Abstract

Background

Complex regional pain syndrome (CRPS) is a rare but debilitating pain disorder. Although the exact pathophysiology of CRPS is not fully understood, central and peripheral mechanisms might be involved in the development of this disorder. To reveal the central mechanism of CRPS, we conducted a proteomic analysis of rat cerebrum using thechronic post-ischemia pain (CPIP) model, a novel experimental model ofCRPS.

Materials and Methods

After generating the CPIP animal model, we performed a proteomic analysis of the rat cerebrum using a multidimensional protein identification technology, and screened the proteins differentially expressed between the CPIP and control groups. Based on these findings, we conducteda confirmation study and determinedcalmodulin(CaM) and Ca^{2+}/CaM kinase II (CaMKII) protein expression byWestern blotting.

Results

A total of 155 proteinswere differentially expressed between the CPIP and control groups: 125 increased and 30 decreased; expressions of proteins related to cell signaling, synaptic plasticity,regulation of cell proliferationand

cytoskeletal formationwere increased in the CPIP group. The expression of CaM (P = 0.030) and CaMKII (P = 0.035) in the rat cerebrum was significantly increased as compared to the control group. However, pro-enkephalin A (P = 0.058), cereblon (P = 0.007) and neuroserpine(P = 0.070) were decreased in CPIP group.

Conclusion

Altered expression of cerebral proteinsin the CPIP modelindicatescerebral involvement in the pathogenesis of CRPS.Further study is required to elucidate the roles of these proteins in the development and maintenance of CRPS.

Keywords: calmodulin, cerebrum, complex regional pain syndrome, protein

Student Number: 2009-30514

Contents

List of Tables

List of Figures

Introduction

Complex regional pain syndrome (CRPS) is a rare but serious and painful disorder. Although CRPS can occur following a minor injury, such as a sprain or even soft-tissue blunt trauma, severe intractable pain from CRPS can impair the quality of life. Symptoms and signs of CRPS include sensory changes (allodynia/hyperalgesia), vasomotor changes (temperature asymmetry/skin color change or asymmetry), sudomotor changes (edema/sweating change or asymmetry) and motor or trophic changes(Harden *et al.*, 2007).Although the exact pathophysiology of CRPS is not fully understood, several pathological mechanisms, including oxidative stress(Eisenberg *et al.*, 2008), neurogenic inflammation(Birklein and Schmelz, 2008), and alteration in theautonomic nervous system(Schattschneider *et al.*, 2006; Schulze and Troeger, 2010)are known to play some roles in itsdevelopment. There is also increasing evidence supporting theconcept that the central nervous system (CNS) is involved in CRPS.Psychophysical studies show that CRPS patients have distorted body image and have difficulty in recognizing the size or the position of the affected extremity(Moseley, 2005).The patients get worse when they think about moving the body part, even if they do not move it (Moseley *et al.*, 2008). Mechanical stimulation of the ‘virtual (unaffected)’ limb reflected in a mirror results in allodynia, which suggests that allodynia and paresthesia can be mediated by the brain(Acerra and Moseley, 2005). Thus, the distorted body representation of CRPS patients

can betreated with mirror therapy (McCabe *et al.*, 2003; Bultitude and Rafal, 2010). Also, the spreading of symptoms and signs of CRPS from the initial site of presentation to another limb is a well-known phenomenon, which may be due to aberrant CNS regulation of neurogenic inflammation (Maleki *et al.*, 2000). These findings highlight the contribution of a cortical pain mechanism in patients with CRPS. Moreover, functional imaging studies providesupporting evidence for the important role of the CNS in the pathogenesis of CRPS (Geha *et al.*, 2008; Schwenkreis *et al.*, 2009; Freund *et al.*, 2010), and recent research suggests that changes in cortical structures can contribute to the pathophysiology of CRPS (Swart *et al.*, 2009).

Thus, the brain seems to play an important role in the development and maintenance of symptoms and signs in patients with CRPS. Some researchers insist thatthe peripheral changes in CRPS must be understood as a manifestation of changes in the brain (Janig and Baron, 2002).Therefore, we postulated that protein expression would be altered in the CRPS-affected brain. However, there have been no studies on the changes of cerebral protein expression in CRPS. Therefore, to verify our hypothesis, we conducted a proteomic analysis using multidimensional protein identification technology (MudPIT) in a chronic post-ischemia perfusion (CPIP) rat model, a novel and widely used experimental model ofCRPStype 1 (Coderre *et al.*, 2004).

Materials and Methods

1. Animals

This study was approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 200–250g had free access to food and water and were housed individually in cages with soft bedding under a 12-h night/day light cycle at a constant temperature of 20–22°C and a humidity level of 55–60%. The animals were acclimatized for at least 1 week prior to the CPIP procedure.

2. CPIP model generation

The CPIP animal model was generatedaccording to previous methods (Coderre *et al*., 2004). Briefly, after induction of anesthesia with isoflurane, a tight fitting O-ring (O-ring West, Seattle, WA,USA) with a 5.5-mm internal diameter was applied tothe left hind limb of each anesthetized rat just proximal to the left ankle joint for 3 h. The O-ring was then removed from the anesthetized rat, allowing reperfusion of the hind limb (Fig. 1). The animals in the control group underwent anesthesia similar to the CPIP animals, but the O-ring was not placed around thehind limb.

3. Behavioral tests

All behavioral tests were performed during the daylight portion of the regulated circadian cycle between 9 am and 3 pm. To assess the mechanical threshold, the rats were placed in individual plastic cages with wire mesh bottoms. After 20-min acclimatization, calibrated von Frey filaments (Stoelting Co., Wood Dale, IL, USA) with logarithmically increasing stiffness of 0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g were applied to the mid-plantar surface of the hind paw. The mechanical threshold was assessed using an up-down statistical method (Chaplan *et al.*, 1994). Then, the change in the mechanical threshold (CMT, %) was calculated. The mechanical threshold was examined during the post-reperfusion period: 1 h, 4 h, 24 h, 48 h, day 7 and day 21. The CMT was calculated by following equation.

$CMT\ (\%) = (M_{post} - M_{pre}) / M_{pre} \times 100$

We used the findings from the neurobehavioral test on day 21 to classify the animals into groups: rats whose CMT was decreased 50% or more after the CPIP procedure were classified as the CPIP group (A), and the animals whose CMT decreased less than 50% were classified into the CPIP-failure group (B). The mechanical threshold of the animals in the control group (C) was also examined. All animals were sacrificed 3 weeks after the CPIP procedure for proteomic analysis and Western blotting.

4. Proteomic analysis

The difference in cerebral protein expression between groups A and C was explored using a MudPIT, as follows.

4-1. Protein extraction

A total of six animals (three each from groups A and C) were used for the mass spectrometry analysis. On the day 21, rat cerebrum was grinded using a mortar in liquid nitrogen. The tissue powder was kept at -80°C. The tissue powder was resolubilized in a small volume of 8 M urea, 100 mM Tris-HCl, pH 8.5, and 1 mM dithiothreitol for two hours. The homogenates were sonicated and centrifuged at 100,000 ×*g* for 1 h. Next, 5 mM dithiothreitol was added to the homogenate for 30 min at 37°Cand alkylated with 25 mM iodoacetamide for 30 min at 37°C in the dark. The samples were then diluted with 2 M urea and with 50 mM Tris-HCl, pH 8.0, and digested at 37°C overnight with sequence grade trypsin (Promega Co., Fitchburg, MA, USA) diluted 1:50 in 5 mM $CaCl_2$.

4-2. MudPIT

Peptides were separated with an Agilent 1100 series™ high-performance liquid chromatography pump (Agilent technologies, Santa Clara, CA, USA) connected to a linear quadruple ion-trap mass spectrometer (MS, LTQ™, Thermo-Finnigan, San Jose, CA, USA) using an in-house-built nano-electrospray ionization interface. To identify peptides, the ion-trap mass

spectrometer was operated in a data-dependent MS/MS mode (m/z 400-2000), in which a full MS scan was followed by 10 MS/MS scans and the temperature of the heated capillary was 200°C. MS/MS spectra were generated in the positive ion mode at an electrospray voltage of 2.5 kV and normalized collision energy of 35%. An analytical column-fused (100-µm internal diameter) silica capillary micro-column (Polymicro technologies, Phoenix, AZ, USA) was pulled to a fine tip using a laser puller and packed with 7 cm of 5-µm C18 reverse-phase resin, which was connected to an internal diameter of 250-µm fused-silica trapping column packed with 2 cm of SCX followed by 2 cm of C18 resin. Each 30-µg peptide mixture was manually loaded onto separate columns using a pressure vessel. A seven-step chromatography run was carried out on each sample and three buffers were used (buffer A: 5% acetonitrile/0.1% formic acid, buffer B: 80% acetonitrile/0.1% formic acid, and buffer C: 5% acetonitrile/0.1% formic acid/500 mM ammonium acetate).

4-3.Data searching and analysis

Acquired MS/MS spectra were searched against an international protein index rat v. 3.78 FASTA-format decoy database downloaded from European Bioinformatics Institute (www.ebi.ac.uk). The SEQUEST algorithm (Eng *et al.*, 1994) was used to find the best matching sequences from the database with BioWorks 3.3 (Thermo Fisher Scientific Inc., Rockford, IL, USA) for fully tryptic peptides. The mass of the amino acid cysteine was statically

modified by +57 Da and the differential modification search was performed for oxidation (+16 Da on Met). Xcorr values were based on tryptic peptides and charge states following 1.8 for singly charged peptides, 2.5 for doubly charged peptides, 3.5 for triply charged peptides, and 0.08 for ΔCn (DTASelect v. 2.0.39). The analysis of protein fold-change was quantified by an overall spectral counting method comparison of label-free methods for quantifying human proteins (Old *et al.*, 2005).

5. Western blotting

Western blot analysis of calmodulin (CaM) and calcium/calmodulin kinase II (CaMKII) was performed on the A, B and C groups (n = 4 per group). The right half of each rat cerebral cortex was sonicated in T-PER® tissue protein extraction buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) and the extracted proteins were quantified and adjusted to 2 mg/mL using the BCA Protein Assay System (Thermo Fisher Scientific Inc., Rockford, IL, USA). Proteins were separated (200 V for 40 min) by 10–16% sodium dodecyl sulfate polyacrylamide gel electrophoresis and separated proteins were electro-transferred (25 V for 100 min) to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA). Then the blots were blocked for 2 h at room temperature in Tris-buffered saline/Tween-20 (TBS/T; 0.1%) containing 5% skim milk. After blocking, blots were probed with primary antibody overnight at 4℃. Then the membranes were washed four times for 10 min in TBS/T and incubated for 2 h at room temperature with

secondary antibody conjugated with horseradish peroxidase. Following four TBS/T washes, proteins were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and band intensities were quantified using the NIH Image-J software v. 1.46. The primary antibodies used were mouse anti-β-actin monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO, USA), rabbit anti-CaM monoclonal antibody (Abcam Plc., Cambridge, UK) and rabbit anti-CaMKII monoclonal antibody (Millipore Corp., Billerica, MA, USA). The secondary antibodies used were peroxidase-labeled anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) and peroxidase-labeled anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA).

6. Statistical analysis

Data are reported as means ± standard error of the mean (SEM). The paw-withdrawal threshold was compared among the groups using repeated-measures analysis of variance. The expression of CaM and CaMKII in each group was compared using the Kruskall–Wallis test. All statistical analyses were performed using a SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA).

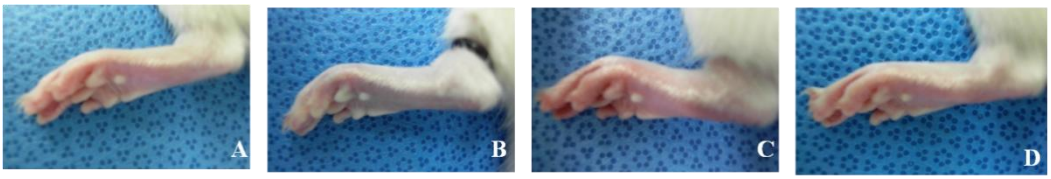


Fig. 1. Plantar skin color changes in chronic post-ischemia pain model rats. (A) Before O-ring application, (B) during O-ring application, (C) 1 hour after reperfusion and (D) 4 h after reperfusion.

Results

1. Behavioral tests

A total of 21 animals (n = 7 per group) were included in the behavioral test. Before the CPIP procedure, there were no differences in the withdrawal mechanical threshold among the groups. However, group A exhibited a significant decrease in the withdrawal mechanical threshold compared to groups B and C after the CPIP procedure ($P< 0.01$, Fig.2). The mean differences of CMT (%) in group A compared to group C were -41.5, -73.2, -92.3, -98.2, -92.2 and -95.3 after CPIP procedure1 h, 4 h, day 1, day 2, day 7 and day 21, respectively.

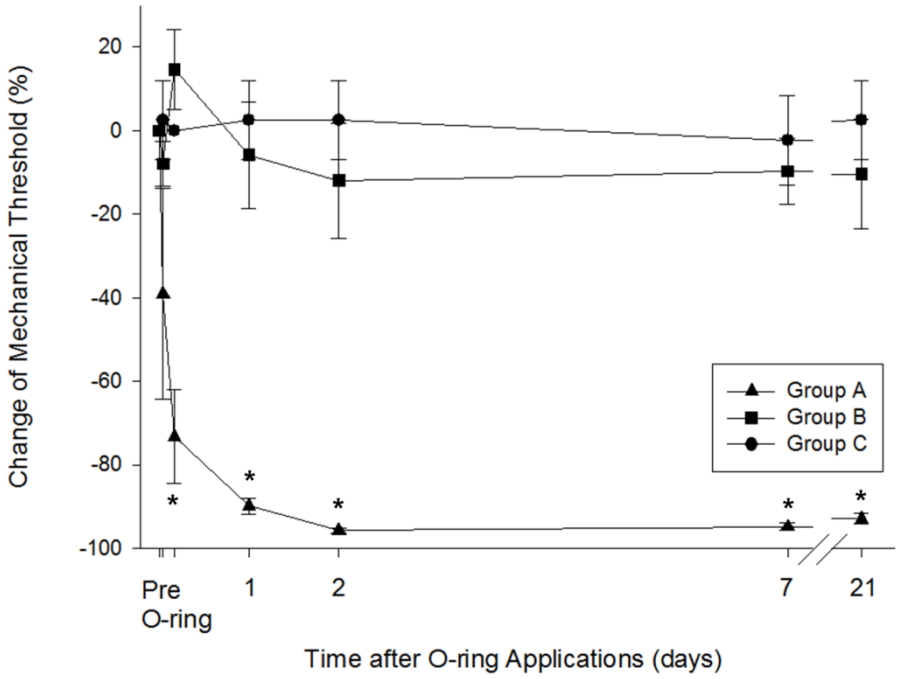


Fig. 2. Change in mechanical threshold (%). Group A showed a significant decrease in mechanical threshold compared to groups B and C (n = 7 in each group). Asterisk (*) indicates $P < 0.05$ at each time point. Group A: CPIP group, group B: CPIP-failure groupand Group C: control group.

2. Differential protein expression in the rat cerebrum

A total of454 proteinswere differentially expressed between groups A and C under the criterion of a *P*-value< 0.1. Among the 454 proteins, we selected those found in the cerebrum of all study animals in either group, and excluded 'uncharacterized proteins' and 'hypothetical proteins.' Finally, we found155differentially expressed proteins between group A and group C:125 increased (appendix 1) and 30 decreased (appendix 2).Specifically, expression of proteins related to cell signaling (table 1), synaptic plasticity (table 2), regulation of cell proliferation(table 3) and cytoskeletal formation (table 4)were increased in group A. Also, expression of a group of protein kinases(calmodulin dependent proteinkinase II beta M isoform, casein kinase 2, phosphoenolpyruvate carboxykinase 2, mitogen-activated proteinkinase 4, protein kinase C delta, N-terminal kinase like protein, uridine kinase-like 1, serine/threonine protein kinase PLK 1, and phosphoinositide 3 kinase regulatory subunit 4) and calcium-related proteins (inositol 1,4,5-triphosphate receptor type 2, annexin A1, annexin A2, annexin A5, voltage-dependent Ca^{2+} channel gamma-2 subunit, and voltage-dependent Ca^{2+}channel beta-3 subunit, and coiled-coil domain-containing protein 47) werealso elevated in group A.However, several proteins were decreased in group A. Specifically, expression of proteins related to cell signaling (table 5), and metabolism of fatty acid (peroxisomal 3, 2-trans-enoyl Co A isomerase, acetyl-CoA acyltransferase 1b and acetyl-CoA acetyltransferase 2) were decreased. Also,

pro-enkephalin A, proteincereblon and neuroserpien were decreased in group A.

Table 1. Increased cerebral proteinsin the chronic post-ischemia pain group; proteins which might be related to cell signaling

No	Symbol	Description	P value
1	Kctd12	Potassium channel tetramerisation domain containing 12	0.004
2	Ecsit	Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial	0.004
3	Tns1	Tensin 1	0.004
4	Ccbp2	Chemokine-binding protein 2	0.004
5	Apba1	Amyloid beta A4 precursor protein-binding family A member 1	0.008
6	Tnc	Tenascin C	0.008
7	Rabl2b	RAB, member of RAS oncogene family-like 2B	0.008
8	Epha4	Eph receptor A4	0.035
9	Rab6a	Ras-related protein Rab-6A	0.038
10	Gpr158	G protein-coupled receptor 158	0.042
11	Anxa2	Isoform Short of Annexin A2	0.043
12	Hgs	Isoform 1 of Hepatocyte growth factor-regulated tyrosine kinase substrate	0.054
13	Prkcd	Isoform 1 of Protein kinase C delta type	0.060
14	Gabarapl2	Gamma-aminobutyric acid receptor-associated protein-like 2	0.065
15	Map2k4	Dual specificity mitogen-activated protein kinase 4	0.066
16	Cacng2	Voltage-dependent calcium channel gamma-2 subunit	0.083
17	Phb2	Prohibitin-2	0.086
18	Camk2b	Calmodulin-dependent protein kinase II beta M isoform	0.086
19	Anxa5	Annexin A5	0.093
20	Scn2a1	Sodium channel Nav1.2	0.095
21	Rab10	Ras-related protein Rab-10	0.097

Table 2. Increased cerebral proteinsin the chronic post-ischemia pain group; proteins which might be related to synaptic plasticity

No	Symbol	Description	P value
1	Itpr2	Inositol 1,4,5-trisphosphate receptor type 2	0.001
2	Kctd12	Potassium channel tetramerisation domain containing 12	0.004
3	Grid2	Glutamate receptor delta-2 subunit	0.004
4	Baiap3	BAI1-associated protein 3-like isoform 2	0.008
5	Atad1	ATPase family, AAA domain containing 1	0.008
6	Pick1	PRKCA-binding protein	0.008
7	Nlgn3	Isoform 1 of Neuroligin-3	0.056
8	Nudc	Nuclear migration protein nudC	0.085
9	Camk2b	Calmodulin-dependent protein kinase II beta M isoform	0.086

Table 3. Increased cerebral proteinsin the chronic post-ischemia pain group; proteins which might be related to regulation of cell proliferation

No	Symbol	Description	P value
1	Pik3r4	Phosphoinositide 3-kinase regulatory subunit 4	0.001
2	Itpr2	Inositol 1,4,5-trisphosphate receptor type 2	0.001
3	Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	0.002
4	Plk1	Serine/threonine-protein kinase	0.004
5	Drg2	Developmentally regulated GTP binding protein 2-like	0.004
6	Dmwd	Dystrophiamyotonica-containing WD repeat motif	0.008
7	Acin1	Apoptotic chromatin condensation inducer1 protein	0.008
8	Pole2	Polymerase (DNA directed), epsilon 2	0.008
9	Cyld	Ubiquitin carboxyl-terminal hydrolase	0.076
10	Csnk2a2	Casein kinase 2, alpha prime polypeptide	0.084
11	Rab10	Ras-related protein Rab-10	0.097

Table 4. Increased cerebral proteinsin the chronic post-ischemia pain group; proteins which might be related to cytoskeletal formation

No	Symbol	Description	P value
1	Krt4	Keratin, type II cytoskeletal 4	0.008
2	Sntb2	Syntrophin, beta 2	0.008
3	Ckap5	Cytoskeleton associated protein 5	0.038
4	Fermt2	Fermitin family homolog 2	0.063
5	Cotl1	Coactosin-like protein	0.065
6	Rps5	40S ribosomal protein S5	0.072
7	Col1a2	Collagen alpha-2(I) chain	0.079
8	Actr10	Actin-related protein 10 homolog	0.080
9	Etl4	Enhancer trap locus 4-like	0.083
10	Farp1	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1	0.094

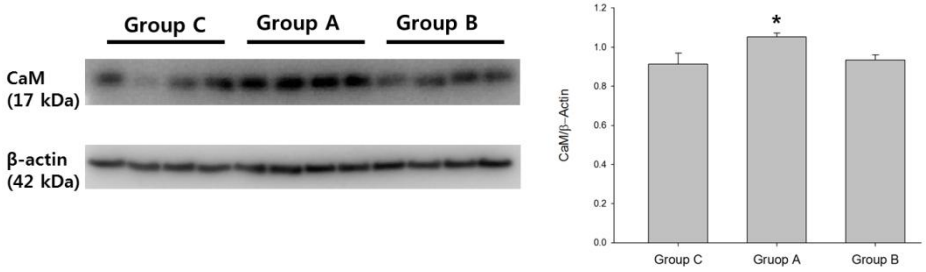
Table 5. Decreased cerebral proteinsin the chronic post-ischemia pain group; proteins which might be related to cell signaling

No.	Symbol	Description	P value
1	Vwa1	Von Willebrand factor A domain-containing protein 1	0.002
2	Ppp1r10	Serine/threonine-protein phosphatase 1 regulatory subunit 10	0.003
3	Slc4a8	Isoform 2 of Electroneutral sodium bicarbonate exchanger 1	0.003
4	Daam2	Dishevelled associated activator of morphogenesis 2	0.003
5	Trim32	Tripartite motif protein 32	0.007
6	Slc1a1	Excitatory amino acid transporter 3	0.007
7	Spn	Sialophorin	0.007
8	Crbn	Cereblon	0.007
9	Thoc1	Da2-19 THO complex subunit 1	0.007
10	Lmo7	Lim domain only protein 7	0.007
11	Rps27a	Ribosomal protein S27a	0.007
12	Sema4d	Sema domain	0.043
13	Sec3l1	SEC3-like 1	0.047
14	Spna2	Alpha II spectrin	0.065
15	Pde10a	Isoform 3 of cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	0.065
16	Snx2	sorting nexin 2	0.067
17	Slc25a3	Phosphate carrier protein, mitochondrial	0.068
18	Cox6a1	Cytochrome c oxidase subunit 6A1, mitochondrial	0.090

3. CaM and CaMKII expression

Cerebral CaM expression was significantly increased in group A as compared to groups B and C ($P = 0.030$, Fig. 3a). Cerebral CaMKII was also significantly increased in group A compared to groups B and C ($P = 0.035$, Fig.3b). There was no significant difference in CaMand CaMKII expression between groups B and C.

(A)



(B)

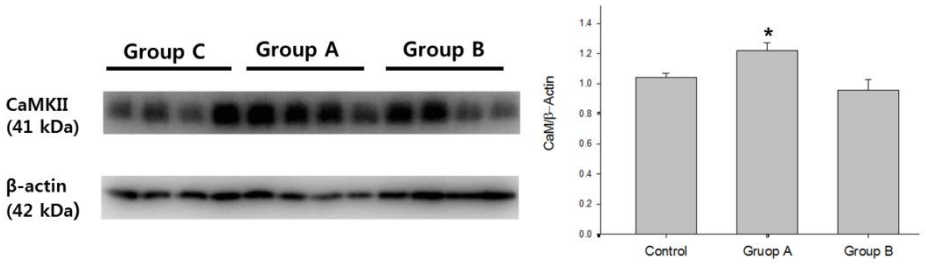


Fig. 3. Expression of calmodulin (CaM, A) and calcium-calmodulin kinase II (CaMKII, B). Group A showed increased expression of CaM(P = 0.030) and CaMKII (P = 0.035) compared to groups B and C (* indicates P < 0.05).

Group A: CPIP group, Group B: CPIP-failure group and Group C: control group.

Discussion

In our study, various proteins weredifferentially expressed in the cerebrum of CPIP animals. Specifically,expressions of proteins related to cell signaling, synaptic plasticity, regulation of cell proliferation and cytoskeletal formationwere increased in the CPIP group. These findings suggest that both functional and structural changes may occur in the cerebrum of CPIP animals, and altered protein expression can be related to the development of CRPS. This is the first study of cerebral protein expression changesin the CPIP rat model. Among the 155 proteins expressed differentially, we conducted confirmation study on the CaM and CaMKII by Western blotting, because they are important calcium-related proteins. Calcium plays acrucial role in many physiological processes, including signal transduction, cell growth and proliferation. Numerous Ca^{2+}-mediated events occur when the released Ca^{2+} binds to and activates the regulatory proteins.Another reason we conducted confirmation study on CaM and CaMKII is that activation of various protein kinase is related to alteration of various protein functions, ultimately results in proteome modification. Therefore, we thought that altered expression of CaM and CaMKII could eventually result in alteration in Ca^{2+}-mediated process and altered protein expression in the rat cerebrum.

CaM is the most well-known calcium-binding protein and is expressed in all eukaryotic cells. CaM is a multifunctional intermediate messenger protein that transduces calcium signals by binding ions and then modifying its interaction

with various target proteins(Balshaw *et al.*, 2002). CaM also mediates many crucial processes, such as regulation of osteoclast(Seales *et al.*, 2006), neuronal plasticity (Sola *et al.*, 2001; Xia and Storm, 2005), and regulation of cell proliferation (Kahl and Means, 2003; Choi and Husain, 2006). Moreover, CaM antagonists can block the TRPV1 channel and NMDA receptor, which results in pain reduction (Olah *et al.*, 2007). Therefore, an increased CaM level suggests that intracellular calcium-mediated processes are increased in the cerebrum of CPIP animals.There have been studies on the altered expression of CaM after nerve injury. In contrast to the results of our study, it was reported that the expression of CaM was decreased in the rat spinal cord after sciatic nerve crushing injury (Jimenez *et al.*, 2005), and that CaM expression wasdecreased in the rat brainstem after spinal nerve ligation(Alzate *et al.*, 2004). The disagreement between the results of these studies and ours might be originated from the anatomical site of the investigation and from the used animal models. Because the cerebrum can be more complex than spinal cord or brainstem, many physiologic changes in the cerebrum can lead to this discrepancy.

Ca^{2+}/CaM complex alsoactivates the CaMKII(Coultrap and Bayer, 2012), which is a serine/threonine-specific protein kinase.It is one of the most prominent protein kinases, present in every tissue, but most concentrated in the brain. CaMKII playsvarious roles, including synthesis and release of neurotransmitter, modulation of calcium channel activity, synaptic plasticity, learning and memory (Sola *et al.*, 2001; Yamauchi, 2005; Coultrap and Bayer, 2012; Halt *et al.*, 2012). Moreover, CaMKII is thought to be important in

central sensitization (Fang *et al.*, 2002; Dai *et al.*, 2005; Crown *et al.*, 2012) and is implicated incentral neuropathic pain (Crown *et al.*, 2012) and long term potentiation (LTP)(Lisman *et al.*, 2012). LTP is initiated when NMDA receptors allow Ca^{2+} into the post-synaptic neuron, and this Ca^{2+} influx activates CaMKII. LTP in nociceptive spinal pathways shares several features with hyperalgesia, and LTP at synapses constitutes a contemporary cellular model for pain(Ruscheweyh *et al.*, 2011; Sandkuhler and Gruber-Schoffnegger, 2012). And it was reported that the overexpression of CaMKII was observed in the dorsal root ganglia in a rat model of type 1 diabetes (Ferhatovic *et al.*, 2013) and the inhibition of CaMKII can reverse the chronic inflammatory pain (Luo *et al.*, 2008). These findings are consistent with the result of our study. Therefore, overexpression of cerebral CaMKII implicates cerebral involvement in CRPS, and CaMKII can be a target for the prevention and treatment of CRPS.

In our study,inositol 1,4,5-triphosphate (IP3) receptor type 2 and phosphoinositide 3 kinase (PI3K) regulatory subunit were also increased in the group A. IP3 receptor is intracellular calcium release channel, and is regulated by calcium and CaM (Nadif Kasri *et al.*, 2002). And it is known that PI3K is animportant mediator of central sensitization in painful inflammatory condition (Pezet *et al.*, 2008) and many tumorous conditions are related to this enzyme (LoRusso and Boerner, 2010; Fung-Leung, 2011).Based on these findings, cerebral overexpression of IP3 receptor type 2 and PI3K can be related to the sustained pain in a rat CPIP model.

In addition, we observed that proteins related to cell signaling and lipid metabolism were decreased in group A. Altered lipid metabolism is thought to be important in CNS injuries or disorders (Adibhatla and Hatcher, 2008). Therefore, the altered expression of peroxisomal 3, 2-trans-enoyl Co A isomerase, acetyl-CoA acyltransferase 1b and acetyl-CoA acetyltransferase 2 in group A can lead to abnormal lipid metabolism in the cerebrum of CPIP animals, which can be another mechanism of CRPS.

Also, we could found the decreased expression of proenkephalin A, cereblon and neuroserpine decreased in CPIP animals. Proenkepalin is an endogenous opioid hormone which produces the enkephalin peptide. Enkephalin provides a role as aninhibitingneurotransmitters in the pathway for pain perception to reduce pain perception. Therefore, decreased proenkephalin A in the cerebrum of CPIP animals seems to reflect the blunted ability to pain modulation and exaggerated response to the pain. For the cereblon, it is known to be related to memory, learning and intelligence(Higgins *et al.*, 2010), and anomalous cereblon expression can lead to memory and learning deficit (Rajadhyaksha *et al.*, 2012). The defect in cereblon gene is associated with mental retardation (Higgins *et al.*, 2004). Therefore, decreased expression of cereblon in our study might be related to the deficit in the learning and memory.Neuroserpine, which was known to be related to neurogenesis (Yamada *et al.*, 2010) was decreased in our study. Neuroserpineplay a role of neuronal protection in pathologic state, and genomic point mutation can cause encephalopathy(Miranda and Lomas, 2006). Also it has been known that deficiency in neuroserpine exacerbates ischemic brain injury (Gelderblom *et*

al., 2013). Therefore, the decreased expression of neuroserpine in our study might be related to the altered or defected cerebral function.

In our study, we used the CPIP model because CRPS develops after a minor injury without distinguishable nerve lesions. This model is considered a novel animal model ofCRPS type 1, in which nerve injury or bone fracture usually does not exist. Although there have been several proteomic studies on the neuropathic pain, those studies usually used the nerve ligation model and nerve crush injury model (Alzate *et al.*, 2004; Jimenez *et al.*, 2005). Since the CPIP model exhibits similar features of human CRPS type 1,our results may havean implication for cerebral involvement in human CRPS.

This study had some limitations. First, the differentially expressed cerebral proteinsmay not be specific to CPIP animals. These proteins may also change in response to peripheral noxious stimuli. However, CPIP animals exhibit many features of human CRPS type 1, and thus ourfindings can be extrapolated to human CRPS. Second, because of the complexity of protein interactions in the many physiologic pathways in the brain, it is still unclear which the key protein in the development of CRPS is.We did not explore the difference of protein expression between group A and group B. The difference of protein expression between group A and group B might be more important for evaluating the key proteins in the pathogenesis of CRPS. Third, inhibitors for the CaM and CaMKII were not used to confirm whether the mechanical threshold change is directly related to the CaM or CaMKII. Further investigationusing specific antagonists of CaM, CaMKII or antagonists of

other proteins expressed differently in our study is required to evaluate the cerebral mechanism of CRPS.

In conclusion, the cerebral proteomeis altered after CPIP injury; many functional and structural changes seem to occur in the cerebrum. These findings support the notion of cerebral involvementin CRPS. Therefore, treatment of CRPS should target not onlythe periphery, but also the brain.

Appendix 1. Cerebral proteinswith increased expression in the chronic post-ischemia pain group.

No.	Symbol	Description	Pvalue
1	Itpr2	Inositol 1,4,5-trisphosphate receptor type 2	0.001
2	Pik3r4	Phosphoinositide 3-kinase regulatory subunit 4	0.001
3	Exoc7	Exocyst complex component 7	0.001
4	Rcor2	REST corepressor 2	0.001
5	Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	0.002
6	Qrich2	Glutamine rich 2-like	0.002
7	Dnah11	Dynein, axonemal, heavy chain 11	0.002
8	Plk1	Serine/threonine-protein kinase PLK1	0.004
9	Ephx1	Epoxide hydrolase 1	0.004
10	Cacnb3	Voltage-dependent L-type calcium channel subunit beta-3	0.004
11	Anxa1	Annexin A1	0.004
12	Tns1	Tensin 1	0.004
13	Hdac4	Histone deacetylase 4	0.004
14	Osbpl7	Oxysterol binding protein like 7	0.004
15	Ecsit	Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial	0.004
16	Sorbs3	Sorbin and SH3 domain containing 3, isoform CRA_b	0.004
17	Kctd12	Potassium channel tetramerisation domain containing 12	0.004
18	Ccbp2	Chemokine-binding protein 2	0.004
19	Drg2	Developmentally regulated GTP binding protein 2-like	0.004
20	Grid2	Glutamate receptor delta-2 subunit	0.004
21	Safb	Scaffold attachment factor B1	0.008
22	Dnm3	Isoform 1 of Dynamin-3	0.008
23	Dnajc16	DnaJ homolog subfamily C member 16	0.008

24	Sntb2	Syntrophin, beta 2	0.008
25	Pnpt1	Polyribonucleotide nucleotidyltransferase 1	0.008
26	Eif3g	Eukaryotic translation initiation factor 3 subunit G	0.008
27	Pole2	Polymerase (DNA directed), epsilon 2	0.008
28	Scyl1	N-terminal kinase-like protein	0.008
29	Atad1	ATPase family, AAA domain containing	0.008
30	Krt4	Keratin, type II cytoskeletal 4	0.008
31	Ctsa	Protective protein for beta-galactosidase	0.008
32	Abca1	5 ATP-binding cassette, sub-family A (ABC1), member 15	0.008
33	Dmwd	Dystrophiamyotonica-containing WD repeat motif	0.008
34	Baiap3	BAI1-associated protein 3-like isoform 2	0.008
35	Znf512b	Uridine kinase-like 1	0.008
36	Gale	Gale protein	0.008
37	Pick1	PRKCA-binding protein	0.008
38	Acin1	Acin1 protein	0.008
39	Chid1	Chitinase domain containing 1	0.008
40	Pcyox1l	Pcyox1l protein	0.008
41	Rabl2b	RAB, member of RAS oncogene family-like 2B	0.008
42	Serpina3k	Serine protease inhibitor A3K	0.008
43	Glg1	Golgi apparatus protein 1	0.008
44	Tnc	Tenascin C	0.008
45	Lysmd1	LysM and putative peptidoglycan-binding domain-containing protein 1	0.008
46	Apba1	Amyloid beta A4 precursor protein-binding family A member 1	0.008
47	Ckap5	cytoskeleton associated protein 5	0.038
48	Ndufab1	Acyl carrier protein	0.035
49	Epha4	Eph receptor A4	0.035
50	Kalrn	Isoform 2 of Kalirin	0.035
51	Myh14	Myosin, heavy chain 14	0.035

52	Anxa2	Isoform Short of Annexin A2	0.043
53	Ccdc47	Coiled-coil domain-containing protein 47	0.043
54	Gpr158	G protein-coupled receptor 158	0.042
55	Cugbp1	CUGBP Elav-like family member 1	0.041
56	Hba2	Hemoglobin alpha 2 chain	0.040
57	Acsl3	Isoform Long of Long-chain-fatty-acid-CoA ligase 3	0.040
58	Rab6a	Ras-related protein Rab-6A	0.038
59	Hbb	Hemoglobin subunit beta-1	0.048
60	Hbb-b1	Zero beta-1 globin	0.044
61	Khsrp	Far upstream element-binding protein 2	0.043
62	Scamp5	Secretory carrier-associated membrane protein 5	0.048
63	Aldh3a2	Fatty aldehyde dehydrogenase	0.049
64	Mesdc2	LDLR chaperone MESD	0.049
65	Rab3d	GTP-binding protein Rab-3D	0.051
66	Vps29	Isoform 2 of Vacuolar protein sorting-associated protein 29	0.051
67	Psma3l	Psma3 Proteasome subunit alpha type-3	0.053
68	Hgs	Isoform 1 of Hepatocyte growth factor-regulated tyrosine kinase	0.054
69	Nlgn3	Isoform 1 of Neuroligin-3	0.056
70	Cygb	Cytoglobin	0.060
71	Pcsk2	Neuroendocrine convertase 2	0.060
72	Prkcd	Isoform 1 of Protein kinase C delta	0.060
73	Fnbp4	formin binding protein 4	0.062
74	Eif2s3x	Eukaryotic translation initiation factor 2 subunit 3	0.063
75	Fermt2	Fermitin family homolog 2	0.063
76	Vps33a	Vacuolar protein sorting-associated protein 33A	0.063
77	SNX3	Sorting nexin-3	0.063
78	Exoc8	Exocyst complex component 8	0.063
79	Thrap3	Thyroid hormone receptor-associated	0.063

		protein 3	
80	Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	0.063
81	Gabarapl 2	Gamma-aminobutyric acid receptor-associated protein-like 2	0.065
82	Cotl1	Coactosin-like protein	0.065
83	Gad1	Glutamate decarboxylase 1	0.065
84	Ehd1	EH domain-containing protein 1	0.066
85	Map2k4	Mitogen-activated protein kinase 4	0.066
86	Mug1	Murinoglobulin (alpha-1-inhibitor 3)	0.070
87	Pck2	phosphoenolpyruvatecarboxykinase 2	0.072
88	Rps5	40S ribosomal protein S5	0.072
89	Ap2s1	Adaptor protein complex 2 subunit sigma	0.075
90	Tpp1	Tripeptidyl-peptidase 1	0.076
91	Cyld	Ubiquitin carboxyl-terminal hydrolase	0.076
92	Nuc	Nucleolin-like protein	0.079
93	Col1a2	Collagen alpha-2(I) chain	0.079
94	Slc6a17	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT4	0.079
95	Actr10	Actin-related protein 10 homolog	0.080
96	Cacng2	Voltage-dependent calcium channel gamma-2 subunit	0.083
97	Ampd3	AMP deaminase 3	0.083
98	Eif5b-ps1	Eif5b Eukaryotic translation initiation factor 5B	0.083
99	Timm9	Mitochondrial import inner membrane translocase subunit Tim9	0.083
100	Etl4	enhancer trap locus 4-like	0.083
101	Csnk2a2	Casein kinase 2, alpha prime polypeptide	0.084
102	Cct6a	Chaperonincontaining TCP1 subunit 6a	0.084
103	Nudc	Nuclear migration protein nud	0.085
104	Ndufa13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 13	0.085
105	Camk2b	Calmodulin-dependent protein kinase II beta M isoform	0.086
106	Clta	Isoform Brain of Clathrin light chain A	0.086
107	Asah1	Acid ceramidase	0.086

108	Phb2	Prohibitin-2	0.086
109	Sod1	Superoxide dismutase [Cu-Zn]	0.088
110	Ndufs8	NADH dehydrogenae (ubiquinone) 1 alpha subcomplex subunit 8	0.090
111	Slc17a7	Isoform 1 of Vesicular glutamate transporter 1	0.091
112	Ugp2	UDP-glucose pyrophosphorylase 2, isoform CRA-b	0.091
113	Rala	Ras-related protein Ral-A	0.091
114	Anxa5	Annexin A5	0.093
115	Hnrph1	Isoform 1 of Heterogeneous nuclear ribonucleoprotein H	0.093
116	Stxbp5l	Syntaxin binding protein 5-like	0.093
117	Abcd3	ATP-binding cassette sub-family D member 3	0.094
118	Farp1	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1	0.094
119	Leng4	Leng4 protein	0.094
120	Scn2a1	Sodium channel Nav1.2	0.095
121	Rab10	Ras-related protein Rab-10	0.097
122	Aldh7a1	Alpha-aminoadipicsemialdehyde dehydrogenase	0.097
123	Cltb	Isoform Brain of Clathrin light chain B	0.097
124	Phyhipl	Isoform 1 of Phytanoyl-CoA hydroxylase-interacting protein-like	0.098
125	Synpo	Isoform 1 of Synaptopodin	0.099

Appendix 2. Cerebral proteinswith decreased expression in the chronic post-ischemia pain group.

No.	Symbol	Description	Pvalue
1	Vwa1	Von Willebrand factor A domain-containing protein 1	0.002
2	Ppp1r10	Serine/threonine-protein phosphatase 1 regulatory subunit 10	0.003
3	Poldip2	DNA-directed polymerase delta interacting protein 2	0.003
4	Slc4a8	Isoform 2 of Electroneutral sodium bicarbonate exchanger 1	0.003
5	Daam2	Dishevelled associated activator of morphogenesis 2	0.003
6	Cep350	Centrosone-associated protein 350	0.003
7	Tra2b	Transformer-2 protein homolog beta	0.007
8	Epb4.1l1	Isoform L of Band 4.1-like protein 1	0.007
9	Trim32	Tripartite motif protein 32	0.007
10	Slc1a1	Excitatory amino acid transporter 3	0.007
11	Spn	Sialophorin	0.007
12	Crbn	Cereblon	0.007
13	Thoc1	Da2-19 THO complex subunit 1	0.007
14	Lmo7	Lim domain only protein 7	0.007
15	Rps27a	Ribosomal protein S27a	0.007
16	Sema4d	Sema domain, immunoglobulin domain (Ig), transmembrane domain	0.043
17	Sec3l1	SEC3-like 1	0.047
18	Ikbkap	Elongator complex protein 1	0.058
19	Peci	Peroxisomal 3,2-trans-enoyl-CoA isomerase	0.058
20	Penk	Proenkephalin-A	0.058
21	Bles03	basophilic leukemia expressed protein	0.058
22	Spna2	Alpha II spectrin	0.065
23	Pde10a	Isoform 3 of cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	0.065
24	Snx2	sorting nexin 2	0.067
25	Slc25a3	Phosphate carrier protein, mitochondrial	0.068
26	Serpini1	Neuroserpin	0.070

27	Acaa1b	Acetyl-CoA acyltransferase 1b	0.077
28	H2afz	Histone H2A.Z	0.079
29	Cox6a1	Cytochrome c oxidase subunit 6A1, mitochondrial	0.090
30	Acat2	Acetyl-CoA acetyltransferase 2	0.094

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국문 초록

서론

복합부위통증증후군(complex regional pain syndrome, CRPS)은 극심한 통증을 유발하는 희귀 난치성 통증 질환이다. 비록 CRPS 의 정확한 발병 기전은 알려지지 않았으나, 중추 및 말초 기전 모두가 CRPS 의 발병에 관여한다고 알려져 있다. 기존의 연구들에 의하면 CRPS 환자들에서 대뇌의 기능적 이상이 발견된다고 보고되었으나 현재까지 CRPS 대뇌 단백질 발현 양상에 대해서는 연구가 이루어진 바 없다. 따라서 본 연구자들은 CRPS 의 중추성 기전 중 대뇌 단백질의 변화를 살펴보기 위해 CRPS 의 새로운 동물 모델이라고 알려져 있는 만성 허혈 후 통증 (chronic post-ischemia pain, CPIP) 모델을 이용하여 렛트 대뇌를 대상으로 단백체학적 분석을 시행하였다.

방법

렛트 21 마리를 대상으로여 CPIP 동물 모델을 확립한 후기계적 역치 검사 결과에 따라 실험군과대조군으로 분류하였다. CPIP 모델 확립 21 일 후 실험 동물의 대뇌를 적출하여 다면적 단백질 인식 기법(multidimensional protein identification technology,

MudPIT)을 이용한 단백체학적 분석을 시행하였다. MudPIT 결과 CPIP 군과 대조군의 대뇌에서 다르게 발현된 단백질을 선별하여 CPIP 군과 대조군 사이의 칼모듈린(calmodulin, CaM)과 칼슘/칼모듈린 제 2 인산화 효소(calcium calmodulin kinase II, CaMKII) 발현 차이를 웨스턴블롯으로 확인하였다.

결과

CPIP 군의 대뇌에서 125 가지 단백질 발현이 증가하였으며 이들은 세포 신호 전달, 신경가소성, 세포 증식, 세포골격 형성과 관련된 단백질이었다. 또한 CPIP 군에서프로엔케팔린(proenkephaline)을 비롯한 30 가지의 단백질 발현이 감소하였다. CaM (P = 0.030)과 CaMKII (P = 0.035)의 발현은 CPIP 군에서 대조군에 비해서 유의하게 증가하였다.

결론

대뇌의 기능적, 구조적 변화가 CRPS 증상 및 징후의 발현 및 유지에 관여한다고 여겨진다. 발현 차이가 있었던 개별 단백질의 CRPS 발생에 미치는 영향에 대한 추가적인 연구가 요구된다.

주요어: 단백질, 대뇌, 복합부위통증증후군, 칼모듈린, 칼모듈린
학 번: 2009-30514

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의과 대학을 졸업하면 해박한 의사가 될 줄 알았습니다.
의학 석사, 박사를 마치면 저도 제 분야에서 정통한 전문가가 되는 줄 알았습니다. 그러나, 공부를 하고, 학위 논문을 준비하면서 점점 더 제 자신이 한없이 작아지는 것을 느꼈다고 고백합니다. 세상에는 정말로 제가 모르는 것이 많고, 공부해야 할 것도 많은 것 같습니다.
학위 논문 준비를 하면서 조금 더 열심히 준비할 수 있었을 텐데 하는 아쉬움도 남지만, 이제야 박사 과정을 마무리하게 되었다는 안도감과 저를 여기까지 이끌어주셨던 여러 고마우신 분들이 머릿속에 떠오릅니다. 논문의 초기 단계부터 마무리 실험까지 도움을 주신, 제가 평생을 존경하는 우리 형 건국대 남상섭 교수님과 proteomics 실험에 크나큰 도움을 주셨던 광주 과기원 박지용 교수님께 깊은 감사를 드립니다. 학위 과정뿐 아니라, 병원 생활 전반에 걸쳐 항상 따뜻한 가르침을 주시는 스승님이신 김용철 교수님과, 의과대학 CaSA 시절부터 저를지켜보시며 사랑으로 돌보아 주셨던김전 교수님께 깊은 존경과 감사를 드립니다.이 논문을 세상에 내놓을 수 있도록 논문의 질적 향상을 위해 조언을 아끼지 않으셨던박성혜 교수님, 임영진 교수님, 심우석 교수님께도

깊은 감사를 드립니다. 항상 가까이서 하루 종일 한 가족같이 저와 얼굴을 맞댄 채생활하시면서 격려해 주시고 이끌어 주시는, 큰 형님 이평복 교수님, 저를 통증 전문의로 이끌어주시고 항상 지도 편달해 주시는 이상철 교수님께도 이 자리를 통해 감사 인사 드립니다.

저를 낳으시고 키워주시며, 막내 아들이 언제 박사 학위를 취득할까 가슴 졸이며 지켜보셨던 아버님, 어머님과 저를 위해 항상 기도해주시는 장모님께도 깊은 감사 드립니다. 부모님들의 무한하신 사랑과 기도 덕분에 제가 이 세상에서 그나마 사람 구실하며 여기까지 걸어올 수 있었습니다. 자주 놀아주지 못해 늘 미안한 사랑하는 아들 남지호미카엘과, 항상 곁에서 힘이 되어준 나의 사랑하는 아내 양성희 카타리나에게미안함과고마움을 보냅니다.

저의 모든 삶을 지켜보시며, 이끌어 주시는 하느님께 감사와 영광을 올립니다. 하느님께서 부르시는 곳에서 당신의 작은 도구로 쓰임 받도록 기도 드립니다.

2013 년 7 월 불곡산 기슭에서

남 상 건