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Polychlorinated biphenyl (118) activates osteoclasts and induces bone resorption in goldfish

Koji Yachiguchi,[1] Noriko Matsumoto,[1] Yuki Haga,[2] Motoharu Suzuki,[2] Chisato Matsumura,[2] Masahiro Tsurukawa,[2] Toshihiro Okuno,[2] Takeshi Nakano,[2] Kimi Kawabe,[3] Kei-ichiro Kitamura,[4] Akira Toriba,[3] Kazuichi Hayakawa,[3] Vishwajit S. Chowdhury,[5] Masato Endo,[6] Atsuhiko Chiba,[7] Toshio Sekiguchi,[1] Masaki Nakano,[8] Yoshiaki Tabuchi,[9] Takashi Kondo,[10] Shigehito Wada,[11] Hiroyuki Mishima[12], Atsuhiko Hattori,[8] and Nobuo Suzuki[1*]

[1]Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Housu-gun, Ishikawa 927-0553, Japan
[2]Hyogo Prefectural Institute of Environmental Sciences, Kobe, Hyogo 654-0037, Japan
[3]Faculty of Pharmaceutical Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan
[4]Faculty of Health Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kodatsuno, Ishikawa 920-0942, Japan
[5]International Education Center, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
[6]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan
[7]Department of Materials and Life Sciences, Sophia University, Tokyo 102-8554, Japan
[8]Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan
[9]Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Sugitani, Toyama 930-0194, Japan
[10]Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani, Toyama 930-0194, Japan
[11]Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of Toyama, Sugitani, Toyama 930-0194, Japan
[12]Kochi Gakuen College, Kochi 780-0955, Japan

Running title: PCB promotes bone resorption in fish

*Correspondence to: Dr. N. Suzuki, Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan. Tel.: 81-768-74-1151; Fax 81-768-74-1644;
e-mail: nobuos@staff.kanazawa-u.ac.jp

Abstract

Purpose: To analyze the effect of Polychlorinated biphenyl (PCB118) on fish bone metabolism, we examined osteoclastic and osteoblastic activities, as well as plasma calcium levels, in the scales of PCB (118)-injected goldfish. In addition, effect of PCB (118) on osteoclasts and osteoblasts was investigated *in vitro*.

Methods: Immature goldfish, in which the endogenous effects of sex steroids are negligible, were used. PCB (118) was solubilized in dimethyl sulfoxide at a concentration of 10 ppm. At 1 and 2 days after PCB (118) injection (100 ng /g body weight), both osteoclastic and osteoblastic activities, and plasma calcium levels were measured. In an *in vitro* study, then, both osteoclastic and osteoblastic activities as well as each marker mRNA expression were examined.

Results: At 2 days, scale osteoclastic activity in PCB (118)-injected goldfish increased significantly, while osteoblastic activity did not change significantly. Corresponding to osteoclastic activity, plasma calcium levels increased significantly at 2 days after PCB (118) administration. Osteoclastic activation was also occurred in the marker enzyme activities and mRNA expressions *in vitro*. Thus, we conclude that PCB (118) disrupts bone metabolism in goldfish both *in vivo* and *in vitro* experiments.

Keywords: PCB (118), bone metabolism, fish scales, osteoclasts, osteoblasts, plasma calcium

1. Introduction

It has been reported that polychlorinated biphenyl (PCB) congeners act as endocrine-disrupting compounds (Lind et al. 2004; Bovee et al. 2011; Nakayama et al. 2011; Ju et al. 2012). As bone formation and resorption are controlled by several hormones and vitamins (see a review, Peacock 2010), PCBs might disturb bone metabolism. In some animals, actually, the bone disruption caused by PCB has been reported (rat: Lind et al. 2004a; bear: Sonne et al. 2004; sheep: Gutleb et al. 2010; alligator: Lind et al. 2004b; turtle: Holliday DK and Holliday CM 2012; salmon: Olufsen and Arukwe 2011; zebrafish: Ju et al. 2012). In humans, changes in bone metabolism associated with exposure to PCBs have also been investigated (Hodgson et al. 2008). However, the direct effects of PCBs on osteoclasts and osteoblasts have not yet been elucidated in any animals.

The teleost scale is a calcified tissue that contains osteoblasts, osteoclasts, and the bone matrix of two layers (bony layer: a thin, well-calcified external layer; a fibrillary layer: a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg 1993; Suzuki et al. 2000; Yoshikubo et al. 2005; Suzuki et al. 2007; Ohira et al. 2007). The bone matrix, which includes type I collagen (Zylberberg et al. 1992), osteocalcin (Nishimoto et al., 1992), and hydroxyapatite (Onozato and Watabe 1979) is present in the scale as well as in mammalian bone. Recently, we detected both cathepsin K and tartrate-resistant acid phosphatase (TRAP) mRNA expression in scale osteoclasts (Azuma et al. 2007). In osteoblasts, we detected osteoblast-specific markers, such as alkaline phosphatase (ALP), runt-related transcription factor 2, osterix, osteocalcin, type I collagen, and the receptor activator of the NF-κB ligand (Thamamongood et al. 2012). Therefore, the features of osteoclasts and osteoblasts in scales are similar to those in mammals.

In fish as well as mammals, plasma calcium level was regulated by hormones such as parathyroid hormone (Suzuki et al. 2011a) and calcitonin (Suzuki et al. 2000; Suzuki et al. 2004a). In an *in vivo* experiment, fugu parathyroid hormone I induced hypercalcemia resulted from the increase of both osteoblastic and osteoclastic activities in the scale and caused to decrease scale calcium contents (Suzuki et al. 2011a). Scale osteoclastic activation was also observed in the prostaglandin E_2 injected-goldfish (Omori et al. 2012). It is reported that the scales are a better potential internal calcium reservoir than the body skeletons, jaws and otolithes, examined by the ^{45}Ca-labelling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe 1977). Thus, we conclude that teleost scale is an active and functional calcium reservoir.

In fish, PCB (118) is the highest congeners compared with PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -169 (Bhavsar et al. 2007). Furthermore, it has been reported that trabecular bone mineral content was almost 30% lower in the PCB (118) (49 μg/kg body wt/day) at the metaphysis in sheep (Gutleb et al. 2010), although the detail mechanism has not yet been elucidated. We therefore analyzed the effect of PCB (118) on scale osteoclastic and osteoblastic activities, as well as plasma calcium levels, in the goldfish scales. In addition, effect of PCB (118) on osteoclasts and osteoblasts was investigated *in vitro*. This is the first to demonstrate that PCB (118) activates osteoclasts and induced bone resorption in fish.

2. Materials and methods

Animals

To examine the effect of PCB (118) on the bone metabolism, immature goldfish (4-6 g), in which the endogenous effects of sex steroids are negligible, were used for the *in vivo* study. A previous study (Suzuki et al. 2000) indicated that the sensitivity for calcemic hormones was higher in mature female than in mature male teleosts. Therefore, female goldfish (*Carassius auratus*) (30 - 40 g) were purchased from commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used for the *in vitro* experiments.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the plasma calcium in goldfish at day-1 and -2 after PCB (118) injection (in vivo experiment)

PCB (118) was solubilized in dimethyl sulfoxide (DMSO) at a concentration of 10 ppm. Goldfish (body weight: 4 - 6 g) were anesthetized with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and taken the blood (about 100 μl) from caudal vessels of each individual into heparinized syringes just before PCB (118) injection. After centrifugation at 15,000 rpm for 3 min, the plasma was immediately frozen and kept at -80 °C until use. In the experimental group (n = 10), thereafter, PCB (118) was intraperitoneally injected (100 ng/g body weight). The goldfish in the control group (n = 10) were injected with DMSO in the same manner. These goldfish were kept in the aquarium for 1 and 2 days. During the experimental periods, these goldfish were not given any food to exclude intestinal calcium uptake from diets. Each day after injection, the scales were collected from each goldfish. At day-2 after injection, blood samples (about 100 μl) were collected from the gill using a heparinized capillary from individual, anesthetized goldfish. After centrifugation at 15,000 rpm for 3 min, the plasma was also immediately frozen and kept at –80 °C until use. The plasma total calcium level (mg/100 ml) was determined using an assay kit (Calcium C, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, we measured the activities of ALP and TRAP activities as respective indicators of each activity in osteoclasts and osteoblasts (Suzuki et al. 2000; Suzuki et al. 2002; Suzuki et al. 2009). The measurement methods (Suzuki et al. 2009) of ALP and TRAP activities were as follows. The incubated scale was transferred to its own well in a 96-well microplate after washing with saline. An aliquot of 100 μl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$) for ALP activity or an acid buffer (0.1 M sodium acetate including 20 mM tartrate, pH 5.3) for TRAP activity was added to each well. This microplate was frozen at -80°C immediately and then kept at -20°C until analysis. After thawing, an aliquot of 100 μl of 20 mM para-nitrophenyl-phosphate in an alkaline buffer or an acid buffer was added to each well. This plate was then incubated at 20°C for 30 min with shaking. After incubation, the reaction was stopped by adding 50 μl of a 3 N NaOH-20 mM EDTA solution. Aliquots of 150 μl of a colored solution were transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP. After measurement of the absorbance, the ALP and TRAP activities were normalized by the surface area (mm^2) of each goldfish scale. The results are shown as the means ± SE of eight scales.

PCB (118) contents in the scales of goldfish (in vivo experiment)

At day-1 and -2 after PCB (118) injection, the scales were collected from goldfish and then immediately frozen and kept at -80 °C until use. The PCB (118) contents were analyzed by the methods of Hirai et al. (2005). Because a single sample volume was very small, we conducted three measurements to obtain a pulled sample. Thus, the mean of three measurements was described in the results.

Effects of PCB (118) on osteoclastic and osteoblastic activities in the cultured scales of goldfish (in vitro experiment)

Scales collected from goldfish (n = 10) after anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) and incubated for 6 and 18 h in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH, USA) supplemented with PCB (118) (0.025, 0.25, and 2.5 ppm). In an *in vivo* experiment, around 0.1 to 0.05 ppm PCB was detected in the PCB-injected scales. Based on these PCB contents in the scales, we decided the administration doses of PCB in an *in vitro* experiment. The PCB concentration in one goldfish was performed using 48 scales from each left or right side. The 48 scales used in the present study were considered to use as follows: 1) 8 scales for TRAP analysis by 0.025 ppm, 2) 8 scales for TRAP analysis by 0.25ppm, 3) 8 scales for TRAP analysis by 2.5 ppm, 4) 8 scales for ALP analysis by 0.025 ppm, 5) 8 scales for ALP analysis by 0.25ppm, 6) 8 scales for ALP analysis by 2.5 ppm. The respective mean for TRAP (obtained from 8 individual scales of one goldfish) and ALP (obtained from 8 individual scales of one goldfish) activities from left side (experimental group) was compared with those of right side (control group). Using 10 individual goldfish, same experiment was done repeatedly. The experiments for 0.25 and 2.5 ppm PCB (118) were carried out in the same manner. After incubation, TRAP and ALP activities were measured using the same methods described above (Suzuki et al. 2009). The results are shown as means ± SEM (n = 10).

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PCB (118)-treated goldfish scales (in vitro experiment)

Scales were collected from goldfish under anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich). To examine changes in TRAP, cathepsin K, and RANKL mRNAs that responded to PCB (118), these scales were incubated for 18 h in Leibovitz's L-15 medium (Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals). In the prostaglandin E_2-treated scales of goldfish, we previously reported that TRAP, cathepsin K, RANKL mRNA expression increased at 18 h of incubation (Omori et al. 2012). Therefore, this incubation period was adopted. After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for TRAP (sense: 5’-AACTTCCGCATTCCTCGAACAG-3’; antisense: 5’-GGCCAGCCACCAGGAGATAA-3’) (Azuma et al. 2007), cathepsin K (sense: 5’-GCTATGGAGCCACACCAAAAGG-3’; antisense: 5’-CTGCGCTTCCAGCTCTCACAT-3’) (Azuma et al. 2007), and RANKL (sense: 5'-GCGCTTACCTGCGGAATCATATC-3'; antisense: 5'-AAGTGCAACAGAATCGCCACAC-3') (Suzuki et al. 2011a) were used. The amplification of β-actin cDNA using a primer set (5': CGAGCGTGGCTACAGCTTCA; 3': GCCCGTCAGGGAGCTCATAG) (Azuma et al. 2007) was performed. The PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki et al. 2011a). The annealing temperature of TRAP, cathepsin K, RANKL, and β-actin was 60 °C. The TRAP, cathepsin K and RANKL mRNA levels were normalized to the β-actin mRNA level.

Statistical analysis

All results are expressed as the means ± SE (n = 10). The statistical significance between control and experimental group was assessed by Student's *t*-test (*in vivo* experiment) or paired *t*-test (*in vitro* experiment). In all cases, the selected significance level was $P < 0.05$.

3. Results

Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the plasma calcium in goldfish at 1 and 2 days after PCB (118) injection in vivo

We measured the activities of ALP and TRAP activities as respective indicators of each activity in osteoclasts and osteoblasts. At day-2, scale TRAP activity in PCB-injected goldfish increased significantly (Fig. 1a), while ALP activity did not change significantly at day-1 and -2 (Fig. 1b).

Corresponding to the elevation of osteoclastic activity, plasma calcium levels increased significantly at day-2 after PCB administration (Fig. 2)

PCB (118) contents in the scales of goldfish in vivo

At day-1 and -2 after PCB (118) injection, PCB (118) was detected in the scales. At day-1, PCB contents in the control and PCB-injected scales were determined as 0.39 and 79 (ng/g-wet), respectively. At day-2, PCB (ng/g-wet) of 0.38 and 55 was detected in the control and PCB-injected scales, respectively.

Effect of PCB (118) on osteoclastic and osteoblastic activities in the cultured scales of goldfish in vitro

PCB (118) significantly increased the TRAP activities of the scales by 6 h of incubation (P < 0.05 for 0.25 ppm) (Fig. 3a). At 18 h of incubation, the TRAP activities in the PCB (118)-treated scales also significantly increased (P < 0.05 for 0.025 and 2.5 ppm; P<0.001 for 0.25 ppm) (Fig. 4a).

In case of the ALP activities, it significantly increased (P<0.05) only by the concentration of 2.5 ppm at the 6 and 18 h incubation (Figs. 3b and 4b).

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PCB (118)-treated goldfish scales in vitro

The mRNA expression of osteoclastic markers (TRAP and cathepsin K) increased significantly by PCB (118) (0.25 ppm) treatment (Figs. 5a and 5b).

Similar results were obtained in RANKL. The mRNA expression of RANKL, an activating factor of osteoclasts, increased significantly in the osteoblasts in the PCB (118)-treated scales (Fig. 5c).

4. Discussion

In the present study, we are the first to demonstrate that PCB (118) induced hypercalcemia resulting from increasing osteoclastic activity *in vivo*. In an *in vitro* experiment, the data were reproduced and osteoclastic marker mRNA expression as well as enzyme activity increased. In fish, PCB (118) is the highest congeners compared with PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -169 (Bhavsar et al. 2007). In aquatic environment, PCB (118) was detected (Hope 2008; Aksoy et al. 2011). Therefore, we paid attention to bone metabolism by PCB (118) pollution.

At day-1 and -2 after PCB (118) injection intraperitoneally, we detect PCB (118) in the scale. As described in the Introduction, the scales are potential internal calcium reservoir than the body skeletons, jaws and otolithes. Lake et al. (2006) reported that the correlation between the total mercury concentration of the scales and that of the muscles was high ($r = 0.89$). In sheep, PCB was accumulated and detected in bone at 2 months after administration (Jan et al. 2006). We therefore suggest that scale PCB content can be used as an environmental PCB monitor to estimate the environmental pollution of PCB.

In the present study, we measured hydroxy-PCB which is a kind of metabolites from PCB because hydroxy-PCB possessed specific and competitive interactions with the plasma thyroid hormone transport protein, transthyretin (Lans et al. 1993). In PCB-treated scales, however, hydroxyl-PCB was not detected. Therefore, this phenomenon of osteogenesis seems to be direct action of PCB (118).

In an *in vivo* experiments, osteoblastic activity increased by the high concentration of PCB (118)(2.5ppm). This indicates that PCB (118) is affected on osteoblasts. Osteogenesis is regulated by osteoblasts (Suda et al. 1999; Teitelbaum 2000; Lacey et al. 2012). RANKL produced by cells in the osteoblast lineage binds to the receptor activator of NF-κB (RANK) in mononuclear hemopoietic precursors and promotes the formation and activity of multinucleated osteoclasts (Suda et al. 1999; Teitelbaum 2000; Lacey et al. 2012). Our present study indicated that RANKL mRNA expression was promoted by PCB (118) treatment. In addition, osteoclastic marker (TRAP and cathepsin K) mRNA expression also increased significantly. Therefore, we strongly suggest that PCB (118) promotes osteoclastogenesis by the RANK-RANKL pathway.

In the present study, we succeeded to analysis the PCB (118) on osteoclasts and osteoblasts. Our results suggest that scale is a good model for analysis of bone metabolism. We previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone and a good model of osteogenesis (Yoshikubo et al. 2005). Using this system, furthermore, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al. 2000) and that melatonin, a major hormone secreted from the pineal gland, suppressed the functions in both osteoclasts and osteoblasts (Suzuki and Hattori 2002). Osteoblasts in the scale responded to estrogen as they do in mammalian bone (Yoshikubo et al. 2005). In addition, the effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori 2003) and tributyltin (Suzuki et al. 2006), and heavy metals, i.e., cadmium and mercury (Suzuki et al. 2004b; Suzuki et al. 2011b), on osteoblasts and osteoclasts have been examined. Moreover, we indicated that cadmium (even at 10^{-13} M) responded to TRAP activity in the scale (Suzuki et al. 2004b).

In conclusion, PCB (118) disrupts bone metabolism in goldfish both *in vivo* and *in vitro* experiments. Our results suggest that PCB (118) promotes osteoclastogenesis by the RANK-RANKL pathway. Furthermore, our previous and present results indicate that scale assay system will be useful for analysis of environmental contaminant on bone metabolism and findings of PCB (118) on bone in fish may be tied in to an overall health issue for mammals in general.

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6. References

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FIGURE LEGENDS

Fig. 1. Effects of PCB (118) injection on scale TRAP (a) and ALP (b) activities in goldfish. Each column and the vertical line represent the mean ±_SEM (n = 10 samples; one sample from one fish). ** indicates statistically significant difference at $P < 0.01$ from the values in the control.

Fig. 2. Effects of PCB (118) injection on plasma calcium level (mg/100 ml) in goldfish. Each column and the vertical line represent the mean ±_SEM (n = 10 samples; one sample from one fish). ** indicates statistically significant difference at $P < 0.01$ from the values in the control.

Fig. 3. Effects of PCB (118) administration on TRAP (a) and ALP (b) activities in the scales of goldfish at the 6 h of incubation. Each column and the vertical line represent the mean ±_SEM (n = 10 samples; one sample from one fish).

* indicates statistically significant difference at $P<0.05$ from the values in the control.

Fig. 4. Effects of PCB (118) administration on TRAP (a) and ALP (b) activities in the scales of goldfish at the 18 h of incubation. Each column and the vertical line represent the mean ±_SEM (n = 10 samples; one sample from one fish). * and *** indicate statistically significant differences at $P<0.05$ and $P<0.001$, respectively, from the values in the control.

Fig. 5. Effect of PCB (118) (0.25 ppm) in the expression of osteoclastic markers: cathepsin K (a), TRAP (b), and RANKL (c) mRNAs in the scale. The cathepsin K, TRAP and RANKL mRNA levels were normalized by the β-actin mRNA level. The values of ordinate indicate relative ratio of cathepsin K/β-actin (a), TRAP/β-actin (b), and RANKL/β-actin (c) respectively. Each column and the vertical line represent the mean ±_SEM (n = 10 samples; one sample from one fish). * and ** indicate statistically significant differences at *P*<0.05 and *P*<0.01, respectively, from the values in the control.

Figure

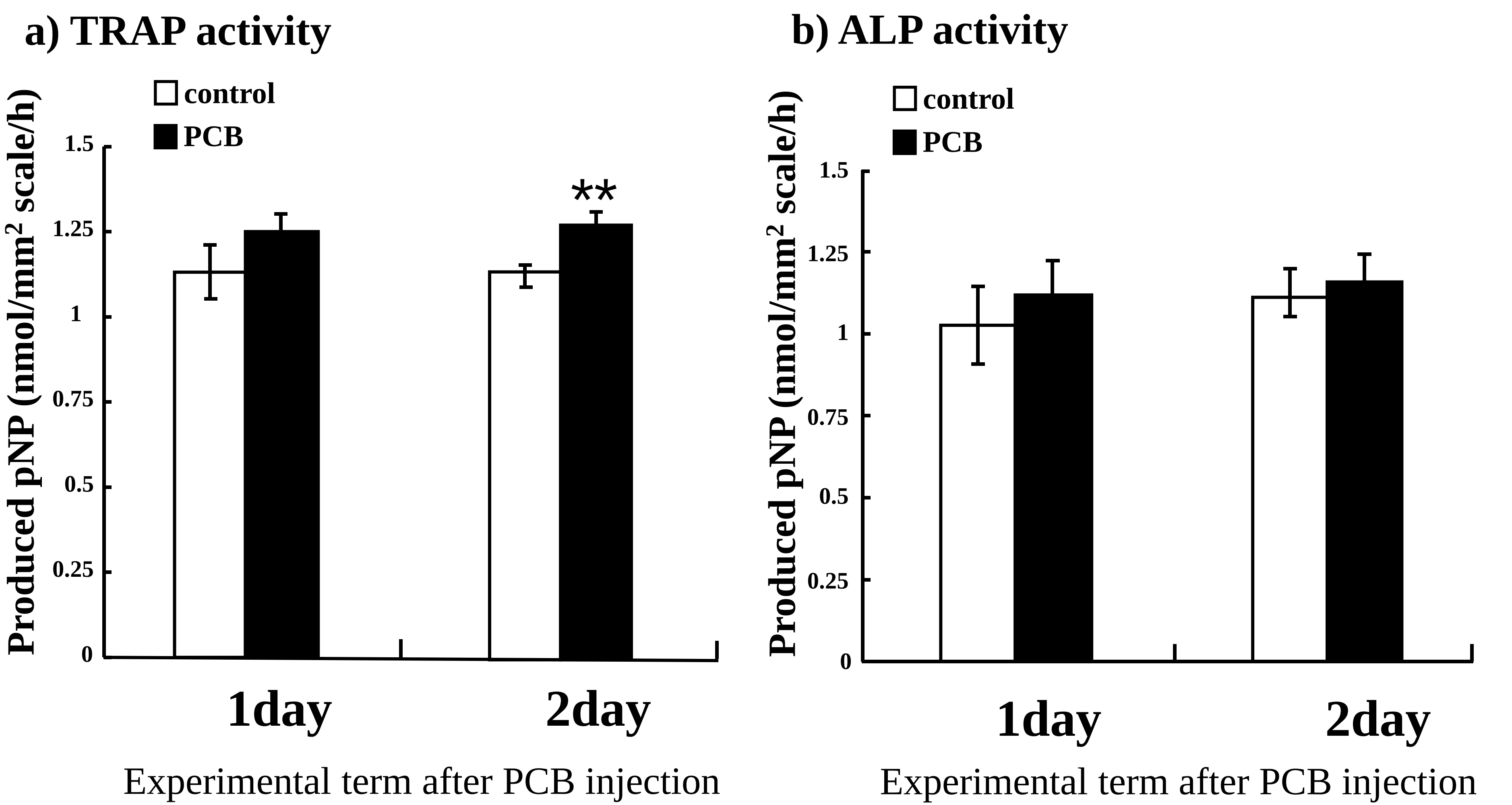
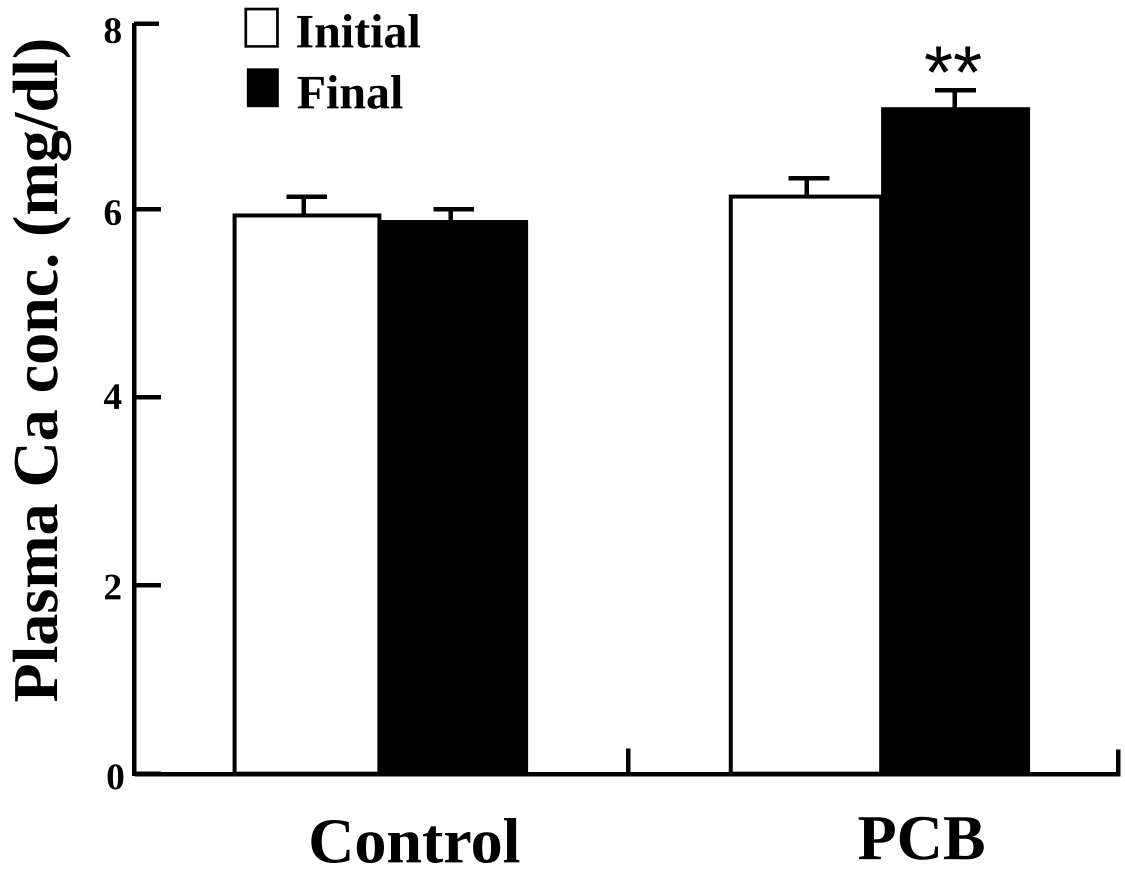


Figure 1 Yachiguchi et al.



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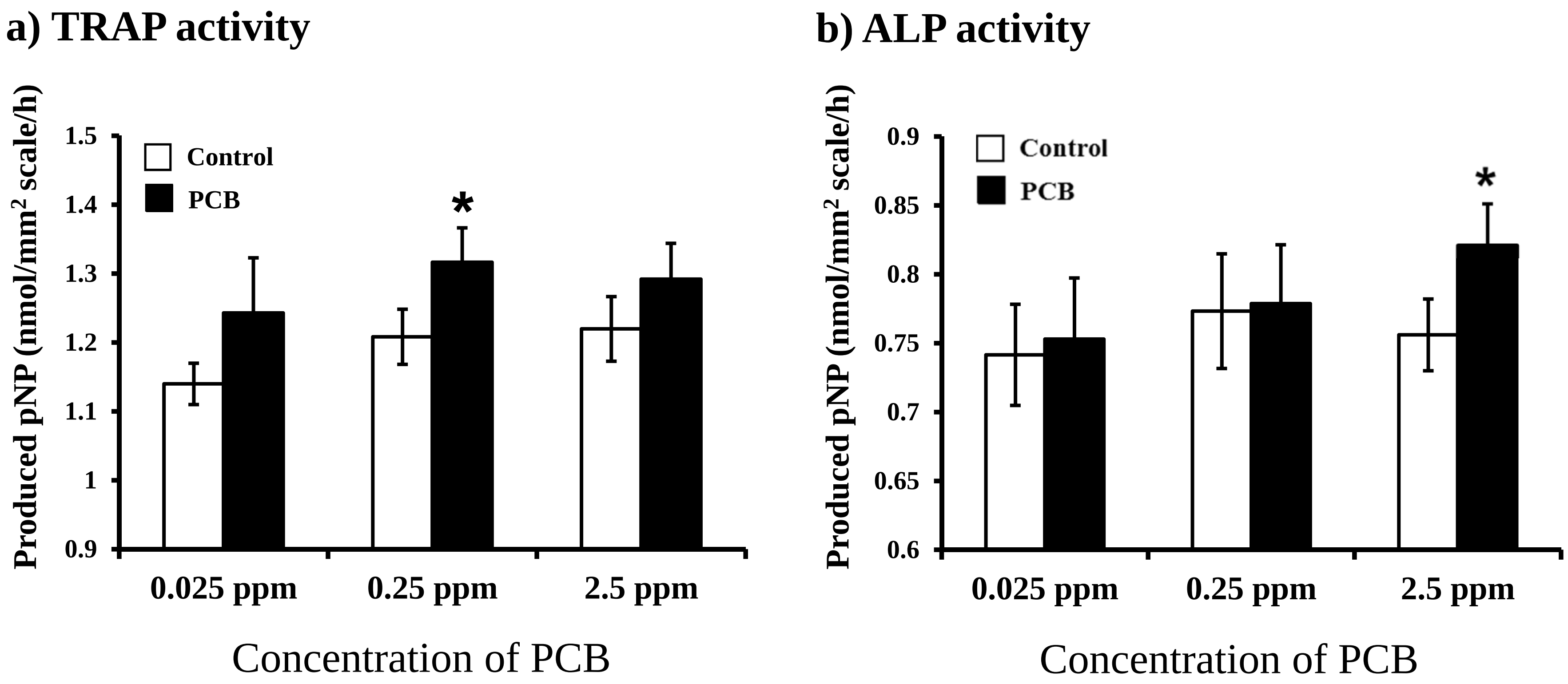

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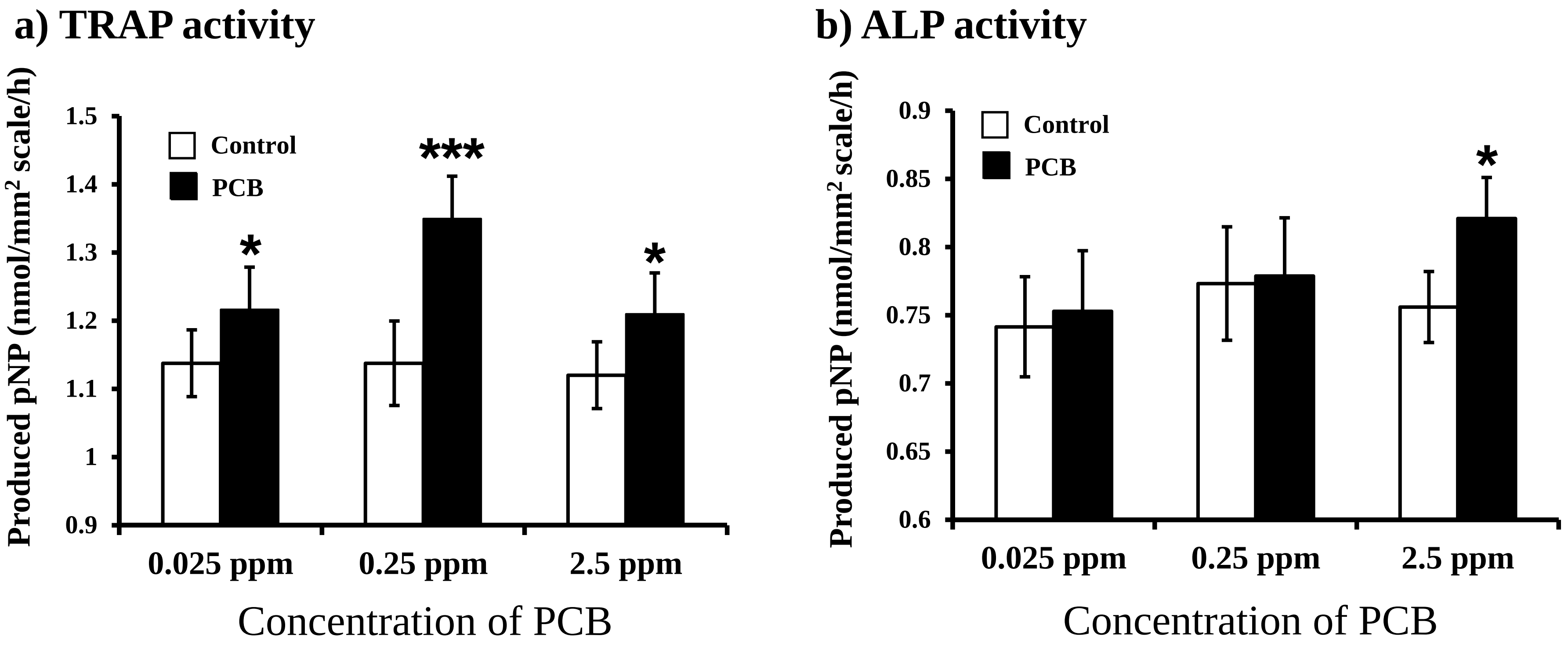

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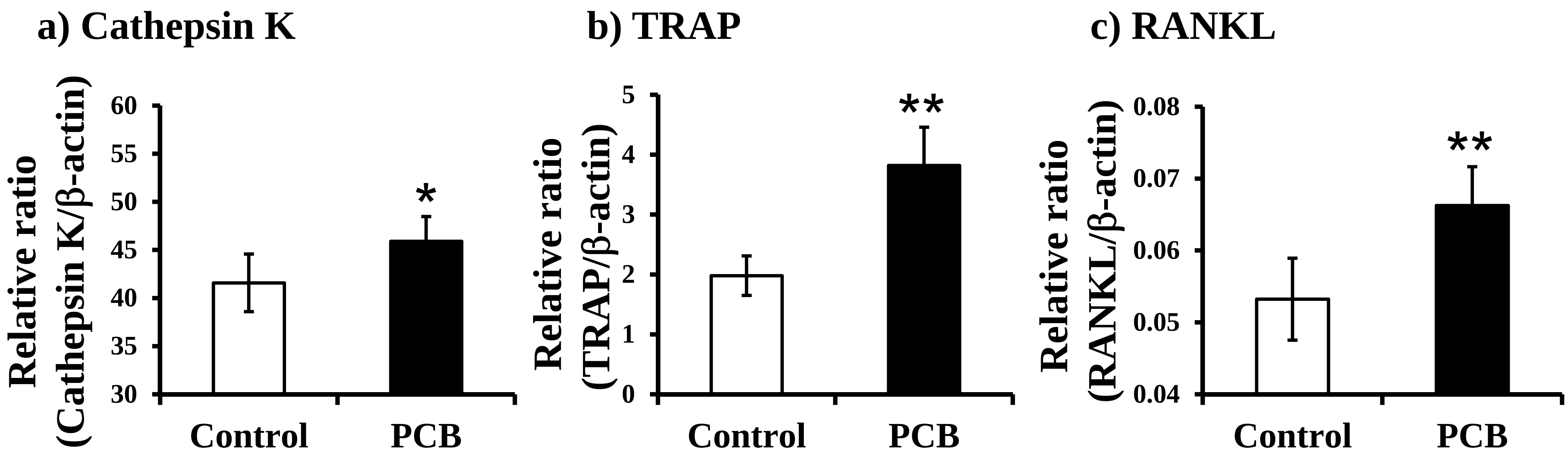

Figure 5 Yachiguchi et al.