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Decrease in intracellular Zn^{2+} level by propranolol: A model experiment using rat thymocytes

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Abstract

Propranolol is a representative β-blocker used for the treatment of cardiovascular diseases. Because the use of propranolol expands for some clinical purposes that are not related to its β-blocking action, it is necessary to further examine cellular actions of propranolol. We revealed that propranolol decreased the intracellular Zn^{2+} level in rat thymocytes by the use of cytometric technique with FluoZin-3, a fluorescent indicator of intracellular Zn^{2+}. Propranolol decreased the influx of extracellular Zn^{2+}. However, the agent decreased the intracellular Zn^{2+} level even in the presence of DTPA, a chelator of extracellular Zn^{2+}. Thus, the decrease in intracellular Zn^{2+} level by propranolol was not due to the decrease in Zn^{2+} influx by propranolol. Propranolol increased the cellular content of nonprotein thiol that was estimated by the use of 5-chloromethylfluorescein fluorescence, an indicator for non-proteinous thiol. Since non-proteinous thiols can make a complex with Zn^{2+}, propranolol may increase the cellular content of nonprotein thiol, resulting in the decrease in intracellular Zn^{2+} level. Since the concentrations of propranolol to affect both intracellular Zn^{2+} level and cellular content of nonprotein thiol are higher than those reported under clinical conditions, it is difficult to emphasize clinical implications from present results.

Key words: Propranolol; Intracellular Zn^{2+} level; FluoZin-3

1. Introduction

Propranolol, a representative β-blocker, is used for the treatment of hypertension and arrhythmia (Nies and Shand, 1975). This agent possesses a potent local-anesthetic activity that confers membrane-stabilizing action (Morales-Aguilerá and Vaughan Williams, 1965). The membrane stabilizing action of propranolol is usually explained by the phenomenon that propranolol suppresses a voltage-dependent Na^{+} channel, resulting in the attenuation of membrane excitability. In our previous studies, tetracaine decreased the intracellular Zn^{2+} level (Kimura et al., 2011) whereas lidocaine increased it (Nishimura and Oyama, 2009, 2012). Although both agents are local anesthetics, they exerted opposite actions. Therefore, we examined the effect of propranolol on intracellular Zn^{2+} level in rat thymocytes by the use of FluoZin-3 fluorescence because of a following reason. Propranolol is relatively safe in clinical use. The use of propranolol expands for some clinical purposes that are not related to its β receptor blocking action. Therefore, it is necessary to further examine cellular actions (or cytotoxic actions) of propranolol.

2. Materials and methods

2.1. Chemicals

Propranolol hydrochloride (*d*,*l*-types) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Tetracaine hydrochloride, NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemicals (Osaka, Japan). FluoZin-3-AM and propidium iodide were obtained from Molecular Probes Inc. - Invitrogen (Eugene, Oregon, USA). FluoZin-3-AM was initially dissolved in DMSO. The final concentration of DMSO in the cell suspension (maximum 0.2 %) did not affect any fluorescence. Diethylenetriamine-pentaacetic acid (DTPA), a Zn^{2+} chelator, was obtained from Dojin Chemical Laboratory (Kumamoto, Japan).

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments at the University of Tokushima

(No. 05279). The cell suspension was prepared in a similar manner to that reported previously (Chikahisa et al., 1996; Matsui et al., 2008; Kinazaki et al., 2011). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 1–2 mm with a blade under cold conditions (3–4°C). The slices were triturated by gently shaking in Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5; pH adjusted to 7.3–7.4 with NaOH) to dissociate the thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (diameter 10 μm) to prepare the cell suspension. The beaker containing the cell suspension was incubated in a water bath at 36–37°C for 1 h before the experiment. Although the Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM zinc derived from the cell preparation (Sakanashi et al., 2009).

Rat thymocytes were used for the study because single cells can be prepared without enzymatic treatment; thus, their cell membranes remain intact. In addition, the modification of cell death (apoptosis and necrosis) by zinc has been studied extensively in murine thymocytes (Barbieri et al., 1992; McCabe et al., 1993; Provinciali et al., 1995; Maclean et al., 2001).

2.3. Fluorescence measurements of cellular and membrane parameters

The methods for measuring the cellular and membrane parameters by using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes were similar to those described previously (Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed by JASCO software (Version 3.06; JASCO).

To assess cell lethality, propidium iodide was added to the cell suspensions to a final concentration of 5 μM. Because propidium stains dead cells, the measurement of propidium fluorescence from the cells provided information on lethality. The fluorescence was measured 2 min after the application of propidium iodide by a flow cytometer. The excitation wavelength used for propidium was 488 nm and the emission was detected at 600 ± 20 nm.

FluoZin-3-AM (Gee et al., 2002) was used as an indicator of intracellular Zn^{2+}. The cells were incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements were taken to estimate the change in the intracellular Zn^{2+} level of the rat thymocytes with intact membranes. FluoZin-3 fluorescence was measured in the cells that were not stained with propidium iodide (Matsui et al., 2008). The excitation wavelength used for FluoZin-3 was 488 nm and the emission was detected at 530 ± 20 nm.

5-CMF-DA was used to monitor changes in the cellular content of nonprotein thiols (Chikahisa et al., 1996). The cells were incubated with 1 μM 5-CMF-DA for 30 min before any fluorescence measurements were taken. 5-CMF fluorescence was measured in the cells that were not stained with propidium iodide. The excitation wavelength used for 5-CMF was 488 nm, and the emission was detected at 530 ± 15 nm.

The agents were added to the cell suspension (2 mL) and the cells were incubated at 36–37°C under room air conditions. The incubation time was dependent on each experiment. The acquisition of the fluorescence data from 2000 or 2500 cells using a flow cytometer required the time of 30 s or less and 100 μL of the cell suspension was used for the fluorescence measurements.

2.4. Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed with Tukey's multivariate analysis. A *P* value of < 0.05 was considered significant.

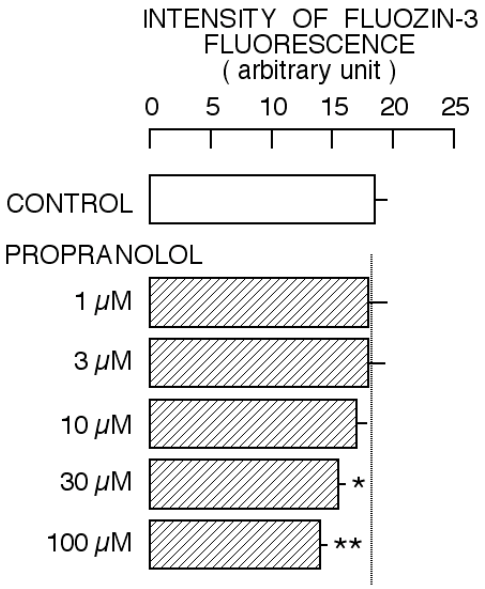

Figure 1. Concentration-dependent change in the intensity of FluoZin-3 fluorescence as a result of propranolol application. Effects were examined 60 min after the start of drug application. The columns and bars indicate the mean and standard deviation, respectively, of 4 experiments. Dotted line indicates control level. Symbols (,**) indicate a significant difference ($P < 0.05$, 0.01) between the control group (CONTROL) and the groups of cells treated with propranolol. The experiments were performed in duplicate and reproduced twice. One representative experiment is shown.*

3. Results

3.1. Effect of propranolol on the FluoZin-3 fluorescence of rat thymocytes

The treatment of cells with 100 μM propranolol for 1 hr slightly shifted the histogram of FluoZin-3 fluorescence to a direction of lower intensity, suggesting the decrease in intracellular Zn^{2+} level by propranolol. The threshold concentration of propranolol to attenuate the FluoZin-3 fluorescence was 10 μM (Fig. 1). Further increases in propranolol concentration (30–100 μM) induced further attenuation of FluoZin-3 fluorescence (Fig. 1B). The attenuation of FluoZin-3 fluorescence by propranolol (30–100 μM) was statistically significant.

3.2. Effect of DTPA on the propranolol-induced change in FluoZin-3 fluorescence

To remove the contribution of extracellular Zn^{2+}, the effect of propranolol was examined in the presence of 10 μM DTPA. As shown in Fig. 2, the incubation of cells with DTPA for 1 hr attenuated the FluoZin-3 fluorescence, suggesting the decrease in intracellular Zn^{2+} level by removing extracellular Zn^{2+}. In the presence of DTPA, 30 μM propranolol still induced significant attenuation of FluoZin-3 fluorescence (Fig. 2).

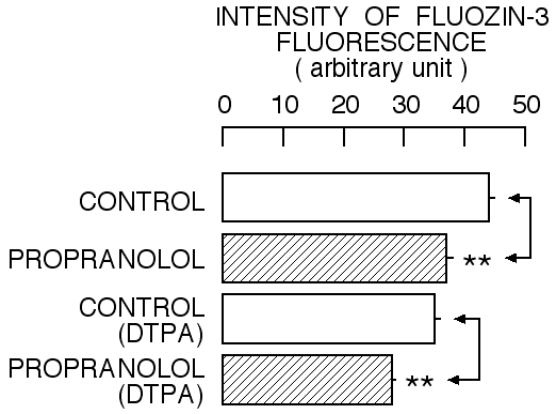

Figure 2. Effect of propranolol on FluoZin-3 fluorescence in the presence of DTPA. The columns and bars indicate the mean intensity and standard deviation, respectively, of 4 experiments. The symbol (**) indicates significant differences (P < 0.01) between the control group and the group of cells treated with propranolol.

3.3. Effect of propranolol on the Zn^{2+} influx induced by the external addition of $ZnCl_2$

The results shown in Fig. 2 suggested that extracellular Zn^{2+} was not involved in the propranolol-induced attenuation of FluoZin-3 fluorescence. However, it was presumably suggested that the Zn^{2+} influx maintained intracellular Zn^{2+} level because DTPA attenuated the FluoZin-3 fluorescence. To test whether propranolol affects Zn^{2+} influx, the effect of propranolol on the augmentation of FluoZin-3 fluorescence induced by external addition of ZnCl2 was examined. The augmentation of FluoZin-3 fluorescence by adding $ZnCl_2$ (presumably Zn^{2+} influx) was diminished under cold condition (1–4°C) (Kimura et al., 2011). The addition of 10 μM $ZnCl_2$ into the cell suspension significantly augmented the FluoZin-3 fluorescence. The treatment of cells with 1–3 μM propranolol for 1 hr did not affect the augmentation of FluoZin-3 fluorescence. Increases in propranolol concentration (up to 100 μM) attenuated the increase in the intensity of FluoZin-3 fluorescence by the addition of $ZnCl_2$ (Fig. 3). The augmentation of FluoZin-3 fluorescence by $ZnCl_2$ was significantly reduced by 30–100 μM propranolol. The reduction by 10 μM propranolol was not statistically significant.

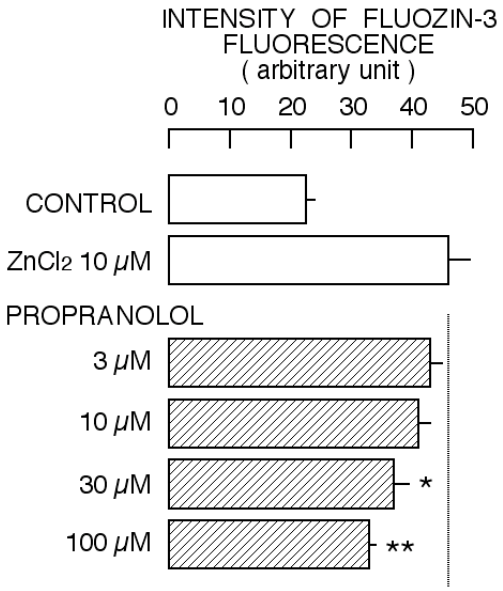

Figure 3. Effect of propranolol on the FluoZin-3 fluorescence augmented by the external application of $ZnCl_2$. The columns and bars indicate the mean intensity and standard deviation, respectively, of 4 experiments. Dotted line shows control level after the application of $ZnCl_2$. The symbols (,**) indicate significant differences (P < 0.05, 0.01) between the control group and the groups of cells treated with propranolol.*

3.4. Effect of propranolol on cellular content of nonprotein thiol

Cellular content of nonprotein thiol is mutually associated with intracellular Zn^{2+} concentration (Kinazaki et al., 2011). Propranolol may change cellular content of nonprotein thiol. In this study, we examined the change in 5-CMF fluorescence, a

parameter of nonprotein thiol, in the cells treated with propranolol. The cells were treated with 10–300 μM propranolol for 60 min and then 5-CMF-DA was applied to the cells. Thereafter, 5-CMF fluorescence was measured at 40 min after the application of 5-CMF-DA (Chikahisa et al., 1996). As shown in Fig. 4, the incubation of cells with propranolol at concentrations ranging from 10 μM to 300 μM induced concentration-dependent augmentation of 5-CMF fluorescence. The augmentation only by 300 μM propranolol was statistically significant. The result suggested that propranolol at 300 μM significantly increased cellular content of nonprotein thiol (not shown in Fig. 4). Control intensity was 52.0 ± 4.1 (arbitrary unit, mean ± SD of four experiments) while it was 75.3 ± 8.2 after the incubation with 300 μM propranolol for 60 min.

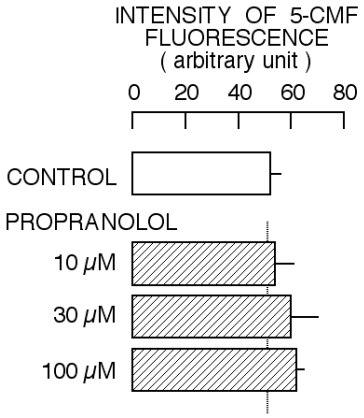

Figure 4. Effect of propranolol on 5-CMF fluorescence. Dotted line indicates control level. The columns and bars indicate the mean intensity and standard deviation, respectively, of 4 experiments.

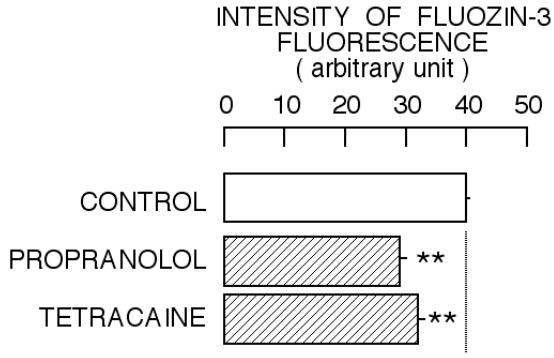

Figure 5. Comparison of propranolol with tetracaine. The columns and bars indicate the mean intensity and standard deviation, respectively, of 4 experiments. Dotted line shows control level. The symbol (**) indicates significant differences ($P < 0.01$) between the control group and the group of cells treated with the drug.

3.5. Comparison with tetracaine

Propranolol possesses a local anesthetic activity (Morales-Aguilerá and Vaughan Williams, 1965). Since tetracaine attenuated the increase in intracellular Zn^{2+} concentration induced by the application of $ZnCl_2$ (Kimura et al., 2011), the effect of propranolol was compared with that of tetracaine. Their effects at the concentration of 100 μM were examined. Propranolol and tetracaine decreased the intensity of FluoZin-3 fluorescence (Fig. 5). The potency of propranolol was greater than that of tetracaine. Therefore, the agents were supposed to attenuate the increase in the intensity of FluoZin-3 fluorescence by the addition of $ZnCl_2$ into the suspension.

4. Discussion

The relation between FluoZin-3 fluorescence and 5-CMF fluorescence showed a negative correlation in the cells under some chemical stresses induced by N-ethylmaleimide (Hashimoto et al., 2009; Kawanai et al., 2009; Oyama et al., 2009; Kinazaki et al., 2011). Decrease in cellular content of nonprotein thiol seemed to induce intracellular Zn^{2+} release. Such a negative relationship between them is true because intracellular Zn^{2+} makes a complex with the thiol group of protein and nonprotein (Jacob et al., 1998), and the modification from thiol to disulfide releases Zn^{2+} from protein and nonprotein (Maret et al., 1994). Propranolol slightly augmented the 5-CMF fluorescence, indicating the increase in cellular content of nonprotein thiol. This phenomenon may decrease intracellular Zn^{2+} release, resulting in the decrease in intracellular Zn^{2+} concentration.

Propranolol possesses local anesthetic action (Morales-Aguilerá and Vaughan Williams, 1965). However, this action is approximately two order of magnitude higher in concentration than that necessary for expression of the β-adrenergic antagonism. The local anesthetic activity of propranolol may be related to its action on intracellular Zn^{2+} level because micromolar concentrations (> 10 μM) were required to affect intracellular Zn^{2+} level (Figs. 1 and 2). However, tetracaine attenuated the FluoZin-3 fluorescence whereas lidocaine augmented it (Kimura et al., 2011; Nishimura and Oyama, 2012). Therefore, local anesthetics seem to be further classified by their actions on intracellular Zn^{2+} level.

The blood concentration of propranolol in human after a single oral dose (80 mg) was less than 1 μM (Mahajan et al., 1984). The concentrations of propranolol to exert the action on intracellular Zn^{2+} level were micromolar in this study (Figs. 1 and 2). The micromolar concentration of propranolol was observed in the blood of fatal case (Hong et al., 1983). Therefore, it is quite difficult to emphasize clinical

implications on the action of propranolol on intracellular Zn^{2+} level.

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Conflict of interest

We had no conflicts of interests in relation to this study.

References

Barbieri, D., Troiano, L., Grassilli, E., Agnesini, C., Cristofalo, E.A., Monti, D., Capri, M., Cossarizza, A., Franceschi, C., 1992. Inhibition of apoptosis by zinc: A reappraisal. Biochem. Biophys. Res. Comm. 187, 1256–1261.

Chikahisa, L., Oyama, Y., Okazaki, E., Noda, K., 1996. Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Jpn. J. Pharmacol. 71, 299–305.

Gee, K.R., Zhou, Z.L., Qian, W.J., Kennedy, R., 2002. Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. J. Am. Chem. Soc. 124, 776–778.

Hashimoto, E., Oyama, T.B., Oyama, K., Nishimura, Y., Oyama, T.M., Ueha-Ishibashi, T., Okano, Y., Oyama, Y., 2009. Increase in intracellular Zn^{2+} concentration by thimerosal in rat thymocytes: intracellular Zn^{2+} release induced by oxidative stress. Toxicol. In Vitro 23, 1092–1099.

Hong, C.Y., Yang, W.C., Chiang, B.N., 1983. Importance of membrane stabilizing effect in massive overdose of propranolol: plasma level study in a fatal case. Hum. Toxicol. 2, 511-517.

Jacob, C., Maret, W., Vallee, B.L., 1998. Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proc. Natl. Acad. Sci. U.S.A. 95, 3489–3494.

Kawanai, T., Satoh, M., Murao, K., Oyama, Y., 2009. Methylmercury elicits intracellular Zn^{2+} release in rat thymocytes: its relation to methylmercury-induced decrease in cellular thiol content. Toxicol. Lett. 191, 231–235.

Kimura, K., Nishimura, Y., Oyama, K., Kawanai, T., Hashimoto, E., Oyama, Y., 2011. Tetracaine decreases intracellular Zn^{2+} concentration by inhibiting Zn^{2+} influx in rat thymocytes. Nat. Sci. Res. Univ. Tokushima 25, 7–13.

Kinazaki, A., Chen, H., Koizumi, K., Kawanai, T., Oyama, T.M., Satoh, M., Ishida, S., Okano, Y., Oyama, Y., 2011. Putative role of intracellular Zn^{2+} release during oxidative stress: A trigger to restore cellular thiol content that is decreased by oxidative stress. J. Physiol. Sci. 61, 403–409.

Maclean, K.H., Cleveland, J.L., Porter, J.B., 2001. Cellular zinc content is a major determinant of iron chelator-induced apoptosis of thymocytes. Blood. 98, 3831–3839.

Mahajan, P., Grech, E.D., Pearson, R.M., Ridgway, E.J., Turner, P., 1984. Propranolol concentrations in blood serum, seminal plasma and saliva in man after a single oral dose. Br. J. Clin. Pharmacol. 18, 849–852.

Maret, W., 1994. Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proc. Natl. Acad. Sci. U.S.A. 91, 237–241.

Matsui, H., Sakanashi, Y., Oyama, T.M., Oyama, Y., Yokota, S., Ishida, S., Okano, Y., Oyama, T.B., Nishimura, Y., 2008. Imidazole antifungals, but not triazole antifungals, increase membrane Zn^{2+} permeability in rat thymocytes: Possible contribution to their cytotoxicity. Toxicology. 248, 142–150.

Matthews, J.C., Baker, J.K., 1982. Effects of propranolol and a number of its analogues on sodium channels. Biochem. Pharmacol. 31, 1681-1685.

McCabe, M.J., Shun A.J., Orrenius, S., 1993. Chelation of intracellular zinc triggers apoptosis in mature thymocytes. Lab. Invest. 69, 101–110.

Morales-Aguilerá, A., Vaughan Williams, E.M., 1965. The effects on cardiac muscle of β-receptor antagonists in relation to their activity as local anaesthetics. Br. J. Pharmacol. Chemother. 24, 332–338.

Nies, A.S., Shand, D.G., 1975. Clinical pharmacology of propranolol. Circulation 52, 6-15.

Nishimura, Y., Oyama, Y., 2009. Cytotoxic actions of lidocaine at sublethal concentrations: A model in vitro experiment using rat thymocytes. Nat. Sci. Res. Univ. Tokushima. 24, 1–5.

Nishimura, Y., Oyama, Y., 2012. Further analysis on lidocaine-induced increase in intracellular Zn^{2+} concentration: Cytometric model study using FluoZin-3, 5-chloromethylfluorescein, and rat thymocytes Nat. Sci. Res. Univ. Tokushima. 26, 7–10.

Oyama, T.B., Oyama, K., Kawanai, T., Oyama, T.M., Hashimoto, E., Satoh, M., Oyama, Y., 2009. Tri-n-butyltin increases intracellular Zn^{2+} concentration by decreasing cellular thiol content in rat thymocytes. Toxicol. 262, 245–249.

Provinciali, M., Di Stefano, G., Fabris, N., 1995. Dose-dependent opposite effect of zinc on apoptosis in mouse thymocytes. Int. J. Immunopharmacol. 17, 735–744.

Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y., Oyama, Y., 2009. Zn^{2+}, derived from cell preparation, partly attenuates Ca^{2+}-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. Toxicol. In Vitro. 23, 338–345.

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