

ORIGINAL ARTICLE

# Upregulation of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter 1 via histone modification in the aortas of angiotensin II-induced hypertensive rats

Hyun-Min Cho<sup>1,4</sup>, Dong-Youb Lee<sup>1,4</sup>, Hye Young Kim<sup>1</sup>, Hae-Ahm Lee<sup>1</sup>, Young Mi Seok<sup>2</sup> and In Kyeom Kim<sup>1,2,3</sup>

The  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter 1 (NKCC1) is upregulated in diverse models of hypertension. We hypothesized that NKCC1 is upregulated via histone modification in the aortas of angiotensin II (Ang II)-induced hypertensive rats. An osmotic mini-pump containing Ang II was implanted in the subcutaneous tissues of the backs of Sprague-Dawley (SD) rats for 7 days. The systolic blood pressure was recorded every day by the tail-cuff method. On days 3 and 7, the mesenteric arteries were excised, cut into rings, mounted in organ baths and subjected to vascular contraction. The levels of *Nkcc1* mRNA and protein in the aortas were measured using real-time PCR and Western blotting, respectively. The histone modifications and recruited proteins at the *Nkcc1* promoter were determined by chromatin immunoprecipitation. The inhibition of concentration-response curves to phenylephrine by bumetanide, an inhibitor of NKCCs, was greater in Ang II-infused rats than in sham-operated (sham) rats. The levels of *Nkcc1* mRNA and protein in the aortas increased gradually as Ang II was infused into the rats. Acetylated histone H3 (H3Ac), an activating histone code, was increased but trimethylated histone H3 at lysine 27 (H3K27me3), a repressive histone code, was greatly decreased in Ang II-infused rats compared with sham. RNA polymerase II was recruited to the *Nkcc1* promoter with increased KDM6b. We conclude that the NKCC1 is upregulated via histone modification in the aortas of Ang II-induced hypertensive rats. Thus, we suggest that this ion transporter is epigenetically upregulated by histone modification or DNA demethylation upon the development of hypertension.

*Hypertension Research* (2012) 35, 819–824; doi:10.1038/hr.2012.37; published online 12 April 2012

**Keywords:** angiotensin II; histone modification; NKCC1

## INTRODUCTION

The  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter (NKCC) belongs to the super-family of cation-coupled  $\text{Cl}^-$  cotransporters and acts as an electro-neutral symporter of one  $\text{Na}^+$ ,  $1\text{K}^+$  and two  $\text{Cl}^-$ .<sup>1</sup> There are two isoforms of the cotransporter in mammals: NKCC1 is expressed in most cells, including vascular smooth muscle cells, endothelial cells, cardiomyocytes, neurons, glial cells and blood cells, whereas NKCC2, a kidney-specific isoform, is exclusively expressed in the apical membranes of the thick ascending limb and macula densa and has a major role in renal handling of salt.<sup>1</sup>

NKCC1 has a role in the regulation of blood pressure (BP), including the maintenance of cell volume and vascular tone.<sup>2,3</sup> Although it is unclear whether the upregulation of NKCC1 is a cause or an effect of high BP, it is clear that NKCC1 is implicated in the maintenance of vascular tone *in vivo*. The intravenous infusion of bumetanide, a specific NKCC inhibitor, causes a rapid reduction in the mean arterial BP and vascular tone in resistance arteries, which is

abolished in *Nkcc1*<sup>-/-</sup> mice.<sup>4</sup> Bumetanide also inhibits contractions and myogenic tone in mesenteric arteries isolated from wild-type mice but not from *Nkcc1*<sup>-/-</sup> mice.<sup>5</sup> The BP of *Nkcc1*<sup>-/-</sup> mice is more sensitive to an increase or decrease in sodium intake.<sup>6</sup> In addition, the expression or activity of NKCC1 is upregulated in a variety of hypertensive models.<sup>4,7–10</sup> Angiotensin II (Ang II) increases the activity of cation-coupled  $\text{Cl}^-$  cotransporters, including NKCC1, through STE20/SPS1-related proline/alanine-rich kinase.<sup>11</sup> However, the mechanism by which Ang II stimulates NKCC1 is not yet clear.

Recently, we reported that the *Nkcc1* promoter is hypomethylated in the aortas and hearts of established spontaneously hypertensive rats compared with normotensive Wistar Kyoto rats and that promoter hypomethylation upregulates the expression of NKCC1.<sup>12</sup> Furthermore, the maintenance of hypomethylation at the *Nkcc1* promoter resulting from low DNA methyltransferase activity has an important role in the upregulation of NKCC1 during the development of spontaneous hypertension.<sup>13</sup> Because the *Nkcc1*

<sup>1</sup>Department of Pharmacology, Kyungpook National University School of Medicine, Daegu, Republic of Korea; <sup>2</sup>Cardiovascular Research Institute, Kyungpook National University School of Medicine, Daegu, Republic of Korea and <sup>3</sup>CMRI, Kyungpook National University School of Medicine, Daegu, Republic of Korea

<sup>4</sup>These two authors contributed equally to this work.

Correspondence: Dr IK Kim, Department of Pharmacology, Kyungpook National University School of Medicine, 101 Dongin-2-Ga, Daegu 700-422, Republic of Korea. E-mail: inkim@knu.ac.kr

Received 23 September 2011; revised 29 January 2012; accepted 1 February 2012; published online 12 April 2012

promoter region in the aortas of SD rats is almost unmethylated, histone modification is another plausible epigenetic mechanism for the upregulation of *Nkcc1* expression during a BP increase. We tested the hypothesis that NKCC1 is upregulated via histone modification in the aortas of Ang II-induced hypertensive SD rats.

## METHODS

### Implantation of the osmotic pump

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals in Kyungpook National University School of Medicine (Supplementary Figure S1). All efforts were made to minimize both the number of animals used and their suffering. Male SD rats weighing 350–400 g were used. Under anesthesia with intraperitoneal injection of ketamine 80 mg kg<sup>-1</sup> and xylazine 10 mg kg<sup>-1</sup>, osmotic mini-pumps (Alzet model 2002; Alza Corp, Palo Alto, CA, USA) containing Ang II (infusion rate 0.7 mg kg<sup>-1</sup> per day) in saline or vehicle alone were implanted subcutaneously in the midscapular region. The rats were euthanized after 3 and 7 days. The aortas were excised and stored at -80 °C until further analysis.

### BP measurement

The systolic BPs were measured by the tail-cuff method using a non-invasive BP controller system (ADInstruments Pty Ltd, Castle Hill, NSW, Australia). Six measurements were obtained and averaged from each rat, which was placed on a warming blanket (37 °C).

### Tension measurement from mesenteric arterial rings

The mesentery bed was removed and the first branches of the mesenteric arteries were used for experiments. The experiments were performed as previously described.<sup>14</sup>

### Quantitative real-time (RT) reverse transcriptase PCR

RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Total RNA (2 µg) was reverse-transcribed into cDNA using RevertAid first strand cDNA synthesis (Fermentas, Glen Burnie, MD, USA). RT-PCR was performed using SYBR Green PCR master mix (TaKaRa, Otsu, Shiga, Japan) and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression level was determined by calculating the values of  $\Delta$  cycle threshold ( $\Delta$ Ct) by normalizing the average Ct value compared with its endogenous control (*Gapdh*) and then calculating  $2^{-\Delta\Delta Ct}$ .<sup>15</sup> The primer sets used in the RT-PCR were as follows: *Nkcc1* (AF\_051561) Forward 5'-TC CTCAGTCAGCCATACCCAAA-3', Reverse 5'-TCCCGGACAACACAAGAA CCT-3'; *Gapdh* (NM\_017008) Forward 5'-AGATCATCAGCAATGCCTCCT G-3', Reverse 5'-ATGGCATGGACTGTGGTCATG-3'; *Ash2l* (NM\_001106089) Forward 5'-TAAAAGGTGGATGGGACTGC-3', Reverse 5'-AGAGGCACAGCAC CAGTTTT-3'; *Ehmt2* (NM\_212463) Forward 5'-CCGCTTCATTAACCACC TGT-3', Reverse 5'-ATGTCCCTGAGCTGAAGAA-3'; *Ezh2* (NM\_001134 979) Forward 5'-CCTGTCCCACTGAGGATGT-3', Reverse 5'-GAGCCGTC CTTTTTCAGTTG-3'; *Kdm6b* (NM\_001108829) Forward 5'-GGCCACCA GGAGAATAACAA-3', Reverse 5'-CCACCAGGAACCAGTCAAGT-3'.

### Protein extraction and western blotting

The frozen tissues were homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors. Western blot analysis was performed as previously described.<sup>12</sup> The expression levels were quantified by optical densitometry using ImageJ software (<http://rsbweb.nih.gov>).

### Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed using EZ ChIP (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocols with certain modifications. Anti-polymerase II (Pol II), anti-H3Ac and anti-H3K9me2 antibodies were obtained from Upstate Biotechnology. Anti-H3K4me3, anti-H3K27me3 and anti-KDM6b were obtained from Abcam (Cambridge, UK).

Anti-BMI-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DNA fragments were amplified using PCR. The quantitative RT-PCR values were normalized by that of the corresponding input. The primer set that was used in the PCR is as follows: *Nkcc1* (AF\_051561) Forward 5'-AGG AGTCCACAGGGTCCGTGC-3', Reverse 5'-CCCACTAAGGAGCCGGGGGA G-3'.

### Drugs

The drugs and chemicals were obtained from the following sources: phenylephrine, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) and Ang II from Sigma Chemicals (St Louis, MO, USA) and bumetanide from Calbiochem (La Jolla, CA, USA). A stock solution of phenylephrine and L-NAME was prepared in double-distilled water, Ang II was prepared in 0.9% NaCl and bumetanide was prepared in dimethyl sulfoxide. All other reagents were analytical grade.

### Statistical analysis

The data are expressed as the mean  $\pm$  s.e. of the mean (s.e.m.) and were analyzed by repeated measures analysis of variance followed by a *post-hoc* Duncan test for tension measurement or analysis of variance followed by a *post-hoc* Duncan test to compare the expression level of NKCC1 or other genes. A *P*-value <0.05 was considered to be statistically significant.

## RESULTS

### Effect of bumetanide on the concentration-response curves to phenylephrine in the mesenteric arteries of sham-operated (sham) rats and Ang II-infused rats

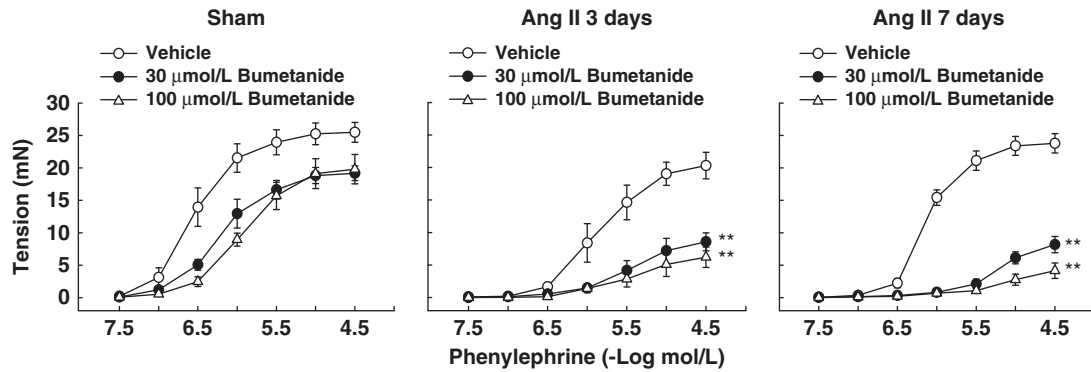
There were significant differences between the sham and the Ang II-infused rats with respect to the contractile response induced by phenylephrine. On days 3 and 7, bumetanide significantly reduced the isometric force response to phenylephrine in Ang II-infused rats (Figures 1b and c) compared with sham (Figure 1a). EC<sub>50</sub> and E<sub>max</sub> on days 3 and 7 were lower in Ang II-infused rats than in sham (Table 1).

### Expression of *Nkcc1* mRNA and protein in aortas from sham and Ang II-infused rats

To confirm whether the expression level of NKCC1 was different between sham and Ang II-infused rats during BP increase, we performed quantitative RT-PCR and Western blotting using aortas. There were gradual increases of both *Nkcc1* mRNA (Figure 2a) and protein (Figure 2b) in Ang II-infused rats. On day 7, the levels of *Nkcc1* mRNA and protein in Ang II-infused rats were higher than those in sham (Figure 2).

### Histone modifications in sham and Ang II-infused rats

To determine whether there were changes in the covalent modifications on histones in the *Nkcc1* promoter region (-367 to +48) during BP increase, we performed ChIP analysis with various antibodies. The Ang II-infused rats showed increased acetylated histone H3, which is an activation mark of gene expression, in the *Nkcc1* promoter region (Figure 3a). Trimethylated H3 at lysine 4, which is another activation mark of gene expression, was not different between the sham and Ang II-infused rats (Figure 3b). On day 7, dimethylated histone H3 at lysine 9, which is a suppressive mark of gene expression, had a tendency to be decreased in Ang II-infused rats compared with sham (Figure 3c). In addition, trimethylated histone H3 at lysine 27, which is also a suppressive mark of gene expression, showed a gradual and profound decrease in Ang II-infused rats (Figure 3d).

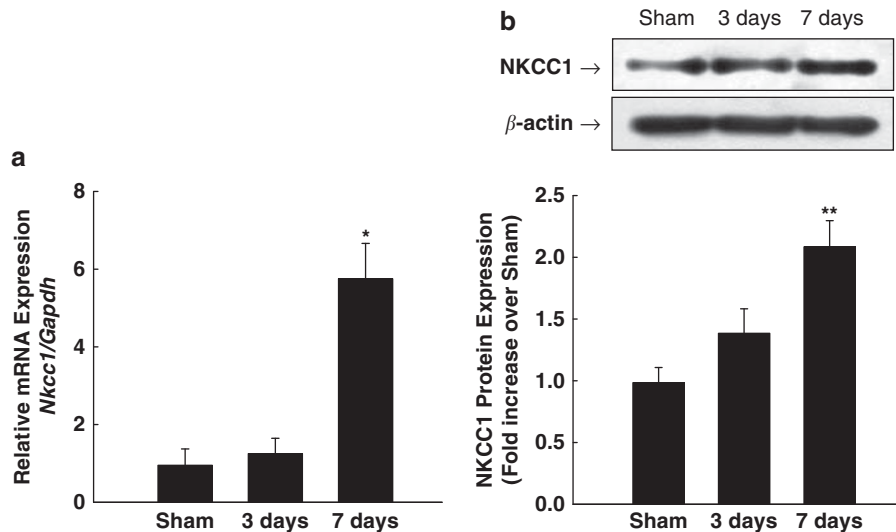


**Figure 1** The effects of bumetanide on the concentration-response curves to phenylephrine in mesenteric arteries from sham or Ang II-infused rats. L-NAME was used to inhibit endothelial nitric oxide synthase function. Phenylephrine was added cumulatively to elicit tension 30 min after pretreatment with bumetanide or vehicle in the first-order branches of the mesenteric arteries from sham or Ang II-infused rats. The developed tension was expressed in mN. The data are expressed as the mean values of five experiments, with the vertical bars showing the s.e.m.  $**P < 0.01$ , bumetanide versus vehicle (repeated measures analysis of variance (ANOVA) followed by *post-hoc* Duncan test).

**Table 1** Effect of bumetanide on phenylephrine-induced contraction in mesenteric arteries from sham or Ang II-infused rats

| Additions                             | $EC_{50}$ ( $-\log \text{mol l}^{-1}$ ) |             |              | $E_{max}$ (mN) |             |             |
|---------------------------------------|---|-------------|--------------|----------------|-------------|-------------|
|                                       | Sham                                    | 3 days      | 7 days       | Sham           | 3 days      | 7 days      |
| Vehicle                               | 6.6 ± 0.1                               | 5.9 ± 0.1** | 6.1 ± 0.03** | 25.0 ± 1.6     | 20.0 ± 1.7  | 23.1 ± 1.5  |
| 30 $\mu\text{mol l}^{-1}$ bumetanide  | 6.2 ± 0.09                              | 5.4 ± 0.3   | 5.2 ± 0.04** | 19.0 ± 1.0     | 8.2 ± 1.1** | 8.5 ± 1.4** |
| 100 $\mu\text{mol l}^{-1}$ bumetanide | 6.0 ± 0.04                              | 5.5 ± 0.1** | 4.9 ± 0.15** | 20.0 ± 2.1     | 4.5 ± 0.9** | 5.8 ± 1.4** |

$EC_{50}$  ( $-\log \text{mol l}^{-1}$ ) is negative logarithm of the molar concentration of phenylephrine causing 50% of the maximal contraction.  $E_{max}$  is expressed as the maximum response (mN); values are expressed as the mean ± s.e.m.  $**P < 0.01$  Ang II-infused rats versus sham (ANOVA).

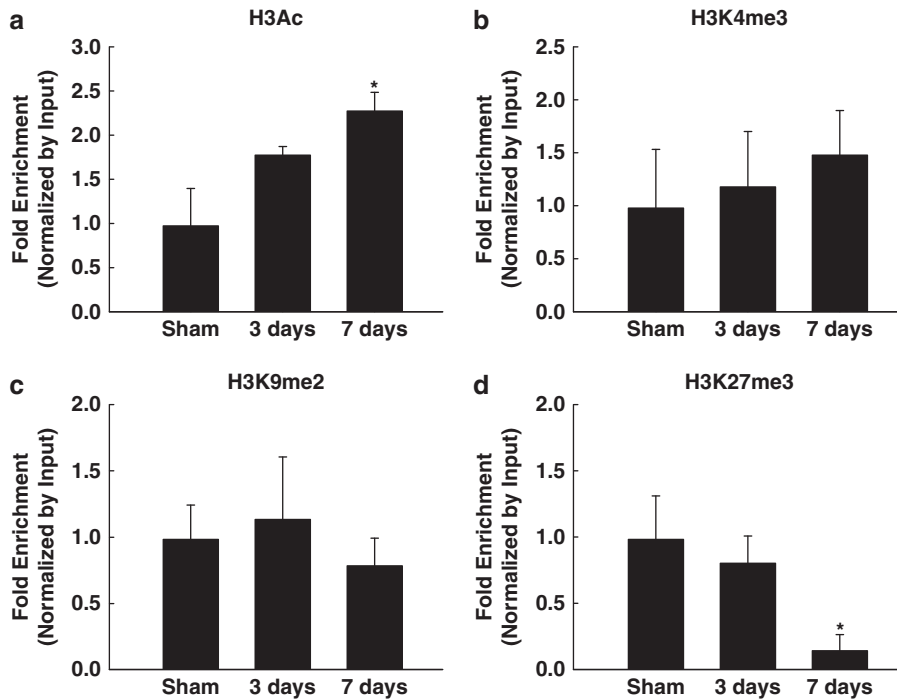


**Figure 2** The differential expression of *Nkcc1* mRNA or protein in aortas. The expression levels of *Nkcc1* mRNA (a) and NKCC1 protein (b) in aortas were measured by RT-PCR and Western blotting, respectively.  $*P < 0.05$  and  $**P < 0.01$ , Ang II-infused rats versus sham (ANOVA).

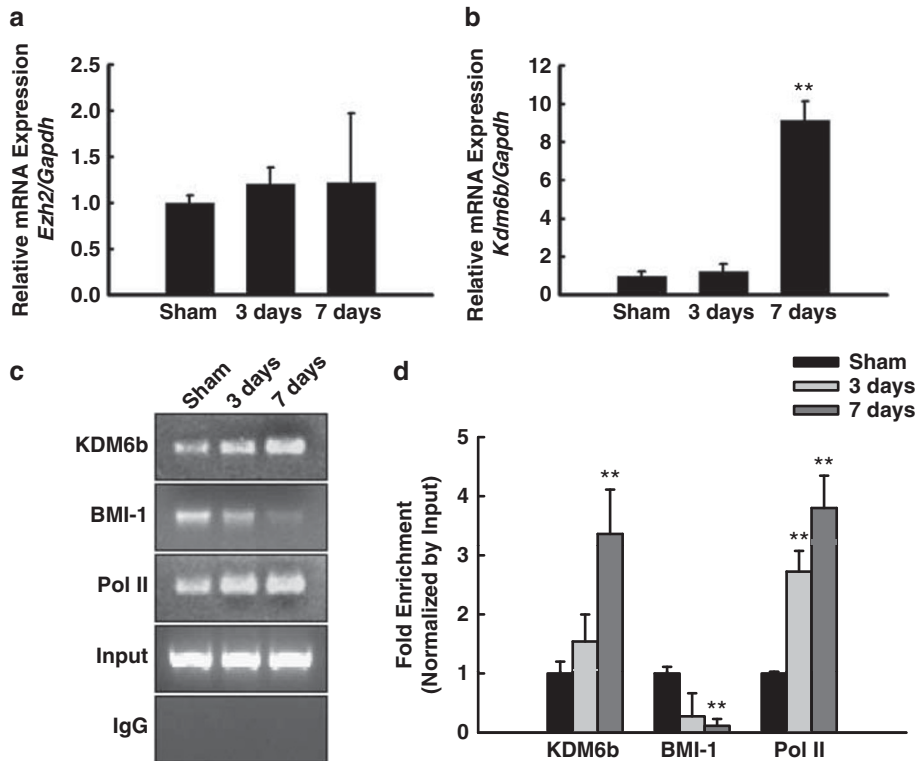
**Expression of histone methyltransferase or demethylase in sham and Ang II-infused rats**

To further validate our ChIP analysis results, the expression levels of H3K27 methyltransferase (enhancer of zeste homolog 2 (*Ezh2*)) and H3K27 demethylase (lysine-specific demethylase

6b (*Kdm6b*)) were analyzed using RT-PCR. The expression levels of *Ezh2* mRNA exhibited no significant difference, whereas the levels of *Kdm6b* mRNA were significantly increased in Ang II-infused rats compared with sham (Figures 4a and b).



**Figure 3** Histone modifications of the *Nkcc1* promoter region in aortas. Chromatin was immunoprecipitated with antibodies against acetyl-H3 (a), trimethyl-H3K4 (b), dimethyl-H3K9 (c) or trimethyl-H3K27 (d) and amplified by quantitative RT-PCR. The quantitative RT-PCR values were normalized by that of the corresponding input. \* $P < 0.05$ , Ang II-infused rats versus sham (ANOVA).



**Figure 4** The expression of H3K27 methylase and demethylase mRNA in aortas and the recruitment of BMI-1, KDM6b and Pol II in the *Nkcc1* promoter region. The expression levels of zeste homolog 2 (*Ezh2*) (a) or lysine-specific demethylase 6b (*Kdm6b*) mRNA (b) were measured by quantitative RT-PCR. Chromatin was immunoprecipitated with anti-BMI-1, KDM6b or anti-RNA Pol II, amplified by quantitative RT-PCR (c) and then normalized by the corresponding input (d). \*\* $P < 0.01$ , Ang II-infused rats versus sham rats (ANOVA followed by *post-hoc* Duncan test).

### Recruitment of BMI-1, KDM6b, or RNA Pol II in the *Nkcc1* promoter region in sham and Ang II-infused rats

To determine the promoter activity of *Nkcc1* during BP increase, we assessed the recruitment of BMI-1, KDM6b or RNA Pol II to the *Nkcc1* promoter region. BMI-1, which is a component of polycomb repressive complex 1 (PRC1), was decreased, whereas KDM6b and Pol II were enriched in the *Nkcc1* promoter region of Ang II-infused rats compared with sham (Figures 4c and d).

### DISCUSSION

This study demonstrates that the NKCC1 is upregulated via histone modification in the aortas of Ang II-induced hypertensive rats. Acetylated histone H3 (H3Ac) was increased but trimethylated histone H3 at lysine 27 (H3K27me3) was greatly decreased in Ang II-infused hypertensive rats compared with sham. H3K27 demethylase, a lysine-specific demethylase 6b (*Kdm6b*), showed a gradual increase in Ang II-infused rats, whereas H3K27 methyltransferase, enhancer of zeste homolog 2 (*Ezh2*), was not significantly different between sham and Ang II-infused rats. Furthermore, RNA Pol II was highly associated with the promoter region of *Nkcc1* in Ang II-infused rats.

H3K27me3 in the promoter region of *Nkcc1* decreased as BP increased (Figure 3d). Increased *Kdm6b* may lead to the demethylation of H3K27me3 (Figure 4b). A study also revealed that the ectopic expression of KDM6b strongly decreased H3K27me3 levels and caused the delocalization of polycomb proteins *in vivo*.<sup>16</sup> Acetylated histone H3 (H3Ac) was increased in Ang II-infused hypertensive rats. Meanwhile, other modifications of the histone tails, such as the methylation of H3K4 and H3K9, did not change significantly between the sham and Ang II-infused rats (Figure 3). These data were supported by the fact that the expression levels of absent, small or homeotic 2-like (*Ash2l*), a subunit of the H3K4 methyltransferase complex, and the euchromatin histone-lysine *N*-methyltransferase 2 (*Ehmt2*), an H3K9 methyltransferase, did not change during BP rise (Supplementary Figure 2). The increase in BP may affect the status of H3K27 methylation in the promoter region of *Nkcc1*. At present, however, we do not know whether histone modifications are caused by oxidative stress, high BP (BP), or related changes or directly through the activation of Ang II receptors (Supplementary Figure S3). Nevertheless, the decreased recruitment of BMI-1 and enrichment of KDM6B and RNA Pol II at the *Nkcc1* promoter may lead to an increased transcriptional level of the *Nkcc1* gene (Figures 4c and d).

Epigenetic modifications are defined as heritable alterations in gene expression patterns without changes in the DNA sequences.<sup>17</sup> Among the epigenetic modifications, the modifications of histone tails, such as acetylation, methylation and phosphorylation, have important roles in regulating various processes, including the replication, repair and transcription of DNA.<sup>18</sup> Histone H3K27 is acetylated or methylated at three different levels (mono-, di- and trimethylation) in the chromatin.<sup>19,20</sup> Acetylated and monomethylated H3K27 are found broadly in euchromatin, whereas di- and trimethylated H3K27 are excluded from active promoters.<sup>21</sup> Methylated H3K27 is recognized by chromodomain-containing proteins, such as PRC.<sup>22</sup> PRC1 and PRC2 are implicated in H3K27 hypermethylation, which is associated with transcriptional silencing.<sup>23</sup> BMI-1-containing PRC1 is recruited to chromatin marked with H3K27me3, resulting in gene silencing through the ubiquitylation of

H2A followed by chromatin compaction.<sup>24,25</sup> *Kdm6b* selectively catalyzes the demethylation of H3K27me3 and H3K27me2 to H3K27me1 without any enzymatic activities on other methylated lysines.<sup>16,26</sup>

Recently, our group reported that CpG hypermethylation on the *Nkcc1* promoter reduced its transcription in the cerebral cortex<sup>27</sup> and in areas of the cardiovascular system, such as the aorta and heart, in normotensive rats.<sup>12</sup> Treatment with 5-aza-cytidine, an inhibitor of DNA methyltransferase, or trichostatin A, an inhibitor of histone deacetylase, upregulated its transcription in the cerebral cortex during postnatal maturation.<sup>27</sup> In this study, however, the *Nkcc1* promoter in SD rats was mostly unmethylated, indicating that the methylation status of the promoter shows species and tissue specificity.

In a bicarbonate-free medium, the elevation of the bumetanide concentration from 10 to 100  $\mu\text{mol l}^{-1}$  sharply suppressed the contraction of endothelium-denuded rat aortas<sup>3,28</sup> and mouse mesenteric arteries<sup>29</sup> evoked by phenylephrine. These data are consistent with our observations that pretreatment with bumetanide inhibited the vascular contraction of rat mesenteric arteries incubated with  $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor. Bumetanide attenuated the  $E_{\text{max}}$  in the mesenteric arteries of Ang II-infused rats to a greater degree than in those of sham (Figure 1 and Table 1). The fact that the vascular tissues of Ang II-infused rats are more sensitive to bumetanide than those of sham indicates that the vascular tissues of Ang II-infused rats have a higher expression level of NKCC1 than those of sham. On day 3, although the level of the NKCC1 protein was not increased, phenylephrine-induced vasoconstriction was significantly inhibited by bumetanide in the mesenteric arteries of Ang II-infused rats compared with sham. It is well known that NKCC1 activity is regulated by the diverse signaling pathways, including the recently discovered with no lysine (WNK) kinase family.<sup>30</sup> The increased sensitivity to bumetanide may be caused by the activation of NKCC1 by protein kinases, such as SPS1-related proline/alanine-rich kinase.<sup>31</sup>

There are five putative Sp1-binding sites in the *Nkcc1* promoter (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The transcription factor Sp1 positively regulates target gene expression upon BP rise in vascular smooth muscle cells.<sup>32</sup> After the demethylation of H3K27me3, Sp1 that is recruited to the promoter may lead to positive regulation of the *Nkcc1* gene with co-activators such as histone acetyltransferases in hypertensive rats. Although Ang II increased the histone acetylation for IGF-II-receptor gene expression,<sup>33</sup> it is still not clear whether the upregulation of the *Nkcc1* gene promoter activity is caused by indirect mechanisms downstream of Ang II, such as oxidative stress and high BP, or by a direct mechanism through the activation of Ang II receptors. This question is very substantial and should be resolved in a future study.

In conclusion, our results indicate that NKCC1 is upregulated via histone modification in the aortas of Ang II-induced hypertensive rats. Nonetheless, these findings imply that the ion transporter has an important role during the development of hypertension. NKCC1 regulation by histone modification or DNA methylation may serve as a potential biomarker in the diagnosis and management of hypertension.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.



## ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology (2011-0014066) and the Brain Korea 21 Project in 2011.

- 1 Gamba G. Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. *Physiol Rev* 2005; **85**: 423–493.
- 2 O'Neill WC, Steinberg DF. Functional coupling of Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransport and Ca<sup>2+</sup>-dependent K<sup>+</sup> channels in vascular endothelial cells. *Am J Physiol* 1995; **269**: C267–C274.
- 3 Anfinogenova YJ, Baskakov MB, Kovalev IV, Kilin AA, Dulin NO, Orlov SN. Cell-volume-dependent vascular smooth muscle contraction: role of Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransport, intracellular Cl<sup>-</sup> and L-type Ca<sup>2+</sup> channels. *Pflugers Arch* 2004; **449**: 42–55.
- 4 Garg P, Martin CF, Elms SC, Gordon FJ, Wall SM, Garland CJ, Sutliff RL, O'Neill WC. Effect of the Na-K-2Cl cotransporter Nkcc1 on systemic blood pressure and smooth muscle tone. *Am J Physiol* 2007; **292**: H2100–H2105.
- 5 Koltsova SV, Kotelevtsev SV, Tremblay J, Hamet P, Orlov SN. Excitation-contraction coupling in resistance mesenteric arteries: evidence for Nkcc1-mediated pathway. *Biochem Biophys Res Commun* 2009; **379**: 1080–1083.
- 6 Kim SM, Eisner C, Faulhaber-Walter R, Mizel D, Wall SM, Briggs JP, Schnermann J. Effect of the Na-K-2Cl cotransporter Nkcc1 on systemic blood pressure and smooth muscle tone in Nkcc1-deficient mice. *Am J Physiol Renal Physiol* 2008; **295**: F1230–F1238.
- 7 Jiang G, Cobbs S, Klein JD, O'Neill WC. Aldosterone regulates the Na-K-2Cl cotransporter in vascular smooth muscle. *Hypertension* 2003; **41**: 1131–1135.
- 8 Jiang G, Akar F, Cobbs SL, Lomashvili K, Lakkis R, Gordon FJ, Sutliff RL, O'Neill WC. Blood pressure regulates the activity and function of the Na-K-2Cl cotransporter in vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 2004; **286**: H1552–H1557.
- 9 Brown RA, Chipperfield AR, Davis JP, Harper AA. Increased (Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup>) cotransport in rat arterial smooth muscle in deoxycorticosterone (doca)/salt-induced hypertension. *J Vasc Res* 1999; **36**: 492–501.
- 10 Owen NE, Ridge KM. Mechanism of angiotensin ii stimulation of Na-K-Cl cotransport of vascular smooth muscle cells. *Am J Physiol* 1989; **257**: C629–C636.
- 11 San-Cristobal P, Pacheco-Alvarez D, Richardson C, Ring AM, Vazquez N, Rafiqi FH, Chari D, Kahle KT, Leng Q, Bobadilla NA, Hebert SC, Alessi DR, Lifton RP, Gamba G. Angiotensin II signaling increases activity of the renal Na-Cl cotransporter through a WNK4-SPAK-dependent pathway. *Proc Natl Acad Sci USA* 2009; **106**: 4384–4389.
- 12 Lee HA, Baek I, Seok YM, Yang E, Cho HM, Lee DY, Hong SH, Kim IK. Promoter hypomethylation upregulates Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter 1 in spontaneously hypertensive rats. *Biochem Biophys Res Commun* 2010; **396**: 252–257.
- 13 Cho HM, Lee HA, Kim HY, Han HS, Kim IK. Expression of Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter 1 is epigenetically regulated during postnatal development of hypertension. *Am J Hypertension* 2011; **24**: 1286–1293.
- 14 Yang E, Cho JY, Sohn UD, Kim IK. Calcium sensitization induced by sodium fluoride in permeabilized rat mesenteric arteries. *Kor J Physiol Pharmacol* 2010; **14**: 51–57.
- 15 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta C<sub>t</sub>) method. *Methods* 2001; **25**: 402–408.
- 16 Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* 2007; **449**: 731–734.
- 17 Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986; **321**: 209–213.
- 18 Kouzarides T. Chromatin modifications and their function. *Cell* 2007; **128**: 693–705.
- 19 Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH, Jenuwein T. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* 2003; **12**: 1577–1589.
- 20 Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ, Zhao K. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 2008; **40**: 897–903.
- 21 Vakoc CR, Sachdeva MM, Wang H, Blobel GA. Profile of histone lysine methylation across transcribed mammalian chromatin. *Mol Cell Biol* 2006; **26**: 9185–9195.
- 22 Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y. Role of histone h3 lysine 27 methylation in polycomb-group silencing. *Science* 2002; **298**: 1039–1043.
- 23 Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. *Cell* 2007; **128**: 735–745.
- 24 Simon JA, Kingston RE. Mechanisms of polycomb gene silencing: Knowns and unknowns. *Nat Rev Mol Cell Biol* 2009; **10**: 697–708.
- 25 Hernandez-Munoz I, Taghavi P, Kuijl C, Neeffjes J, van Lohuizen M. Association of Bmi1 with polycomb bodies is dynamic and requires PrC2/Ezh2 and the maintenance DNA methyltransferase DNMT1. *Mol Cell Biol* 2005; **25**: 11047–11058.
- 26 Rotili D, Mai A. Targeting histone demethylases: a new avenue for the fight against cancer. *Genes & Cancer* 2011; **2**: 663–679.
- 27 Lee HA, Hong SH, Kim JW, Jang IS. Possible involvement of DNA methylation in Nkcc1 gene expression during postnatal development and in response to ischemia. *J Neurochem* 2010; **114**: 520–529.
- 28 Akar F, Skinner E, Klein JD, Jena M, Paul RJ, O'Neill WC. Vasoconstrictors and nitrovasodilators reciprocally regulate the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter in rat aorta. *Am J Physiol* 1999; **276**: C1383–C1390.
- 29 Koltsova SV, Luneva OG, Lavoie JL, Tremblay J, Maksimov GV, Hamet P, Orlov SN. HCO<sub>3</sub>-dependent impact of Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransport in vascular smooth muscle excitation-contraction coupling. *Cell Physiol Biochem* 2009; **23**: 407–414.
- 30 Richardson C, Alessi DR. The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. *J Cell Sci* 2008; **121**: 3293–3304.
- 31 Sid B, Miranda L, Vertommen D, Viollet B, Rider MH. Stimulation of human and mouse erythrocyte Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransport by osmotic shrinkage does not involve AMP-activated protein kinase, but is associated with STE20/SPS1-related Prolin/Alanine-rich kinase activation. *J Physiol* 2010; **588**: 2315–2328.
- 32 Negoro N, Kanayama Y, Haraguchi M, Umetani N, Nishimura M, Konishi Y, Iwai J, Okamura M, Inoue T, Takeda T. Blood pressure regulates platelet-derived growth factor A-chain gene expression in vascular smooth muscle cells *in vivo*. An autocrine mechanism promoting hypertensive vascular hypertrophy. *J Clin Inv* 1995; **95**: 1140–1150.
- 33 Chu CH, Lo JF, Hu WS, Lu RB, Chang MH, Tsai FJ, Tsai CH, Weng YS, Tzang BS, Hung CY. Histone acetylation is essential for Ang II-induced IGF-1R gene expression in H9c2 cardiomyoblast cells and pathologically hypertensive rat heart. *J Cell Physiol* 2012; **227**: 259–268.

Supplementary Information accompanies the paper on Hypertension Research website (<http://www.nature.com/hr>)