

Heparin Attenuates the Expression of TNF α -induced Cerebral Endothelial Cell Adhesion Molecule

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Heparin is a well-known anticoagulant widely used in various clinical settings. Interestingly, recent studies have indicated that heparin also has anti-inflammatory effects on neuroinflammation-related diseases, such as Alzheimer's disease and meningitis. However, the underlying mechanism of its actions remains unclear. In the present study, we examined the anti-inflammatory mechanism of heparin in cultured cerebral endothelial cells (CECs), and found that heparin inhibited the tumor necrosis factor α (TNF α)-induced and nuclear factor kappa B (NF- κ B)-dependent expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are crucial for inflammatory responses. Heparin selectively interfered with NF- κ B DNA-binding activity in the nucleus, which is stimulated by TNF α . In addition, non-anticoagulant 2,3-O desulfated heparin (ODS) prevented NF- κ B activation by TNF α , suggesting that the anti-inflammatory mechanism of heparin action in CECs lies in heparin's ability to inhibit the expression of cell adhesion molecules, as opposed to its anticoagulant actions.

Key Words: Heparin, NF- κ B, ICAM-1, VCAM-1, Cerebral endothelial cells, Anti-inflammation

INTRODUCTION

Inflammatory responses in the brain play an important role in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, as well as classical neuroinflammatory conditions, such as meningitis and encephalitis (Minghetti, 2005; Mrak and Griffin, 2005). Brain endothelial cells are of crucial importance in the development of inflammation. In response to proinflammatory cytokines, such as tumor necrosis factor- α (TNF α) and interleukin-1 (IL-1), cerebral endothelial cells express adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Osborn, 1990). These adhesion molecules are critical players in the process of leukocyte adhesion and migration into the brain, which eventually exacerbates the brain's inflammatory responses (de Vries et al, 1997; Osborn, 1990).

Nuclear factor kappa B (NF- κ B) is a well-known proinflammatory transcription factor. It is activated in response to proinflammatory cytokines, such as TNF α and IL-1 (Allan and Rothwell, 2001.). NF- κ B activation mediates TNF α - and other inflammatory cytokine-induced expression of ICAM-1 and VCAM-1 in endothelial cells (Hou et al, 1994; Jahnke and Johnson, 1994; Neish et al, 1995; Read et al, 1995). Therefore, preventing NF- κ B activation or inhibiting the expression of adhesion molecules in brain endo-

thelial cells is considered to be a promising therapeutic target for neuroinflammatory diseases (Turowski et al, 2005).

Heparin has widely been used as an anticoagulant drug in the treatment of ischemic heart disease and stroke. Interestingly, there have been reports that heparin has also anti-inflammatory effects (Elsayed and Becker, 2003) and can alleviate the progression of inflammatory diseases, such as meningitis (Weber et al, 1997) and inflammatory bowel disease (Gaffney et al, 1991). Recently, it has been shown that heparin mitigates Alzheimer's disease by reducing inflammatory activity (Bergamaschini et al, 2002; Bergamaschini et al, 2004). However, heparin's anti-inflammatory mechanisms remain unclear.

In the present study, we hypothesized that heparin would show anti-inflammatory effects by inhibiting the expression of adhesion molecules in mouse cerebral endothelial cells (bEnd.3), showed that heparin inhibited TNF α -induced transcriptional activations of ICAM-1 and VCAM-1 by inhibition of NF- κ B DNA-binding activity.

METHODS

Cell culture and reagents

A mouse cerebral endothelial cell line, bEnd.3 (CRL-2299, ATCC), was maintained in low glucose Dulbecco's modified

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ABBREVIATIONS: CEC, cerebral endothelial cell; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ODS, 2,3-O desulfated heparin.

Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C. Heparin was purchased from USB Corporation (Cleveland, OH) and TNF α was purchased from Sigma (St. Louis, MO).

Alkaline lyophilization of heparin

Heparin was chemically modified to 2,3-O desulfated heparin (ODS) by lyophilization under alkaline conditions as previously described (Fryer et al, 1997). Briefly, aqueous solutions (0.4~5.0%) of heparin in ddH₂O were alkalized to pH 13.0 by addition of NaOH. The alkalized solutions were frozen and lyophilized to dryness. The crusty and yellowish precipitates were dissolved in 10 mL of 1 M NaOH and adjusted to pH 9 with 20% acetic acid. The solutions were then dialyzed against ddH₂O for 2 days and lyophilized again to dryness.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Cells were washed twice with cold PBS, and nuclear extracts were prepared according to the method described previously (Lee et al, 2007). Oligonucleotides with NF- κ B and Sp-1 (Promega, Madison, WI) consensus binding sequences were used in the EMSA. Procedures for EMSA and for labeling oligonucleotides with [γ -³²P]-ATP have been detailed previously by Lee et al (Lee et al, 2007). For the supershift assay, 1 μ l each of anti-p65, anti-p50, and anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were incubated with nuclear extract for 20 min at room temperature prior to the reaction with [γ -³²P]-labeled oligonucleotides. For the *in vitro* direct binding assay, heparin or ODS was directly added to nuclear extracts obtained from TNF α -treated bEnd.3 cells, and the mixtures were incubated with [γ -³²P]-labeled oligonucleotides for 30 min at room temperature before sample loading.

RNA isolation and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the modified protocol for polysaccharide rich sources (Chomczynski and Mackey, 1995). Next, cDNA was synthesized by reverse transcription from 2.5 μ g of total RNA to a final volume of 20 μ l using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The amounts of cDNAs of ICAM-1, VCAM-1, and β -actin were measured by real-time RT-PCR, as described previously (Lee et al, 2007). Briefly, 20 μ l of PCR reactions were set up with 3 μ l of cDNA and 10 μ l of SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA) for each reaction. The final concentration of the ICAM-1 (sense: 5'-CTCGAGAGTGGACCCAACT-GGAAG-3', antisense: 5'-CTCAGCTGGACTCTCCGG AAAC-3'), VCAM-1 (sense: 5'-CTCACCTTCGCGTTAGTGGCTGTCTATC-3', antisense: 5'-CTC ACCTTCGCGT-TTAGTGGGCTGTCTATC-3'), and β -actin (sense: 5'-ACC-AACTGGGACATGGAG-3', antisense: 5'-GTCAGGATCTT-CATGAGGTAGTC-3') primers was 250 nM each. Amplification was performed on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster city, CA) using the following cycling conditions: 10 min at 95°C, and 40 cycles of 20 s at 95°C, 40 s at 60°C, and 30 s at

70°C. Triplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values were averaged. According to the comparative Ct method, the gene expression level was calculated as $2^{-\Delta\Delta Ct}$ (Fold change). The values of ICAM-1 and VCAM-1 were normalized to β -actin level.

Immunoblotting

Cells were rinsed with ice-cold PBS and harvested using a cell scraper. They were then pelleted by centrifugation, and the cell pellet was resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100) containing Complete protease inhibitor cocktail (Roche, Indianapolis, IN). After centrifugation (13,000 x g for 15 min at 4°C), equal amounts of protein from each supernatant were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Anti-p65 (Serotec, Raleigh, NC), anti-p50 (Abcam, Cambridge, MA), anti-Sp1, and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as primary antibodies, and horseradish peroxidase-conjugated anti-rabbit antibody was used as a secondary antibody. ECL reagent (Pierce, Rockford, IL) was used for antigen detection.

Statistics

Data were analyzed with one-way ANOVA and subsequently Tukey-Kramer test. Significance was accepted at $p < 0.05$. Results are reported as mean \pm standard error of the mean (S.E.M).

RESULTS

Heparin showed anti-inflammatory effects on cerebral endothelial cells

Previously, we observed that 30 min treatment of CECs with TNF α induced 2-fold increase in mRNA level of ICAM-1, which was blocked by heparin (Lee et al, 2007). Therefore, to investigate the underlying mechanism of heparin inhibition of adhesion molecule expression, we treated CECs with TNF α for prolonged period of time. As expected, 4 h treatment with TNF α induced more robust increase in mRNA expression of ICAM-1 and VCAM-1, which was measured using real-time RT-PCR (Fig. 1A). Next, we examined whether heparin inhibits the expression of adhesion molecules in CECs induced by 4 h treatment with TNF α . After a 24 h pretreatment with heparin, cells were incubated with TNF α for 4 h. Fig. 1A shows that heparin significantly suppressed the transcriptional activation of ICAM-1 and VCAM-1 stimulated by TNF α . Heparin itself didn't have any effect on either of the transcripts. Next, we examined whether heparin inhibits TNF α -induced NF- κ B activation that is linked to the expression of ICAM-1 and VCAM-1. Thus, DNA-binding activity of NF- κ B was determined using EMSA, and heparin was found to inhibit TNF α -induced DNA-binding activity of NF- κ B, showing about 290% decrease of NF- κ B DNA-binding activity compared to the TNF α treated group (Fig. 1B). Using supershift assay, we confirmed that the NF- κ B bands consisted of p50/p65 heterodimer or p50/p50 homodimer (Fig. 1B). To examine the selective action of heparin, DNA-binding activ-

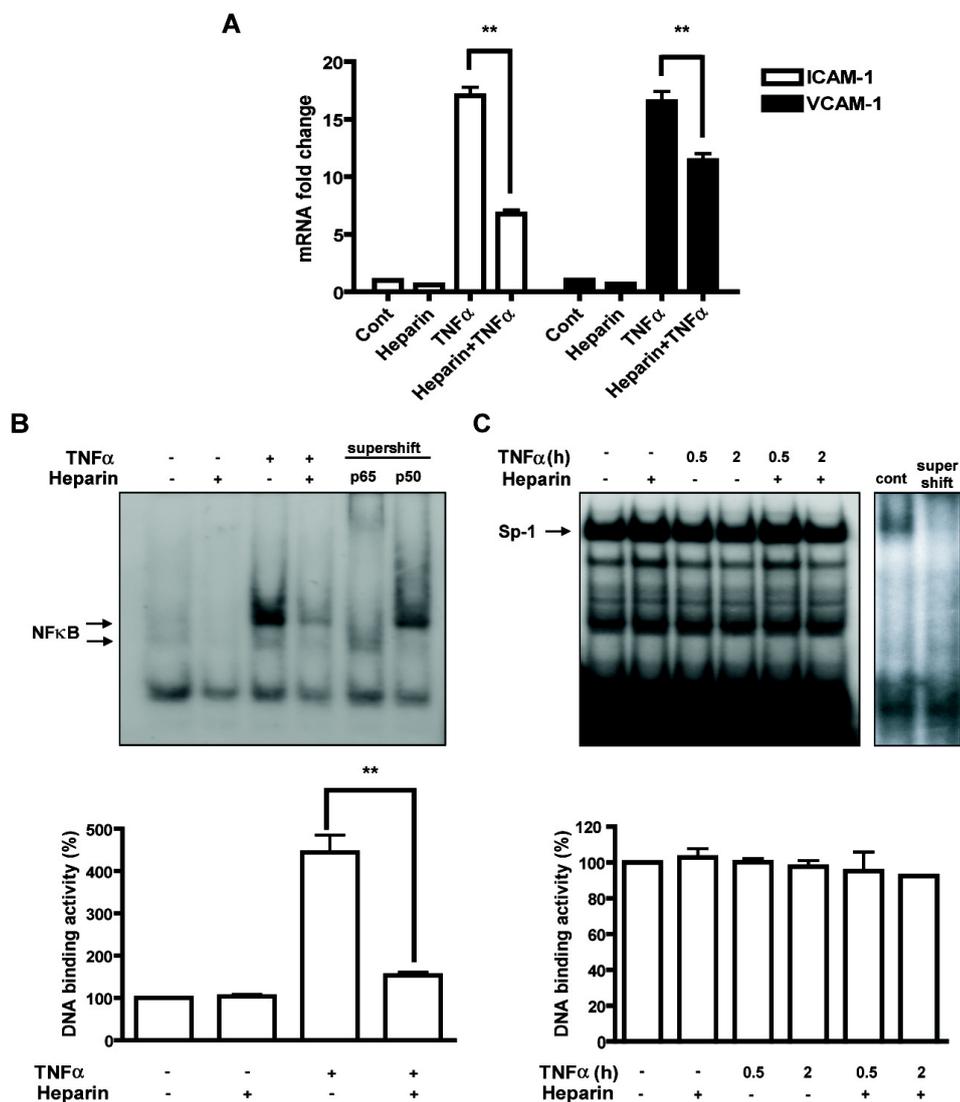


Fig. 1. Heparin inhibited TNF α -induced expression of adhesion molecules and NF- κ B activation in cerebral endothelial cells. (A) Mouse cerebral endothelial cells (bEnd.3) were pretreated with heparin (1 mg/ml for 24 h) and then exposed to TNF α (10 ng/ml) for 4 h. mRNA levels of ICAM-1 and VCAM-1 were determined using real-time RT-PCR. **p<0.01 (B) The NF- κ B DNA-binding activity of nuclear extracts of TNF α -stimulated cells (10 ng/ml for 30 min) in the presence or absence of pretreatment with heparin was analyzed using electrophoretic mobility shift assay (EMSA). Super-shift assay showed bands containing p65 and p50, which are major components of NF- κ B. **p<0.01 (C) DNA-binding activity of Sp-1 was observed. Super-shift assay indicated a Sp-1 specific band.

ity of a constitutive transcription factor, Sp-1, was also measured. In contrast to NF- κ B, the DNA-binding activity of Sp-1 was not affected by heparin, indicating that heparin selectively inhibits NF- κ B DNA-binding activity (Fig. 1C). Therefore, these findings suggest that heparin blocks NF- κ B activation, accounting, at least in part, for the subsequent inhibition of adhesion molecule expression.

Heparin directly inhibited NF- κ B DNA-binding activity without affecting nuclear translocation

We previously reported that heparin was taken up by endocytosis in bEnd.3 cells and was present in the nucleus as well as in the perinuclear cytoplasm (Lee et al, 2007). Heparin is a highly anionic mucopolysaccharide, whereas the DNA-binding domain of NF- κ B is mostly composed of basic amino acids (Muller et al, 1995). Therefore, we examined whether heparin directly inhibits NF- κ B DNA-binding activity. To do so, we mixed nuclear extracts of TNF α -induced cells with heparin and analyzed the DNA-binding activities of NF- κ B and Sp-1. Fig. 2A shows that heparin

selectively decreased up to ~90% of NF- κ B DNA-binding activity, but not Sp-1. Next, we examined whether heparin affects TNF α -induced I κ B degradation and nuclear translocation of NF- κ B subunits p65 and p50; both processes are essential for NF- κ B activation (Li and Verma, 2002). Thus, cells were pretreated with heparin for 24 h and then stimulated by TNF α . The amounts of p65 and p50 in nuclear extracts and I κ B α in whole cell lysates were analyzed using immunoblotting with anti p65, p50, and I κ B α antibodies. We found that TNF α increased p65 and p50 nuclear translocation by about 4 or 5 times, respectively, and enhanced I κ B degradation by ~5 times compared to control groups. Heparin affected neither I κ B degradation nor nuclear translocation of p65 and p50 (Fig. 2B and C). Therefore, these findings suggest that heparin directly inhibits NF- κ B DNA-binding activity.

Non-anticoagulant 2,3-O desulfated heparin inhibited NF- κ B DNA-binding activity

In order to determine the relation of heparin's anti-

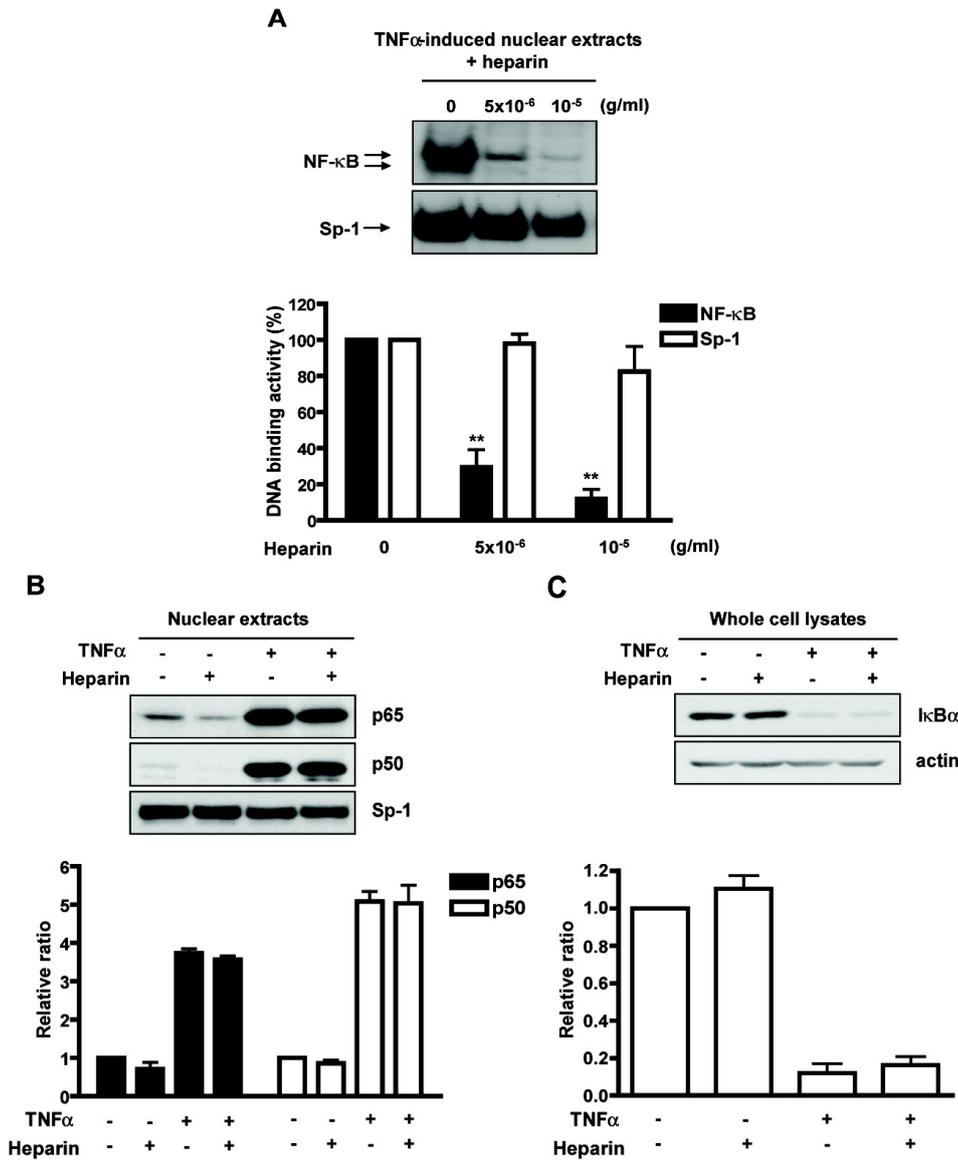


Fig. 2. Heparin directly inhibited NF- κ B DNA-binding activity *in vitro* without affecting nuclear translocation. (A) An aliquot of nuclear extracts from TNF α -induced bEnd.3 cells was directly mixed with heparin *in vitro*. DNA-binding activities of NF- κ B and Sp-1 were analyzed using EMSA. **p < 0.01 versus no heparin treatment group. (B) Cells were pretreated with heparin (1 mg/ml for 24 h) and then exposed to TNF α (10 ng/ml) for 30 min. An aliquot of nuclear extracts was immunoblotted with anti p65 and p50 antibodies. Sp-1 protein in the nuclear extracts was used as a loading control. (C) TNF α -stimulated CEC lysates with or without pretreatment with heparin were separated by polyacrylamide gel electrophoresis and transferred to a PVDF membrane, which was successively probed with I κ B α antibody. Actin protein in the whole cell lysates was used as a loading control.

coagulant action to its inhibitory effect on NF- κ B, we chemically modified heparin to 2,3-O desulfated heparin (ODS), a derivative of heparin that lost its anticoagulant function due to alkaline lyophilization (Fryer et al, 1997). Cells were pretreated with ODS for 24 h before treatment with TNF α . Intriguingly, ODS also showed ~85% of reduction in TNF α -induced NF- κ B activation (Fig. 3). These findings demonstrate that the anticoagulant function of heparin is independent of its inhibitory effects on NF- κ B activation, suggesting a beneficial role for modified heparin as an anti-inflammatory agent.

DISCUSSION

In many inflammatory diseases, prevention of leukocyte adhesion and transendothelial migration may be of clinical value (Ulbrich et al, 2003). Consequently, there have been many efforts to develop modulators for the adhesion mole-

cules that play a central role in inflammatory diseases (Goedkoop et al, 2004; Kavanaugh et al, 1997). This is also true for CECs, which are major components of the blood-brain barrier (BBB) and intimately involved in the pathogenesis of many inflammatory brain diseases. CECs have characteristic features that are distinct from peripheral endothelial cells. They demonstrate minimal endocytosis, thereby decreasing nonspecific flux of ions, proteins, and other substances into the brain parenchyma (Gloor et al, 2001). In addition, many drug transporters that can efflux various iatrogenic molecules out of the brain, such as P-glycoprotein (Tsuji and Tamai, 1999) and organic anionic transporting polypeptide type 2 (oatp 2) (Asaba et al, 2000), are abundant in CECs (Sun et al, 2003). Because our previous research indicated that heparin is taken up by endocytosis and inhibits proinflammatory NF- κ B activation, our logical next step to evaluate the usefulness of heparin as an anti-inflammatory drug in the brain was to examine the effect of heparin on inflammation signaling in CECs.

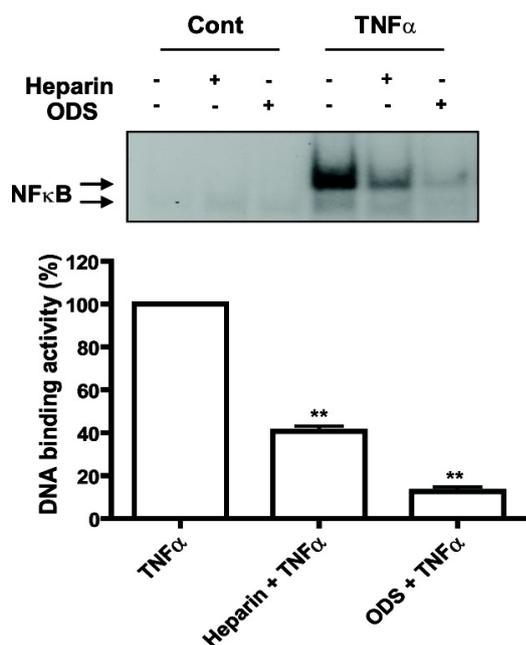


Fig. 3. Non-anticoagulant 2,3-O desulfated heparin (ODS) inhibited TNF α -induced NF- κ B DNA-binding. Cells were pretreated with ODS (1 mg/ml for 24 h) or heparin (1 mg/ml for 24 h). NF- κ B DNA-binding activity of nuclear extracts of TNF α -induced cells (10 ng/ml for 30 min) were analyzed using EMSA. ** $p < 0.01$ versus TNF α treatment group.

Considering many evidences to indicate that NF- κ B activation is a critical mechanism regulating TNF α -induced expression of adhesion molecules in endothelial cells (Hou et al, 1994; Jahnke and Johnson, 1994; Neish et al, 1995; Read et al, 1995), heparin's inhibitory effects on NF- κ B activation and subsequent decreases of ICAM-1 and VCAM-1 expression in CECs raise the possibility of developing heparin as an anti-inflammatory drug for inflammatory brain diseases.

In the present study, heparin inhibited NF- κ B DNA-binding activity without affecting I κ B α degradation or NF- κ B nuclear translocation, suggesting that heparin activity occurs in the nucleus, rather than at other upstream levels of the NF- κ B activating pathway, such as I κ B kinase (McIntyre et al, 2003; Node et al, 1999) and proteasome (Meng et al, 1999; Read et al, 1995). Since heparin is a highly anionic glycosaminoglycan, that it is present in the nucleus of cerebral endothelial cells, and that the NF- κ B DNA-binding domain consists largely of basic amino acids (Muller et al, 1995), it seems likely that heparin's anti-inflammatory properties lie in its ability to directly inhibit NF- κ B DNA-binding activity (Lee et al, 2007). However, it is also possible that other mechanisms controlling nuclear NF- κ B DNA-binding activity may be acting simultaneously. Phosphorylation of NF- κ B subunits is required for nuclear NF- κ B DNA-binding and trans-activation activity (Guan et al, 2005; Vermeulen et al, 2003). In response to TNF α , several kinases, including PKA catalytic subunits (PKAc), can induce phosphorylation of p65 and regulate NF- κ B transcriptional activity (Jamaluddin et al, 2007; Vermeulen et al, 2003). Specifically, siRNA-mediated PKAc knockdown and p65 mutation

analysis show that TNF α -activated PKAc induces the phosphorylation of serine 276 on p65 that is required for NF- κ B DNA-binding activity and consequent trans-activation of target genes (Jamaluddin et al, 2007; Zhong et al, 1998). In addition, phosphorylation of serine 337 on p50 by PKAc is also crucial for NF- κ B DNA-binding activity (Guan et al, 2005). Taken together, heparin's mechanism of action may lie in its ability to affect phosphorylation of NF- κ B subunits, thereby inhibiting NF- κ B DNA-binding activity.

It should be noted that despite having non-anticoagulant properties, ODS inhibited TNF α -induced NF- κ B activation in CECs as much as heparin. This finding is consistent with other reports indicating that non-anticoagulant heparin shows anti-inflammatory effects on different cell types (Gao et al, 2005; Wang et al, 2002). In addition, ODS seems to inhibit TNF α -induced NF- κ B activation more efficiently than heparin, and modification of sulfate group in ODS might help it to enter into cell more than heparin or enhance its inhibitory effect on NF- κ B DNA-binding in the nucleus. However, these hypotheses should be confirmed by further experiments. Although heparin has widely been used for several decades in the treatment of ischemic stroke (Marsh et al, 1989) and myocardial infarction (Commerford 1997), its anticoagulant function and accompanying high hemorrhage risk have hindered its development as an anti-inflammatory drug (Levine et al, 2004). Our findings provide preliminary evidence for the anti-inflammatory effects of non-anticoagulant ODS heparin in CECs, suggesting a potential usefulness for non-anticoagulant heparin in treating neuroinflammation-related diseases.

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