A Thesis

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Immumohistochemical localization of protein kinase C (PKC) beta I in the pig retina during postnatal development

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Abstract

Immunohistochemical localization of protein kinase C (PKC) beta I in the pig retina during postnatal development

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To investigate the expression of protein kinase C (PKC) beta I in the retinas of pigs during postnatal development, we analyze the retinas sampled from 3-day-old and 6-month-old pigs by Western blotting and immunohistochemistry.

Western blot analysis detected the expression of PKC beta I in the retinas of 3-day-old piglets and it was increased significantly

in the retinas of 6-month-old adult pigs.

Immunohistochemical staining showed PKC beta I in the retinas of both groups. Immunohistochemistry of 3-day-old retinas revealed weak PKC beta I reactivity in the ganglion cell layer, inner plexiform layer, inner nuclear cell layer, and outer plexiform layer and rod and cone cell layer. In the 6-month-old pig retina, the cellular localization of PKC beta I immunostaining was similar to those of 3-day-old retina, where PKC beta I was localized in some glial fibrillary acidic protein-positive cells, glutamine synthetase-positive cells, parvalbumin-positive cells, and PKC alpha-positive cells in the retina.

This is the first study to show the expression and cellular localization of PKC beta I in the retina of pigs with development, and these results suggest that PKC beta I, in accordance with PKC alpha, play important roles in signal transduction pathways in the pig retina with development.

Key words: Postnatal development; Protein kinase C; Pig; Retina

CONTENTS

I. Introduction

The protein kinase C (PKC) family of serine/threonine kinases is expressed in all vertebrate tissues, and they play divergent roles in signal transduction pathways, elicited by a variety of extracellular stimuli, including hormones, growth factors, and cytokines [Azzi et al., 1992; Steinberg, 2008]. The PKC is important in the control of brain functions including neurotransmitter release, neuronal survival [Battaini, 2001]. The PKC isoforms have been classified into three groups: conventional PKCs (alpha, beta I, beta II, and gamma), novel PKCs (delta, theta, epsilon, and eta), and atypical PKCs (lambda, iota, and zeta) [Steinberg, 2008]. PKC-alpha and beta I are calcium-dependent isoforms that are abundant in the central nervous system (CNS) [Tanaka et al., 1992]. But PKC alpha and beta I have different structural domain residues [Steinberg, 2008]. PKC beta I is expressed in the retina, which is part of the CNS, in the rat [Zhang et al., 1991; Kosak et al., 1998; Johansson et al., 2000], cat [Fyk-Kolodziej et al., 2002], monkey [Kolb et al., 1997], rabbit [Osborne et al., 1992], and cow [Shin et al., 2006], but not in the pig.

The pig eye is a good model for experimental studies of human retinal diseases, as the anatomy, size, and vasculature of the pig retina share many similarities with the human retina [Garcá et al., 2005; Ruiz-Ederra et al., 2004, 2005; Guduric-Fuchs et al., 2009]. The histological characteristics of the developing retina have been examined in fetal pigs [De Schaepdrijver et al., 1990] and an immunohistochemical study of the pig retina with development has been reported

[Guduric-Fuchs et al., 2009]. The number of pig retinal ganglion cells decreases with aging [De Schaepdrijver et al., 1990], probably due to oxidative stress [Militante et al., 2004].

Previously, we examined the expression of PKC alpha in the retinas of pigs during postnatal development. We demonstrated that the level of retinal PKC alpha protein increased with maturation [Ahn et al., 2009]. However, little is known about PKC beta I in the retinas of pigs with development.

The aim of this study was to examine the expression and cellular localization of PKC beta I in the retinas of 3-day-old and 6-month-old pigs during postnatal development, and compared its results with those of PKc alpha [Ahn et al., 2009].

II. Materials and Methods

1. Tissue sampling

The retinas of adult (6 months old; n=3) pigs were obtained from a local slaughterhouse. To compare the PKC expression, newborn retinas were used (postnatal, 3 days old; n=3). The anterior segments of the eyeballs were removed, and the retinas were dissected carefully. For Western blot analysis, retinal tissues were detached from the eyecups and kept in a deep freezer. For histology, the anterior segments of the eyes were dissected. The eyecups without anterior segments were fixed by immersion in 10% neutral-buffered formalin in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 24 h and processed for routine paraffin embedding.

2. Antisera used in this study

Rabbit polyclonal anti-PKC beta I was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Mouse monoclonal anti-glial fibrillary acidic protein was obtained from Sigma (St. Louis, MO). Mouse monoclonal anti-glutamine synthetase (GS) was obtained from Chemicon International (Temecula, CA). Mouse monoclonal anti-PKC alpha was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Parvalbumin was obtained from Sigma (St. Louis, MO).

3. Western blot analysis

Western blot analysis of the porcine retina was performed as reported previously [Ahn et al., 2009; Kim et al., 2009]. In brief, retinal tissues were thawed in lysis buffer (40 mM Tris, 120 mM NaCl, 0.1% Nonidet 40, 2 mM Na_3VO4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After incubation in an ice-cold bath, the homogenates were centrifuged, and the lysate supernatant was obtained. Equal amounts of the lysate (40 μg) were then dissolved in the SDS sample buffer and boiled. The homogenized tissue samples were electrophoresed under denaturing conditions in 10% SDS-polyacrylamide gels. Then, the proteins were electrotransferred in transfer buffer to a nitrocellulose transfer membrane (Schleicher and Schuell; Keene, NH, USA) for 2 h at 100 V. The residual binding sites on the membrane were blocked by incubation with 5% nonfat milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 1 h and then incubated with anti-PKC beta I for 2 h. The blots were washed three times in TBS containing 0.1% Tween-20 and probed with horseradish peroxidase-conjugated anti-rabbit IgG (Vector; Burlingame, CA, USA) for 1 h. The membrane blots were developed using an enhanced chemiluminescence reagent kit (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. After imaging, the membranes were stripped and reprobed using monoclonal anti-beta-actin antibody as the primary antibody (Sigma, St. Louis, MO, USA). The density (OD/mm^2) of each band was measured with a scanning laser densitometer (GS-700; Bio-Rad, Hercules, CA, USA) and was reported as the mean ± S.E. The ratios of the density of the band to that of the beta-actin band were compared using Molecular Analyst software (Bio-Rad). The results were

analyzed statistically using one-way analysis of variance (ANOVA) followed by the post hoc Student-Newman-Keuls t-test for multiple comparisons. In all cases, $p < 0.05$ was considered significant.

4. Immunohistochemistry

Paraffin sections (5 μm) of retinas were deparaffinized and allowed to react with rabbit anti-PKC beta I for immunohistochemistry, as described previously [Ahn et al., 2009]. The sections were incubated with the appropriate biotinylated second antibody, followed by formation of the avidin-biotin peroxidase complexes using an Elite kit (Vector). The peroxidase reaction was developed with a diaminobenzidine substrate kit (Vector). Before mounting, the sections were counterstained with hematoxylin. As negative controls, the primary antiserum was omitted from a few test sections in each experiment, and no specific labeling of cell bodies or fibers was detected in these sections.

5. Double immunofluorescence

For double immunofluorescence, the sections were incubated in the following order: 10% normal goat serum for 1 h at RT, rabbit anti-PKC beta I and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG (Sigma). Then, they were washed and blocked with 10% normal horse serum for 1 h at RT, incubated with mouse monoclonal

anti-glial fibrillary acidic protein (GFAP) (Sigma), mouse monoclonal anti-glutamine synthetase (Chemicon), mouse monoclonal anti-parvalbumin (Sigma), and mouse monoclonal anti-PKC alpha (Santa Cruz) overnight at 4°C, biotinylated anti-mouse IgG and washed and incubated with fluorescein isothiocyanate (FITC)-labeled streptabidin IgG (Zymed, USA). To reduce or eliminate lipofuscin autofluorescence, the sections were washed in PBS (three times for 1 h) at RT and then dipped briefly in distilled H_2O, treated with 10 mM $CuSO_4$ in ammonium acetate buffer (50 mM CH_3COONH_4, pH 5.0) for 20 min, dipped briefly again in distilled H_2O, and returned to PBS. The double immunofluorescence-stained specimens were examined under an FV500 laser confocal microscope (Olympus, Tokyo, Japan).

III. Results

1. Western blot analysis

Western blot analysis has shown that PKC beta I was present constitutively in the retinas of 3-day-old piglets and the amount was increased in the retinas of 6-month-old pigs (2.4-fold increases; 3-day-old vs. 6-month-old retinas, $p<0.05$) (Fig. 1).

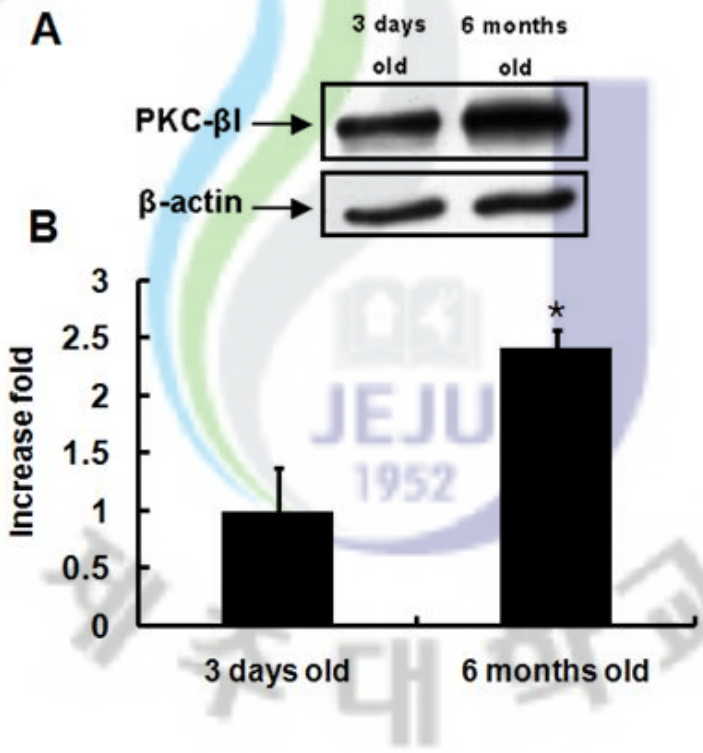

Figure 1. Western blot analysis of PKC-beta I in 3-day-old and 6-month-old pig retinas. (A) A shows a representative Western blot for PKC beta I (approximately 79 kDa) and beta-actin (approximately 45 kDa). (B) Densitometric analysis of PKC-beta I (mean±S.E., n=3 per group). The data are presented as the mean fold increase in the level of

PKC-beta I, relative to that of beta-actin. The intensity of the PKC-beta I immunoreactive band in the retinas of the 6-month-old pigs was approximately two times greater than that in the retinas of the 3-day-old piglets. Values for the retina of 3-day-old piglets were arbitrarily defined as 1. * $p<0.05$ vs. 3-day-old retinas.

2. Immunohistochemistry of PKC beta I

In 3-day-old piglet retinas, PKC beta I immunoreactivity was weak in the ganglion cell layer (GCL), intense in the synaptic vesicles of the inner plexiform layer (IPL) (Fig. 2A, arrowheads) and in the cells in the outer half of the inner nuclear cell layer (INL) (Fig. 2A, arrows), weak in the outer plexiform layer (OPL), and very sparse in the outer nuclear cell layer (ONL) and the rod and cone cell layer (RCL). PKC beta I was also present in some amacrine cells in the INL. In the INL, bipolar cells were intensely immunopositive for PKC beta I (Fig. 2A, arrows).

In the 6-month-old pig retina, the PKC beta I cellular localization was similar to that in the retinas of 3-day-old piglets and was increased in each layer. PKC beta I was also detected in some cells in the INL (Fig. 2B, arrows). By contrast, PKC beta I immunostaining was observed in amacrined cells in the INL and rod and cone cells (Fig. 2B, arrowheads) in the RCL.

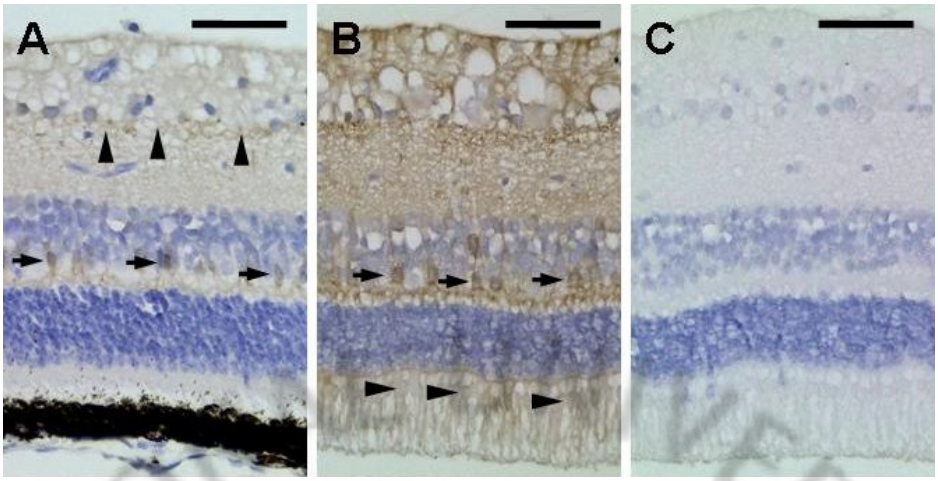

Figure 2. Immunohistochemical localization of PKC-beta I in the retinas of 3-day-old (A) and 6-month-old (B) pigs, and a negative control (C). (A) In the retina of a 3-day-old piglet, PKC-beta I immunostained some cells (arrows) in the INL and their synaptic terminals (arrowheads) in the IPL. (B) In the retina of a 6-month-old pig, the pattern of PKC-beta I immunoreactivity was similar to that seen in the retina of the 3-day-old piglet shown in (A). PKC-beta I immunostained bipolar cells (arrows) in the outer half of the INL and optic nerve fibers in the GCL. Particularly intense PKC-beta I immunostaining was seen in some cells in the rod cells (arrowheads) in the RCL, compared with that in the 3-day-old piglet. (C) Negative control (6-month-old retina). No specific reaction product is seen in sections incubated with non-immune sera. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RCL, rod and cone layer. Counterstained with hematoxylin. Scale bars = 50 μm.

3. Double-labeling experiment

A double-labeling experiment was performed to determine the cell phenotype of PKC beta I expression in the pig retinas. PKC beta I immunoreactivity (Fig. 3A, arrowheads) was co-localized with GFAP (Fig. 3B, arrowheads), suggesting that some astrocytic processes crossing from the GCL to the INL express PKC beta I (Fig. 3C, merged). PKC beta I (Fig. 3C, arrows) was co-localized with anti-GS (Fig. 3D, arrows), suggesting that Müller cells (Fig. 3E, merged) in the INL are positive for PKC beta I. PKC beta I (Fig. 4A, arrowhead) was co-localized with parvalbumin (Fig. 4B, arrowhead), suggesting that amacrine cells contain parvalbumin (Fig. 4C, merged). In addition, PKC beta I (Fig. 4D, arrows) was also co-localized with PKC alpha (Fig. 4E, arrows), suggesting that bipolar cells are positive for PKC alpha (Fig. 4F, merged).

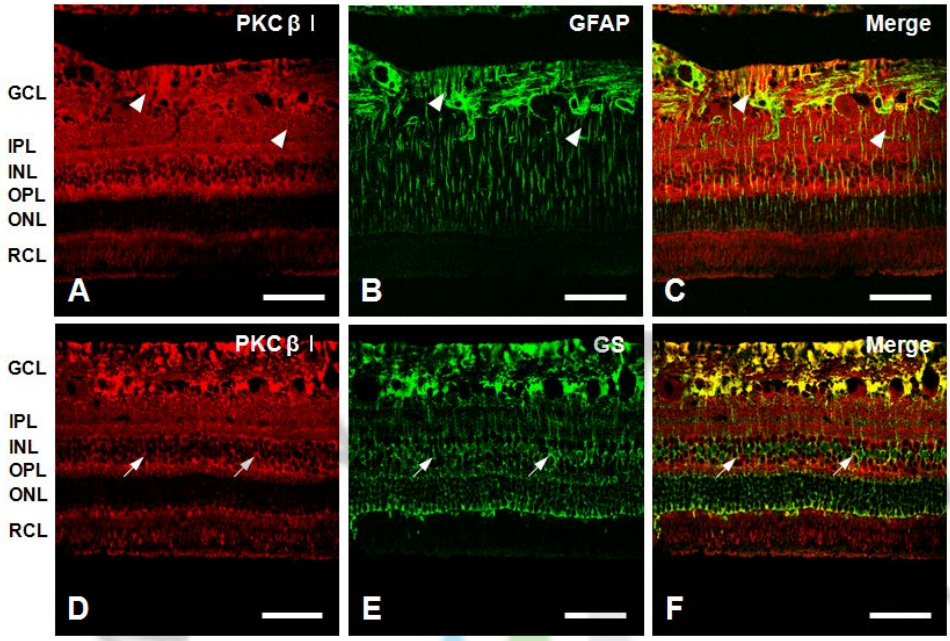

Figure 3. Double immunofluorescence co-localization of PKC beta I (A and D) with GFAP (B) or GS (E) in the retina of a 6-month-old pig. PKC beta I (A; red, arrowheads) is co-localized in some GFAP-positive cells (B; green fluorescence, arrowheads). The co-localization of PKC beta I and GFAP is shown in C (C; merged image, arrowheads). PKC beta I (D; red fluorescence, arrows) was also observed in the GS-positive cells (E; green fluorescence, arrows) in the retina (co-localization shown in F; merged image, arrows). Scale bars = 50 μm.

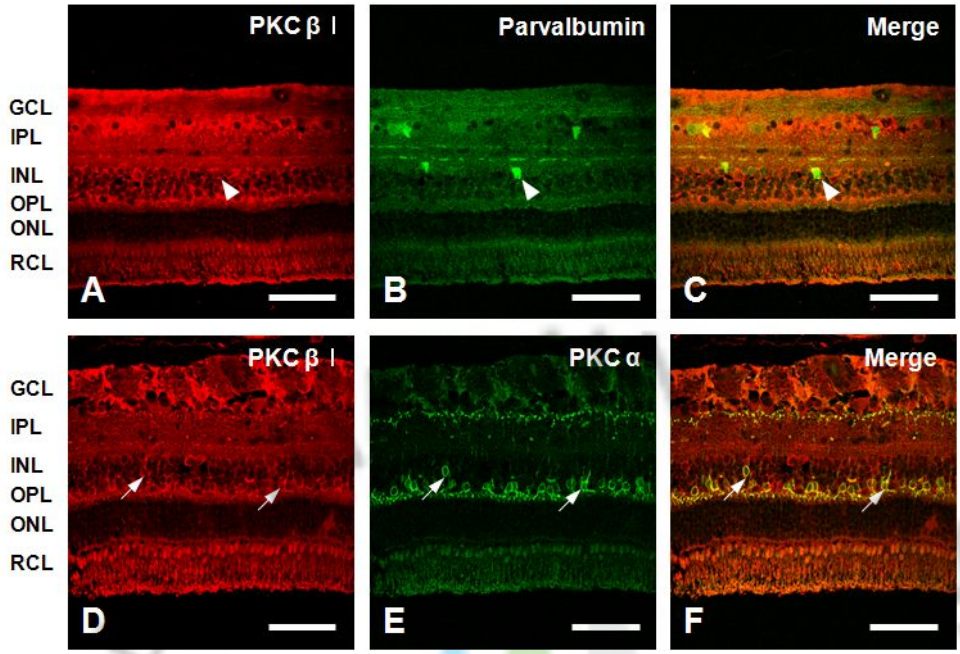

Figure 4. Double immunofluorescence co-localization of PKC beta I (A and D) with parvalbumin (B) or PKC alpha (E) in the retina of a 6-month-old pig. PKC beta I (A; red, arrowhead) is co-localized in some parvalbumin-positive cells (B; green fluorescence, arrowheads). The co-localization of PKC beta I and parvalbumin is shown in C (C; merged image, arrowhead). PKC beta I (D; red fluorescence, arrows) was also observed in the PKC alpha-positive cells (E; green fluorescence, arrows) in the retina (co-localization shown in F; merged image). Scale bars = 50 μm.

Ⅳ. Discussion

This is the first study to demonstrate PKC beta I expression in the pig retina and that its expression increases with maturation of the retina. The expression of PKC beta I was more intense in the retinas of 6-month-old pigs than in those of 3-day-old piglets. PKC alpha and beta I belong to the conventional PKC family and have similar structures [Steinberg, 2008], although the three C2 domains of PKC alpha and beta differ in calcium affinity and stoichiometry [Kohout et al., 2002]. We postulate that the expression of PKC alpha and beta I is different, and that they play different roles in pig retinas with development.

We previously found a similar finding in the pig retina: PKC alpha expression was increased during postnatal development, suggesting that the increased PKC alpha level was related to retinal development [Ahn et al., 2009]. In this study, we found similarities and differences in the cellular localizations of PKC alpha and beta I. Generally, the cellular localizations were similar in the newborn and adult retinas. The ganglion cells in the GCL, synaptic terminals in the IPL, bipolar cells in the outer half of the INL, astrocytic processes in the GCL, and Müller cells in the INL were positive for both PKC alpha and beta I. By contrast, PKC beta I immunoreactivity was seen in amacrine cells in the INL and rod and cone cells in the RCL, whereas these were not immunoreactive for PKC alpha.

PKC-positive immunoreactivity was observed throughout the cytoplasm of bipolar cells and plays an important role in morphology and synaptogenesis in both rat and rabbit retinas [Osborne et al., 1991]. PKC alpha and beta I might play roles in the morphology and synaptogenesis

in the pig retina with maturation.

Astrocytes and Müller cells are the major cells types affected in retinal injury and light damage [De Raad et al., 1996; Bodeutsch et al., 1999]. A previous study showed that PKC alpha immunoreactivity is associated with astrocytes and Müller cells [Ahn et al., 2009]. We also detected PKC beta I in astrocytes and Müller cells in the pig retinas.

Amacrine cells are intervening neurons that pass the rod signal through the cone bipolar cell to ganglion cells [Park et al., 2008]. PKC alpha is modulator of bipolar cell signal transduction in the retina [Ruether et al., 2010]. We detected PKC beta I in the amacrine cells and bipolar cells. Particularly, the expression of PKC alpha and PKC beta I differed in the INL and RCL. The expression of PKC beta I, but not PKC alpha activated the differentiation of rod photoreceptors in the mouse retina [Pinzon-Guzman et al., 2010]. Vision required a visual pigment in rod and cone photoreceptors produced in light [Arshavsky et al., 2002]. PKC phosphorylations of rhodopsin have been studied to have roles in downregulating the sensitivity of rod photoreceptors to light [Xiong et al., 1997]. These findings support the hypothesis that PKC beta I contributes to the differentiation and visual pathways by light of photoreceptors in the porcine retina with postnatal maturation.

Collectively, this study suggests that PKC beta I, as does PKC alpha, is uppregulated in the pig retina with development, and that PKC beta I, in particular, is associated with photoreceptor differentiation and visual pathway by light in postnatal development of porcine retina.

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감사의 글

지난 석사과정 시간을 돌이켜보면 마치 꿈을 꾼 것만 같습니다. 앞만 바라보며 정신없이 달려온 제가 드디어 졸업을 앞두게 되었습니다. 지금까지 저를 있게 한 많은 분들께 감사의 마음을 전하고자 합니다.

2004년 겨울, 골격표본을 제출하기 위해 수의해부학교실 문턱을 넘은 인연이 저의 20대를 함께하는 필연이 되었습니다. 2005년 실험실 여름엠티 때, 두 번의 실험실패로 상심하고 있는 저를 달래주셨던 신태균 교수님의 말씀이 지금도 잊혀지지 않습니다. 그 때의 순수했던 열정과 집념을 늘 잊지 않도록 아낌없는 조언과 가르침을 주셨고, 결과에 집착하기 보단 과정에 더 충실한 제가 되게 하여 주셨습니다. 진심으로 감사드립니다. 신태균 교수님을 만난 건 제 인생에 행운이였습니다.

수의학이란 학문과 생명의 소중함을 가르쳐주신 김희석 교수님, 박전홍 교수님, 배종희 교수님, 이두식 교수님, 이경갑 교수님, 임윤규 교수님, 우호춘 교수님, 이영재 교수님, 정종태 교수님, 손원근 교수님, 김재훈 교수님, 황규계 교수님, 주홍구 교수님, 윤영민 교수님, 이주명 교수님, 강태영 교수님, 박현정 교수님, 한창훈 교수님, 지영흔 교수님, 그리고 고 이국경 교수님께 감사의 마음을 전합니다.

공중방역수의사로써 제주특별자치도 동물위생시험소에서 군복무를 이행하며 학생에서 사회인으로 한 걸음 나아갈 수 있도록 도와주신 많은 분들께 감사의 마음을 전하고 싶습니다. 김명원 소장님, 김병학 소장님, 조순여 과장님, 최성두 계장님, 김익천 계장님, 김병수 계장님, 병리진단계의 문성환 계장님, 형석이형, 경옥누나, 현화누나, 민경누나, 용준이형, 정훈이형, 축산물분석계의 김황룡 계장님, 종철형, 성훈형, 정헌이형, 성근이형, 혜진누나, 수현누나, 현주, 늘 따뜻한 점심밥을 해주신 수경·희선이모, 1기 선배 으뜸이형, 2기 동기 경용이형, 형준이, 3기 후배 영곤이, 상훈이, 4기 후배 정식이까지 모두 잊지 못할 것 같습니다.

실험실 생활에서 아낌없는 후원과 사랑으로 감싸주셨던 김철 선배님, 김승준 교수님, 강재윤 선배님, 강종철 선배님, 안미정 박사님, 허승담 선배님, 박달수 선배님, 이기현 선배님, 이광협 선배님, 정경숙 선배님 진심으로 감사합니

다. 졸업 동기 태기형과 지성이. 일본의 후배 정태형, 지훈, 주연, 규영이형, 석원형, 준우, 성영, 원호, 원준, 철호, 한슬, 소희, 창남, 그리고 든든한 진우 함께해서 좋았습니다.

늘 리더가 되어주었던 교회동생 지혜와 영적 버팀목이 되어주신 조용진 형제님, 뉴질랜드에 있는 친구 상훈이, 전화통화로 늘 힘이되어준 도현형, 서울에서 따뜻하게 재워준 준곤, 광주에서 추억을 만들어준 준영이.

마지막으로 매일 새벽기도로 후원해주신 할아버지·할머니, 지금의나를 있게 한 사랑하는 나의 가족 아빠·엄마, 아우재우·용우, 그리고 내가 이 땅에 태어난 목적과 소명을 허락하신 하나님께 감사를 드립니다.

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정찬우