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The effect of hypothermia on the NLRP3 inflammasome expression in a rat model of focal cerebral ischemia

백서 국소뇌허혈 모델에서 저체온요법이 NLRP3 인플라마좀 발현에 미치는 영향

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The effect of hypothermia on the NLRP3 inflammasome expression in a rat model of focal cerebral ischemia

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Abstract

The effect of hypothermia on the NLRP3 inflammasome expression in a rat model of focal cerebral ischemia

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Background: Hypothermia is generally known to reduce brain injury following cerebral ischemia by various mechanisms including anti-inflammatory effects. However, the exact anti-inflammatory mechanism of hypothermia is not well known. Recently, several evidence have suggested that NLRP3 inflammasome is involved in the pathogenesis of sterile inflammatory response by processing caspase-1 and Interleukin (IL)-1β to an active stage following cerebral ischemia. The association between hypothermia and inflammasome in the cerebral ischemia animal model is not reported. So we hypothesized that hypothermia can attenuate the expression of NLRP3 inflammasome in a rat model of focal cerebral ischemia.

Methods and Results: For *in vitro* study, BV-2 cells (immortalized mouse microglial cell line) were divided by two groups, control and oxygen-glucose deprivation groups. Each groups were re-divided by different temperatures,

normothermia (37 °C) and hypothermia (33 °C). Hypothermia was maintained for 3 h in the beginning of study. Western blotting with antibodies to IL-1β, IL-18 and NLRP3 inflammasome complexes including NLRP3 protein, ASC, and caspase-1 and immunohistochemical staining with caspase-1 antibody were done. Western blotting showed increased expression levels of IL-1β, IL-18, NLRP3, ASC, and caspase-1 after OGD group and reduced significantly by hypothermia. Caspase-1 expressed in the cytosol of BV-2 cell in all groups and showed relatively decreased expression in OGD with hypothermia group compared with OGD with normothermia group.

For *in vivo* study, transient middle cerebral artery occlusion (MCAO) model was induced in male Sprague–Dawley (SD) rats using the suture occlusion technique. Sixteen SD rats underwent left MCA occlusion for 2 h followed by reperfusion for 22 h. For the hypothermia, rats were cooled by alcohol spraying and evaporating methods within 15 min at the occlusion of MCA. During 2 h with occlusion period, rectal temperature was maintained with 33 °C (hypothermia group) or 37 °C (normothermia group). Rats were sacrificed at 24 h after ischemia. Nissl staining to evaluate the infarct volume showed reduced infarct volume in hypothermia group compared with the normothermia group (normothermia, 248 ± 52 mm3; hypothermia, 128 ± 27 mm3; $p<0.001$; n=8 per group). To analyze the effects of hypothermia on the expression of IL-1β, and NLRP3 proteins in ischemic brain tissue, we measured IL-1β, and NLRP3 levels in ischemic or contralateral non-ischemic brain tissues. Compared with contralateral non-ischemic hemisphere, Western blot with IL-1β and NLRP3 antibodies showed significantly increased

expression levels in ischemic hemisphere ($p<0.001$ in normothermia groups; $p<0.05$ in hypothermia groups). IL-1β and NLRP3 proteins were significantly decreased in ischemic brain tissue with hypothermia than ischemic brain tissue with normothermia ($p<0.05$ by Western blot with IL-1β antibody; $p<0.001$ with NLRP3 antibody).

Conclusion: Our study revealed that NLRP3 inflammasome is overexpressed in the BV-2 microglial cells with OGD models, and hypothermia attenuates the NLRP3 inflammasome expression and infarct volume in a rat model of focal cerebral ischemia.

Key words: hypothermia, cerebral ischemia, inflammasome

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Introduction

Stroke is a major health burden in Korea as well as worldwide, accounts for roughly 1 of every 10 deaths.(1) Despite the social burden of stroke is large, knowledge about the mechanism of brain injury by ischemia and treatment methods are negligible. There is evidence for efficacy of over 500 treatment strategies in animal models of focal cerebral ischemia, (2) but only recombinant-tissue plasminogen activator (rt-PA), aspirin and stroke unit care have convincingly demonstrated efficacy in clinical trials of acute ischemic stroke.(3) The failure of most of neuroprotective therapies may be explained by the fact that most neuroprotectants inhibit only a single step in the broad cascade of events that leads to cell death.(4, 5)

Hypothermia is generally known to reduce brain injury following cerebral ischemia in animals(6) and humans. (7, 8) There are some theories presume that hypothermia enhances the tolerance for cerebral ischemia by reducing the cerebral metabolic rate and the release of neurotoxic excitatory amino acids (9, 10). When considering that the major mechanism of cell death in ischemic stroke is the inflammatory cascade followed by necrosis or apoptosis induced by ATP loss, hypothermia may have neuroprotective effect by inhibition of expression of inflammatory factors in cerebral ischemia. Some inflammatory factors inhibited by hypothermia in cerebral ischemia, such as NF-κB and macrophage inflammatory protein-3alpha (MIP-3α), are reported recently, but the exact anti-inflammatory mechanism of hypothermia is not known.(11, 12)

Recently, several evidence have suggested that inflammasome, one of the components of the innate immune system, is involved in the pathogenesis of sterile inflammatory response by processing caspase-1 and Interleukin (IL)-1β to an active stage following human CNS disorders (such as: spinal cord injury, traumatic brain injury, hemorrhagic stroke and ischemic stroke).(13–16) The inflammatory pathway induced by inflammasome is considered to be a new inflammatory cascade separate from classic NF-κB pathway. There are eight inflammasome subtypes have been identified, and nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is recently reported as the important factor involved in ischemic stroke.(16–18) The NLRP3 inflammasomes are cytosolic macromolecular complexes composed of the NLRP3 receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), precursor caspase-1, precursor caspase-11 and/or XIAP (X-linked inhibitor of apoptosis).

There is an report that hypothermia have neuroprotective effect by inhibition of the expression of inflammasome in the traumatic brain injury animal model,(14) but the association between hypothermia and inflammasome in the cerebral ischemia animal model is not reported. So we hypothesized that hypothermia can attenuate the expression of NLRP3 inflammasome in a rat model of focal cerebral ischemia.

Methods

Cell culture

The BV-2 cell is an immortalized mouse microglial cell line that exhibits the morphological and functional characteristics of microglia. The cells were grown in monolayers in 89 % RPMI 1640 with L-glutamine (300 mg/L), 25 mM HEPES, and 25 mM NaHCO3 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; Gibco) in a humidified 5% CO2 atmosphere at 37 °C. Cells were cultured in 80-cm2 flasks overnight to 80–90 % confluence. For oxygen-glucose deprivation (OGD), BV-2 cells were incubated in glucose-free Locke's buffer in an oxygen-free chamber for 3 h. Hypothermia *in vitro* were maintained during OGD, and re-warmed at 37 °C in the following reperfusion period.

Animal models

All experimental procedures were carried out with institutional approval in accordance with the National Institutes of Health Guide of the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Animals were allocated to normothermia (37 °C, n = 16) and hypothermia (33 °C, n = 16) groups. Transient middle cerebral artery occlusion (MCAO)

model was induced in male Sprague–Dawley (SD) rats (250–300 g; Orient Bio, Korea) using the suture occlusion technique established in our laboratory (19–21). Briefly, male SD rats were anesthetized using 2% isoflurane in a gas mixture of 70% nitrogen oxide and 30% oxygen. After cleaning of skin with povidone iodine, a midline incision was made. The left common carotid artery and external carotid artery were exposed and ligated. The 3-0 monofilament nylon suture was gently inserted and the left internal carotid artery was temporarily ligated to avoid further bleeding from the puncture site. After closing the incision, the animal was kept in the cage to recover from the anesthesia. Two hours after the occlusion, nylon suture were removed to recover blood flow. Rats were sacrificed at 24 h after ischemia.

For the hypothermia *in vivo*, animals were cooled by spraying with alcohol and evaporating this with an electric fan at the onset of ischemia. Rats were subsequently rewarmed on a heating pad under a lamp. Cooling and rewarming were achieved within 15 min. The rectal temperature was monitored by rectal temperature probe and maintained at 33 °C in hypothermia group and 37 °C in normothermia group. Hypothermic condition was maintained for 2 h of transient occlusion period.

Western blot analysis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide (SDS) (10%) gel electrophoresis using a Tris-glycine running buffer. Gels were then electro-blotted using a transfer apparatus in transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% methanol for

1.5 h at 15 V onto a nitrocellulose membrane. The membrane was then incubated in blocking buffer for 1 h at 23 °C. The membrane was then incubated overnight at 4 °C with primary antibodies including those that selectively bind NLRP3 (Novus Biologicals), ASC (Abcam), caspase-1 (Abcam), IL-1β (Abcam), IL-18 (Bioworld Technology), and β-actin (Sigma-Aldrich). After washing three times, the membrane was incubated with secondary antibodies against the primary antibody and β-actin for 1 h at room temperature.

In a rat model of focal cerebral ischemia, brains were homogenated and centrifuged at 13,000 rpm for 10 min at 48 hours after MCAO (n = 8 per group). 20 μg samples of proteins were separated by SDS-PAGE and analyzed by Western blot using primary antibodies to described as above. Quantification of protein levels was achieved by densitometry analysis using Image J software.

Immunohistochemical staining

BV-2 cells were fixed in 4% buffered paraformaldehyde in PBS for immunohistochemistry. Fixed cells were incubated at 4 °C overnight and stained with primary antibodies that selectively bind caspase-1 (Abcam) and fluorescent-tagged secondary antibody. The nuclei were counterstained with DAPI. Slides were analyzed with a fluorescence microscope (Leica DM5500 B, Leica Microsystems, Germany) and LAS AF software.

Measurement of infarction volumes

At 24 hours after MCAO, brains (n = 8 per group) were removed after cardiac perfusion-fixation with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS). Using a freezing microtome, brains were cut into 30 μm coronal sections. A total of 8 brain sections were mounted onto glass slides and processed for Nissl staining. Infarct volumes were determined using Photoshop 12.0 (Adobe Systems Inc., San Jose, CA).

Statistics

Significance was determined by Student's t-test or one-way analysis of variance (ANOVA) as appropriate. P values <0.05 were considered significant. Data analysis was performed using SPSS 19.0 (Chicago, IL).

Results

Hypothermia reduced the expression levels IL-1β and IL-18 in vitro

BV-2 cells were divided by four groups, control with normothermia (group C), control with hypothermia (group H), OGD with normothermia (group N+O), and OGD with hypothermia (group H+O) groups. Hypothermia with 33 °C was maintained during 3 h of OGD period and then normothermia with 37 °C was maintained during 24 h of reperfusion period. Hypothermia in group H was maintained during 3 h. After 3h of OGD period and followed reperfusion of 24 h, Western blotting showed significantly elevated expression levels of IL-1β and IL-18 in Western blotting compared with control groups. Control with hypothermia did not affect the expression levels of IL-1β and IL-18. OGD with hypothermia group showed significantly reduced expression levels of IL-1β and IL-18 compared with OGD with normothermia group (Figure 1A).

Hypothermia reduced the expression levels precursor caspase-1 and caspase-1 in vitro

BV-2 cells were divided by four groups as above and Western blotting with antibodies to caspase-1 was done with same settings. After OGD 3h and followed reperfusion of 24 h, the expression levels of precursor caspase-1 and caspase-1 (activated forms of precursor caspase-1) were elevated significantly. Control with hypothermia group and control with normothermia group

showed no differences. OGD with hypothermia group showed significantly decreased expression levels of precursor caspase-1 and caspase-1 compared with OGD with normothermia group (Figure 1B).

The expression of NLRP3 and ASC proteins were attenuated by hypothermia in vitro

Protein samples, obtained by BV-2 cells with same experimental settings described as above, were subjected to Western blotting by antibodies to NLRP3 and ASC proteins. NLRP3 and ASC, the components of NLRP3 inflammasome complexes, were significantly increased after OGD, and be suppressed by hypothermia during 3 h of OGD (Figure 2).

Immunohistochemistry of caspase-1 showed cytosolic expression in BV-2 cell

Immunohistochemical staining was done with antibodies to caspase-1 (green) and DAPI (blue) for staining of nuclei. Caspase-1, which was one of the components of activated NLRP3 inflammasome complexes, showed cytosolic expression. If compare OGD with normothermia group and OGD with hypothermia group, caspase-1 was relatively decreased in OGD with hypothermia group (Figure 3).

Hypothermia reduced infarct volume in a rat model of focal cerebral ischemia

To investigate the neuroprotective effect of hypothermia in a rat model of focal cerebral ischemia, we induced transient MCAO and reperfusion with SD rats in two groups (normothermia and hypothermia groups). Examination of animals at 24 h after MCAO showed that hypothermia reduced infarct volume significantly as compared with the normothermia group (normothermia, 248 ± 52 mm3; hypothermia, 128 ± 27 mm3; $p<0.001$; n=8 per group) (Figure 4).

Hypothermia reduced the expression levels IL-1β and NLRP3 in vivo

To analyze the effects of hypothermia on the expression of inflammatory cytokines, IL-1β, and NLRP3 proteins in ischemic brain tissue, we measured IL-1β, and NLRP3 levels in homogenated and centrifuged ischemic or contralateral non-ischemic brain tissues. IL-1β and NLRP3 proteins were significantly decreased in ischemic brain tissue with hypothermia than ischemic brain tissue with normothermia (Figure 5).

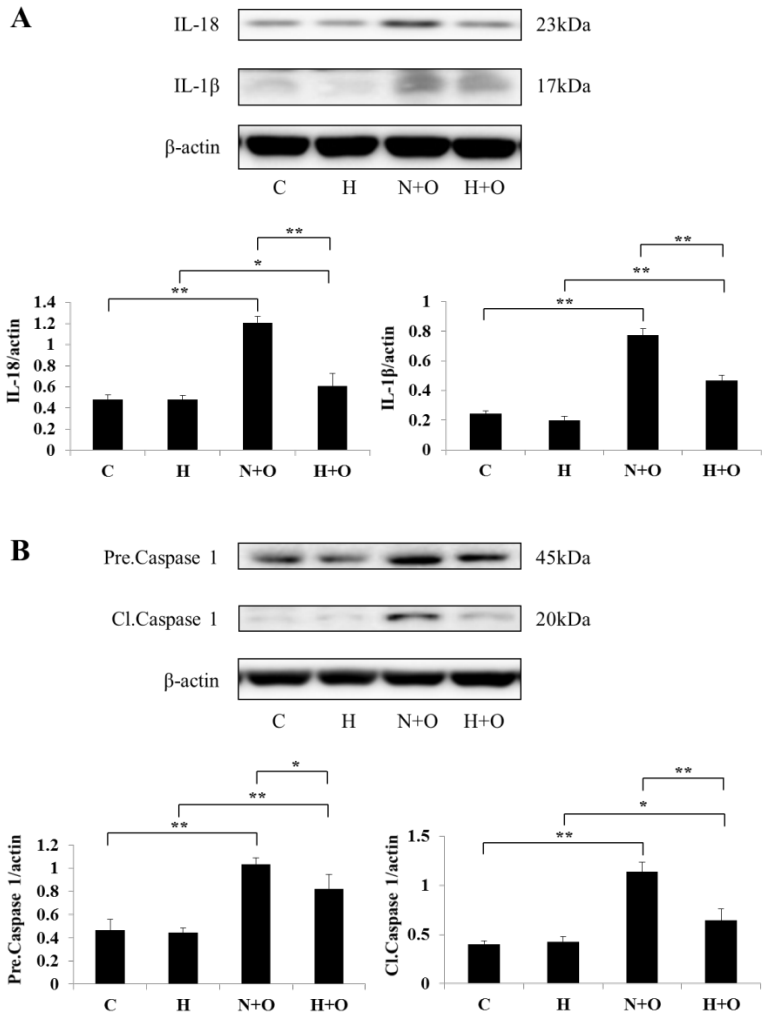

Figure 1. The expression levels of IL-18, IL-1β, precursor caspase-1, and caspase-1 were suppressed by hypothermia *in vitro*. (A) The expression levels of IL-1β and IL-18 were increased after OGD significantly, and be suppressed by hypothermia during 3 h of OGD. (B) Western blot of precursor caspase-1 and caspase-1 results showed same as above. (Abbreviations: C, control with normothermia; H, control with hypothermia; N+O, OGD with normothermia; H+O, OGD with hypothermia). (*$P < 0.05$, **$P < 0.001$).

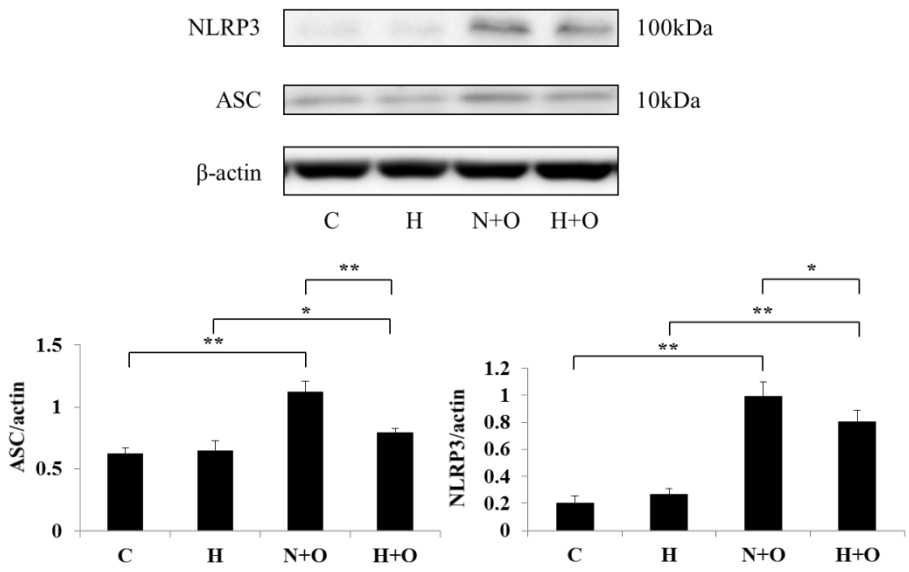

Figure 2. The expression of NLRP3 and ASC proteins were attenuated by hypothermia *in vitro*. NLRP3 and ASC, the components of NLRP3 inflammasome complexes, were significantly increased after OGD, and be suppressed by hypothermia during 3 h of OGD. (*$P < 0.05$, **$P < 0.001$).

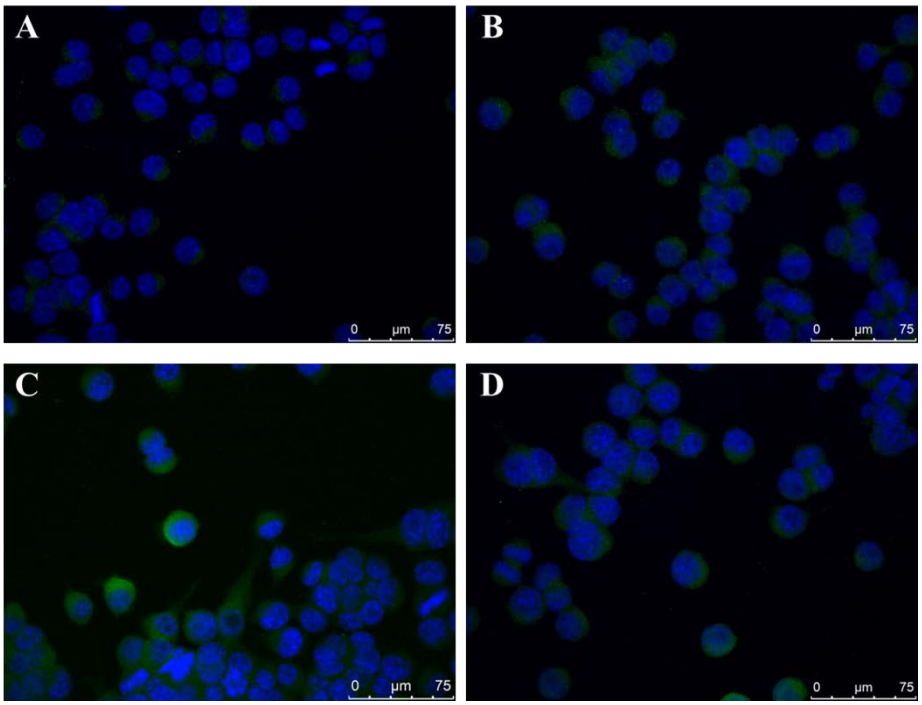

Figure 3. Immunohistochemistry of caspase-1 showed cytosolic expression in BV-2 cell. Immunohistochemical staining by DAPI (blue) and caspase-1 antibody (green), which was one of the components of activated NLRP3 inflammasome complexes, showed increased expression of caspase-1 by OGD and reduced by hypothermia. (A) Control with normothermia. (B) Control with hypothermia. (C) OGD with normothermia. (D) OGD with hypothermia.

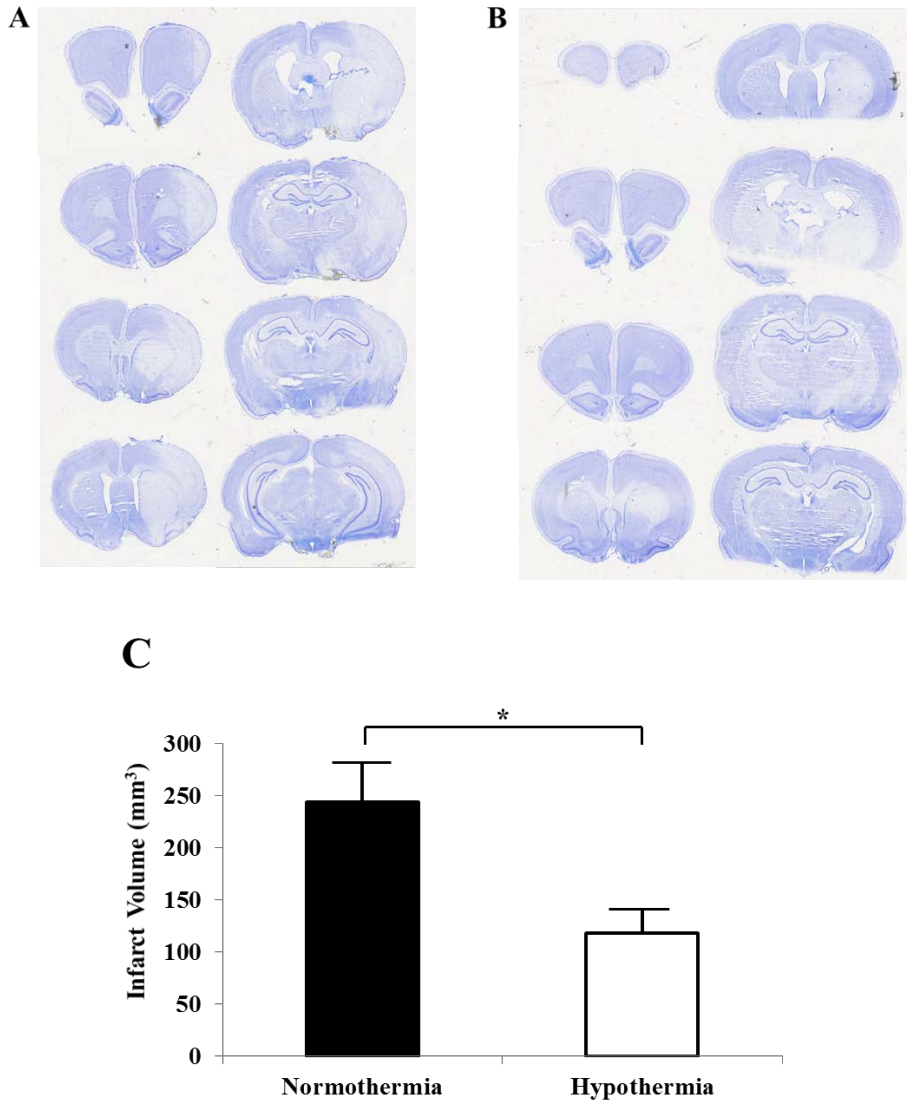

Figure 4. Effects of hypothermia on ischemic damage in a rat model of focal cerebral ischemia. Nissl stain of ischemic rat brain showed grossly reduced infarct volume in hypothermia group. (A) Representative brain slices of normothermia group. (B) Hypothermia group. (C) Comparisons of infarct volume between normothermia and hypothermia groups after transient middle cerebral artery occlusion. The symbols and bars represent mean ± standard deviation of 8 rats per group. (*$P < 0.001$).

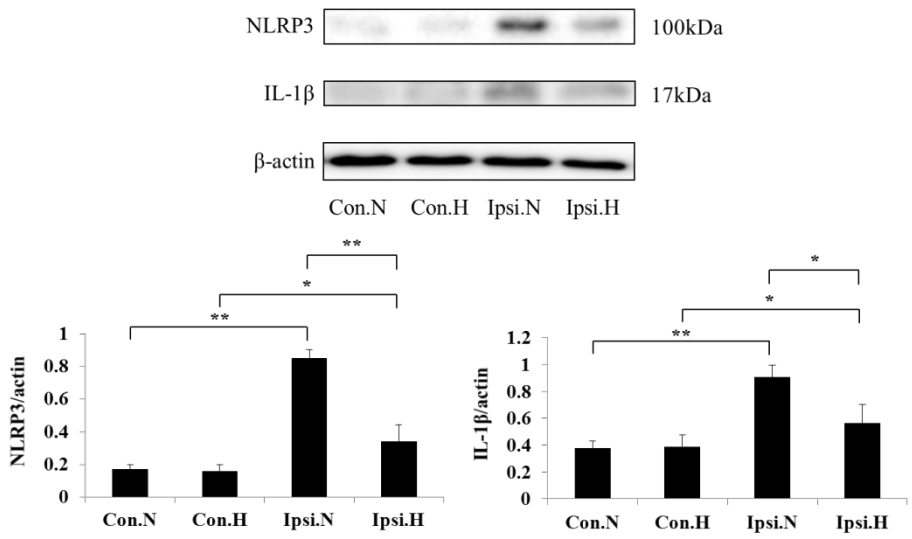

Figure 5. The expression levels of IL-1β and NLRP3 were attenuated by hypothermia *in vivo*. Western blot of IL-1β and NLRP3 results showed significantly increased expression levels in the ipsilateral hemisphere of transient middle cerebral artery occlusion compared with contralateral hemisphere. In the hypothermia group, IL-1β and NLRP3 were significantly suppressed. (Abbreviations: Con.N, contralateral hemisphere of MCAO with normothermia; Con.H, contralateral hemisphere of MCAO with hypothermia; Ipsi.N, ipsilateral hemisphere of MCAO with normothermia; Ipsi.H, ipsilateral hemisphere of MCAO with hypothermia). (*$P < 0.05$, **$P < 0.001$).

Discussion

Our study revealed that hypothermia attenuates infarct volume and NLRP3 inflammasome activation which aggravates brain inflammation in a rat model of focal cerebral ischemia. This is the first report which demonstrated the association of hypothermia and NLRP3 inflammasome in cerebral ischemia.

Ischemic brain injury results from the complex interplay of multiple pathways including excitotoxicity during the first several minutes after onset of the ischemia followed by post-ischemic inflammation and apoptosis.(22) After excitotoxicity period, inflammation is the most important factor that aggravates brain injury and influences patients' clinical outcome. Damaged cells by excitotoxicity release danger signals, designated danger-associated molecular patterns (DAMPs). Types of DAMPs have ATP, reactive oxygen species, monosodium urate crystals, UVB, glucose and more.(23) Most of them expressed without ischemic condition and maintained with balance by anti-inflammatory pathways. Therefore, control group without OGD or focal cerebral ischemia showed slightly elevated expression of NLRP3 inflammasome in our study. When DAMPs are upregulated by ischemic condition, NLRP3 inflammasome can be over-expressed and activate inflammatory cytokines, such as IL-1β.

Several inflammasome sensor molecules can trigger the formation of inflammasomes. Most of the inflammasomes that have been described to date contain a NOD-like receptor (NLR) sensor molecule, namely NLRP1 (NOD-,

LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4 (NOD-, LRR- and CARD-containing 4).(24) Among them, NLRP1 and NLRP3 inflammasomes are recently reported which have an important role in CNS disorders including cerebral infarction and intracerebral hemorrhage.(15, 16). Inflammasomes is located in an upper portion of inflammatory pathways. Therefore inhibition of inflammasome can influence of variety of inflammatory cytokines and more powerful anti-inflammatory effects can be resulted. Our study showed that NLRP3 inflammasomes are associated with ischemic brain injury and can be novel therapeutic target for neuroprotectants.

Hypothermia influences multiple aspects of brain physiology in the acute, subacute and chronic stages of ischemia. It affects pathways leading to excitotoxicity, apoptosis, inflammation and free radical production, as well as blood flow, metabolism and blood–brain barrier integrity.(25) The neuroprotective mechanisms of hypothermia are slowly beginning to be understood. Moreover it is not well known that which pro-inflammatory factors including inflammatory cytokines can be inhibitied by hypothermia. In the traumatic brain injury model, there was one report that suggested the effects of hypothermia on NLRP1 inflammasome signaling after traumatic brain injury.(26) Our study first reported the association between NLRP3 inflammasome and hypothermia in a rat model of focal cerebral ischemia.

Hypothermia has many side effects so can be indicated to patients with strict criteria. Side effects include immunosuppression with increased infection risk, cold diuresis and hypovolemia, electrolyte disorders, insulin resistance,

impaired drug clearance, and mild coagulopathy.(27) These are known to be aggravated by prolonged treatment periods.(28) Therefore NLRP3 inflammasome can be novel adjuvant therapeutic targets with hypothermia in cerebral ischemia and may attenuate the side effects by reducing the treatment periods of hypothermia.

Our study had limitations including clinical applicability and causal relationship. Additional studies including inhibition of NLRP3 inflammasome should be needed for the evaluation of causal relationship between reduced infarct volume by hypothermia and decreased expression of NLRP3 inflammasome. But reduced NLRP3 inflammasome expression may contributes to attenuate the infarct volume by hypothermia as compared with previous report.(29)

Furthermore, we examined the expression levels of NLRP3 inflammasome 24 h after the onset of cerebral ischemia. Additional studies including various time points, such as several hours and days after the onset of ischemia, should be examined for the evaluation of the temporal expression levels of NLRP3 inflammasome. In the previous other report, the expression levels of NLRP1 inflammasome were significantly increased at 6 h after ischemia and maintained at 14 days.(30) Given the fact that the effects of the NLRP1 and NLRP3 inflammasomes are similar, the expression of NLRP3 inflammasome may be upregulated at several hours after ischemia and maintained about 14 days.

In the time point of the initiation of hypothermia, it can be initiated after the onset of cerebral ischemia in the real clinical world. In our study, hypothermia

was induced right before carotid artery occlusion, so this might augment the effect of hypothermia compared with the real clinical setting.

In conclusion, our study revealed that hypothermia attenuates infarct volume and inhibits NLRP3 inflammasome activation which aggravates brain inflammation in a rat model of focal cerebral ischemia. This study may provide new insight into understanding the mechanisms of neuroprotective effects of hypothermia in cerebral ischemia.

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초 록

배경: 저체온요법은 일반적으로 뇌경색 후 발생하는 신경 손상을 감소시켜주는 것으로 알려져 있으나, 구체적인 저체온요법의 항염증 기전을 통한 신경보호효과에 대해서는 밝혀진 바가 미미하다. 최근 NLRP3 인플라마좀이 뇌경색 동물 모델에서 caspase-1 활성화를 통한 IL-1β 생성을 촉진하여 뇌경색 후 발생하는 염증성 신경 손상과 연관되어 있음이 보고되었다. 저체온요법이 항염증 효과가 있다는 점을 고려할 때 저체온요법과 NLRP3 인플라마좀의 연관성에 대해 유추할 수 있으나 아직 서로의 관련성에 대해서는 보고된 바 없다. 이에 백서 국소뇌허혈 모델에서 저체온요법을 적용하였을 때 NLRP3 인플라마좀의 발현에 어떠한 영향을 미치는가를 본 논문에서 확인하고자 하였다.

방법과 결과: BV-2 세포(미세아교세포)를 이용한 산소-포도당 결핍 실험을 진행하였다. 산소-포도당 결핍 없이 정상온도(37 도)로 유지한 군, 산소-포도당 결핍 없이 저온(33 도)으로 유지한 군, 3 시간 산소-포도당 결핍 동안 정상온도로 유지한 군, 3 시간 산소-포도당 결핍 동안 저온으로 유지한 군으로 나누어 실험을 진행하였다. 산소-포도당 결핍을

하였을 때 유의하게 IL-1β, IL-18, NLRP3 단백, ASC, caspase-1 의 발현 정도가 증가하였고, 산소-포도당 결핍 중 저온으로 유지한 군에서는 산소-포도당 결핍 중 정상온도로 유지한 군에 비해 유의하게 발현 정도가 감소하였다. Caspase-1 항체를 통한 면역형광염색 검사를 하였을 때, 세포질에서 caspase-1 이 발현됨을 확인하였고, 마찬가지로 산소-포도당 결핍 중 저온으로 유지한 군에서 산소-포도당 결핍 중 정상온도로 유지한 군에 비해 상대적으로 염색 정도가 감소하였다.

2 시간 결찰 동안 저체온(33 도) 또는 정상체온(37 도)을 유지하며 유도한 백서 국소뇌허혈 모델에서 결찰 후 22 시간 동안 재관류를 유지하였다. 저체온 유지는 알코올 분무를 통해 체온을 하강시키고, 직장 온도를 측정하여 유지하였다. 모델 제작 후 24 시간 뒤 Nissl 염색을 진행하였을 때, 저체온을 유지한 군에서 정상체온 군에 비해 유의하게 뇌경색 크기가 감소하였다 (정상체온 군, 248 ± 52 mm^3; 저체온 군, 128 ± 27 mm^3; $p<0.001$). 또한 뇌경색 유도 반구에서 체온 변화 상관없이 뇌경색을 유도하지 않은 반구에 비해 유의하게 NLRP3 단백과 IL-1β의 발현 정도가 증가하였고 ($p<0.001$, 정상체온 군 간 비교; $p<0.05$, 저체온 군 간 비교), 저체온 군과 정상체온 군의 뇌경색 유도 반구를 비교하였을 때, 저체온 군에서 NLRP3 단백과 IL-1β의 발현 정도가 유의하게 감소하였다 ($p<0.01$, IL-1β; $p<0.001$, NLRP3).

결론: 우리는 이 연구를 통해 미세아교세포에서 산소-포도당 결핍시 NLRP3 단백의 발현이 증가하고, 백서 국소뇌허혈 모델에서 저체온요법이 NLRP3 인플라마좀 발현을 억제하며, 뇌경색 크기를 감소시키는 신경보호효과가 있음을 확인하였다.

주요어: 저체온요법, 뇌경색, 인플라마좀

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