were treated with dispase (1000 protease units/ml; Godo Shusei, Tokyo) for 60 min at a temperature of 36°C. After enzymatic treatment, cerebellar neurons were dispersed by gentle trituration in Tyrode's solution. The characteristic small size (diameter of 10 \(\mu\)m or less) and spherical shape of the neurons in cell suspension under microscopic observation indicated that they were cerebellar granule cells. Neurons were also selected from the cytogram (forward-angle light scatter versus side light scatter, cell diameter versus cell density) for fluorescence measurement.

To monitor the changes in the [Ca\(^{2+}\)]\(_i\) of neurons with intact membranes, two fluorescent probes, fluo-3-AM (Dojindo Laboratory, Kumamoto) and ethidium bromide (Katayama Chemical Industries, Osaka) were used. The respective dye was added into the cell suspension to achieve a final concentration of 500 nM for fluo-3-AM or 5 \(\mu\)M for ethidium bromide (6). Before measurement of fluo-3 fluorescence, the neurons were incubated with fluo-3-AM for at least 60 min. Fluo-3 fluorescence is predominantly used for monitoring the changes in [Ca\(^{2+}\)]\(_i\), because this dye would be fluorescent if bound to intracellular Ca\(^{2+}\) (8). Ethidium which is highly impermeant to intact membranes can not stain live cells (9). Therefore, live neurons show strong fluorescence of fluo-3 while damaged or dead neurons show ethidium fluores-
To estimate the cellular redox status of neurons, two fluorescent dyes, 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probe, Inc., Eugene, OR, USA) and 3-chloromethylfluorescein diacetate (CMF-DA; Molecular Probe, Inc.), were used (7, 9-12). DCFH-DA and CMF-DA are respectively hydrolyzed to DCFH and CMF by cellular esterases. DCFH is oxidized to DCF, a fluorescent form, by cellular peroxides including hydrogen peroxide (10, 12). DCF fluorescence was measured at least 60 min after application of 100 μM DCFH-DA. CMF-DA is used to estimate the cellular level of non-protein thiols (7, 11). The measurement of CMF fluorescence was done at 30–60 min after the application of 1 μM CMF-DA because the CMF fluorescence attained a peak intensity during this period.

Dual measurements for respective fluorescence (fluo-3, DCF or CMF) and ethidium fluorescence from neurons were made by a flow cytometer (Cyto ACE-150; Japan Spectroscopic Co., Tokyo). The excitation wavelength for all fluorescent dyes was 488 nm produced by an argon laser. Emissions were detected at a wavelength of 530 ± 20 nm for fluo-3, DCF and CMF and 600 ± 20 nm for ethidium. Fluorescence cytograms (Fig. 1A) obtained from a programmed number of neurons were analyzed by software (Jasco Ver.3XX, Japan Spectroscopic Co.) and a personal computer (PC-9801RX; NEC, Tokyo). Tentative calibration of fluo-3 fluorescence for [Ca^{2+}]_i was made by a method using ionomycin and Mn^{2+} (8). In brief, the [Ca^{2+}]_i was calculated by the following equation: [Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F), where K_d is 400 nM at vertebrate ionic strength, F_{max} represents the maximum fluorescence intensity from the Ca^{2+}-saturated cellular dye, F_{min} represents the minimum intensity predicted from the metal-free dye fluorescence and background fluorescence, and F is the fluorescence intensity obtained during the experiment. Fluorescence intensities were obtained from the mean intensity of fluo-3 fluorescence histogram (Fig. 1B) constructed from 4000 or 5000 live neurons that were not stained with ethidium. Statistical analysis was performed with the two-sample t-test. P values of <0.05 were considered to be significant.

There was a large population of cells stained predominantly with fluo-3, but not with ethidium, in the control fluorescence cytogram, while a large population of cells was predominantly stained with ethidium after lysis of the cells with 50 μM digitonin that released fluo-3 and permitted ethidium to permeate the membranes (Fig. 1A: a and d). Digitonin was reported to release essentially all of the lactic dehydrogenase, a standard cytosolic mark-

![Fig. 1. Flow-cytometric measurements of fluo-3 and ethidium fluororescences from a programmed number of neurons (5000 cells). A: panels show the fluorescence cytograms, fluo-3 fluorescence (abscissa) versus ethidium fluorescence (ordinate), before (a, CONTROL), 60 min after application of 2.5 μM ionomycin (b, IONOMYCN) and 1 mM hydrogen peroxide (c, H_2O_2), and 2 min after application of 50 μM digitonin (d, DIGITONIN). The intensity of fluo-3 fluorescence indicated with arrows shows the mean intensity of fluo-3 fluorescence measured from the neurons that were not stained with ethidium. B: effects of 1 mM hydrogen peroxide on histogram of fluo-3 fluorescence obtained from a programmed number of rat neurons (4000 cells) that were not stained with ethidium. Superimposed histograms were obtained before (CONT) and 10 min (a), 30 min (b) and 60 min (c) after application of 1 mM hydrogen peroxide and 60 min after application of 2.5 μM ionomycin (d, IONO).]
er (8). Thus, the cells stained predominantly with ethidium in the cytogram are dead or have compromised membranes (9). In the continued presence of 2.5 μM ionomycin or 1 mM hydrogen peroxide, there was another cell population consisting of neurons exerting both fluo-3 and ethidium fluorescence (Fig. 1A: b and c), suggesting neurons with compromised membranes. Since such neurons partly lost fluo-3, the fluo-3 fluorescence was measured only from the neurons that were not stained with ethidium as indicated with the arrow in Fig. 1A. Hydrogen peroxide at a concentration of 1 mM shifted the fluorescence histogram to a higher intensity direction in a time-dependent manner (Fig. 1B: a to g). Ionomycin at a concentration of 2.5 μM greatly shifted the histogram (Fig. 1B: d). A prolonged exposure of neurons to hydrogen peroxide (100 μM or higher) produced a great decrease in cell viability. Therefore, the dose-dependent effect of hydrogen peroxide on the [Ca²⁺], was examined at 60 min after application of hydrogen peroxide at concentrations ranging from 1 μM to 1 mM (Fig. 2A). Hydrogen peroxide started to increase the [Ca²⁺], at the concentration of 10 μM. Increasing the concentration of hydrogen peroxide produced a further increase in the [Ca²⁺],. Application of hydrogen peroxide also dose-dependently increased the intensity of DCF fluorescence by oxidation of cellular DCFH (12). As shown in Fig. 2B, the hydrogen peroxide-induced increase in [Ca²⁺], was greatly correlated with the oxidative effect of hydrogen peroxide on cellular DCFH.

Application of 2.5 μM ionomycin induced the saturation of fluo-3 fluorescence in almost all neurons, indicating a great increase in the [Ca²⁺], of intact neurons. Although it was impossible to estimate the mean value of [Ca²⁺], because of saturation, the [Ca²⁺], after application of ionomycin was estimated to be greater than 3 μM in some neurons. Ionomycin also augmented the intensity of DCF fluorescence significantly as shown previously (1, 13). However, the potency of ionomycin in augmenting DCF fluorescence (the potency of oxidative effect of ionomycin) was unknown. Therefore, the effect of ionomycin on DCF fluorescence was compared with that of hydrogen peroxide. As shown in Fig. 3A, the ionomycin-induced augmentation of DCF fluorescence was almost equivalent to those of 100–300 μM hydrogen peroxide. Furthermore, hydrogen peroxide at the concentrations of 100 μM to 1 mM greatly attenuated the CMF fluorescence intensity of neurons (Fig. 3B), which represents the cellular level of nonprotein thiols, presumably glutathione (7, 11). Ionomycin also decreased the intensity of CMF fluorescence, indicating an ionomycin-
induced decrease in cellular content of nonprotein thiols.

Fluo-3, DCF and CMF are converted respectively from fluo-3-AM, DCF-DA and CMF-DA by cellular esterases and retained within the neurons if neurons possess intact membranes (9). Neurons with compromised membranes are unable to retain the respective dyes, resulting in a decreased intensity of fluorescence that leads to underestimation. In the present study, the intensity of fluo-3, DCF or CMF fluorescence was measured in combination with ethidium fluorescence. Ethidium is highly impermeant to the membranes of intact neurons (9). Ethidium is permeant following membrane disruption (loss of membrane integrity), resulting in augmentation of cellular ethidium fluorescence. Therefore, since the neurons exerting ethidium fluorescence were not suitable for measurements of fluo-3, DCF and CMF fluorescences, fluorescence measurements of the respective dyes were performed on intact neurons that retained the dye by neglecting dead or damaged neurons that were stained with ethidium.

Excessive influx of Ca²⁺ into neurons may result in an increased formation of reactive oxygen species that are involved in ischemic brain damage (3, 4). In fact, ionomycin that greatly increases the [Ca²⁺]; of neurons augmented DCF fluorescence (Fig. 3A), suggesting an oxidation of cellular DCFH, depending on the [Ca²⁺]; increased by ionomycin (1). The potency of ionomycin in increasing the intensity of DCF fluorescence was almost equivalent to that of hydrogen peroxide at concentrations of 100–300 μM (Fig. 3A). These concentrations of hydrogen peroxide seem to be toxic for brain neurons because 100–300 μM hydrogen peroxide increased [Ca²⁺]; (Fig. 2A). It is likely that ionomycin causes oxidative stress to neurons. Furthermore, ionomycin reduced the intensity of CMF fluorescence (Fig. 3B), indicating a decrease in the cellular content of nonprotein thiols. It is likely that the cellular content of glutathione is greatly reduced during exposure of neurons to ionomycin because glutathione is a major component of cellular nonprotein thiols. The potency of 2.5 μM ionomycin in decreasing the intensity of CMF fluorescence is almost equivalent to that of 5 mM diethylmaleate (14) (Y. Oyama et al., unpublished observation). Therefore, from our results, it is also suggested that an abnormal elevation of [Ca²⁺]; increases the formation of reactive oxygen species that induce oxidative stress to the neurons. As shown in Fig. 2A, hydrogen peroxide at concentrations of 10 μM or greater dose-dependently increased the [Ca²⁺]; of neurons. The effect of hydrogen peroxide on [Ca²⁺]; is closely correlated with its effect on the intensity of DCF fluorescence resulting from oxidation of cellular DCFH (Fig. 2B). The relation between elevation of [Ca²⁺]; and oxidative stress seems to have a pathological implication since both are proposed to be linked in neuronal death (2–5, 15). Thus, there is a possibility that uncontrolled and sustained elevation of [Ca²⁺]; increases the formation of reactive oxygen species that induce a further increase in [Ca²⁺];. If so, such a cycle would be responsible for neuronal death.

---

**Fig. 3.** Effects of hydrogen peroxide (100 μM to 1 mM) and ionomycin (2.5 μM) on the intensities of DCF fluorescence (A) and CMF fluorescence (B). Each symbol and bar indicate the average of mean intensity and S.D. of 4 experiments. Asterisks indicate significant changes when compared with the control (***P < 0.005).
Acknowledgments

This study was supported by Grants-in-Aid from the Ciba-Geigy Foundation (Japan) for the Promotion of Science.

REFERENCES

1. Oyama Y, Hayashi A and Ueha T: Ca^{2+}-induced increase in oxidative metabolism of dissociated mammalian brain neurons: Effect of extract of Ginkgo biloba leaves. Jpn J Pharmacol 61, 367–370 (1993)

2. Flamm ES, Demopoulos HB, Seligman ML, Poser RG and Ransohoff J: Free radicals in cerebral ischemia. Stroke 9, 445–447 (1978)

3. Choi DW: Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci 11, 465–469 (1988)

4. Orrenius S, McConkey DJ, Bellomo G and Nicotera P: Role of Ca^{2+} in toxic cell killing. Trends Pharmacol Sci 10, 281–285 (1989)

5. Whittemore ER, Loo DT, Watt JA and Cotman CW: A detailed analysis of hydrogen peroxide-induced cell death in primary neuronal culture. Neuroscience 67, 921–932 (1995)

6. Oyama Y, Chikahisa L, Ueha T, Hatakeyama Y and Kokubun T: Change in membrane permeability induced by amyloid β-protein fragment 25–35 in brain neurons dissociated from rats. Jpn J Pharmacol 68, 77–83 (1995)

7. Chikahisa L, Oyama Y, Okazaki E and Noda K: Fluorescent estimation of H$_2$O$_2$-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Jpn J Pharmacol 71, 299–305 (1996)

8. Kao JPY, Harootunian AT and Tsien RY: Photochemically generated cytosolic calcium pulses and their detection by fluo-3. J Biol Chem 264, 8179–8184 (1989)

9. Haugland RP: Fluorescent dyes for assessing vital cell functions. In The Handbook of Fluorescent Probes and Research Chemicals, Edited by Haugland RP, pp 172–180, Molecular Probe, Inc, Eugene (1992)

10. Keston AS and Brandt R: The fluorometric analysis ultramicro quantities of hydrogen peroxide. Anal Biochem 11, 1–5 (1965)

11. Poot M, Kavanagh TJ, Kang HC, Haugland RP and Rabinovitch PS: Flow cytometric analysis of cell cycle-dependent changes in cell thiol level by combining a new laser dye with Hoechst 33342. Cytometry 12, 184–187 (1991)

12. Oyama Y, Hayashi A, Ueha T and Mackawa K: Characterization of 2′,7′-dichlorofluorescin fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide. Brain Res 635, 113–117 (1994)

13. Oyama Y, Furukawa K, Chikahisa L and Hatakeyama L: Effect of N,N-diethyldithiocarbamate on ionomycin-induced increase oxidation of cellular 2′,7′-dichlorofluorescin in dissociated cerebellar neurons. Brain Res 660, 158–161 (1994)

14. Plummer JL, Smith BR, Sies M and Bend JR: Chemical depletion of glutathione in vivo. Methods Enzymol 77, 50–59 (1981)

15. McCord JM: Oxygen-derived free radicals in the post ischemic tissue injury. N Engl J Med 312, 159–163 (1985)