

Short Communication

Antioxidant Effects of Stereoisomers of *N*-Acetylcysteine (NAC), L-NAC and D-NAC, on Angiotensin II-Stimulated MAP Kinase Activation and Vascular Smooth Muscle Cell Proliferation

Moe Kyaw¹, Masanori Yoshizumi¹, Koichiro Tsuchiya², Yuki Izawa¹, Yasuhisa Kanematsu¹, Yoshiko Fujita¹, Nermin Ali¹, Keisuke Ishizawa¹, Aiko Yamauchi³, and Toshiaki Tamaki^{1,*}

¹Department of Pharmacology, The University of Tokushima Graduate School of Medicine, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Departments of ²Clinical Pharmacology and ³Pharmaceutical Information Science, Graduate School of Pharmaceutical Sciences, The University of Tokushima, 1-78 Sho-machi, Tokushima 770-8505, Japan

Received April 6, 2004; Accepted July 1, 2004

Abstract. We examined the effects of the stereoisomers of *N*-acetylcysteine (NAC), L-NAC and D-NAC, on cellular glutathione (GSH) concentration and whether NAC-regulated cellular GSH levels are directly associated with angiotensin II (Ang II)-induced intracellular signaling events in vascular smooth muscle cells (VSMC). Both L-NAC and D-NAC similarly increased intracellular GSH concentration. We found that L-NAC and D-NAC both inhibited Ang II-induced c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and [³H]-thymidine incorporation in VSMC. Our present study indicates the comparable effects of NAC stereoisomers in regulating intracellular GSH and the redox-dependent intracellular signaling mechanisms in VSMC.

Keywords: *N*-acetylcysteine, glutathione, vascular smooth muscle cell

A variety of external stimuli such as growth factors, hormones, cytokines, and mechanical stresses are known to trigger reactive oxygen species (ROS) generation in vascular smooth muscle cells (VSMC) and subsequent activation of various cellular signaling events such as mitogen-activated protein kinases (MAPKs), gene expression, activation of transcription factor, and cellular proliferation (1).

Glutathione (GSH) has been known to have multifaceted cellular functions including antioxidant defense, modulation of redox-regulated signal transduction, gene regulation, regulation of cell proliferation, and synthesis of deoxyribonucleotides (2). *N*-Acetylcysteine (NAC), which acts as a precursor of GSH synthesis (3, 4), has frequently been shown to be an effective antioxidant in scavenging ROS and in suppressing the activity of ROS-dependent signaling molecules in VSMC (5–7). Although many studies have reported that NAC inhibits ROS-sensitive intracellular signaling in different cell

types, it is still unclear whether it exerts its effects directly or through modulation of cellular GSH.

In the present study, we first determined and compared the effects of stereoisomers of NAC, L-NAC and D-NAC, on cellular GSH concentration in VSMC. We utilized buthionine-*SR*-sulfoximine (BSO) as an inhibitor of γ -glutamyl-cysteine synthetase to elucidate whether NAC acted via modulation of cellular GSH. Then we studied the effects of these stereoisomers on angiotensin II (Ang II)-induced extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK activation and [³H]-thymidine incorporation in VSMC.

Human Ang II was purchased from Peptide Institute, Inc. (Osaka). *N*-Acetyl-L-cysteine (L-NAC) and L-buthionine-*SR*-sulfoximine (BSO) were from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Acetyl-D-cysteine (D-NAC) was synthesized according to the method of Sheffner et al. (8). In brief, D-NAC was chemically synthesized from D-cysteine and acetic anhydride. The molecular rotation of the product was confirmed as that of the D-isomer estimated by circular

*Corresponding author. FAX: +81-88-633-7062
E-mail: tamaki@basic.med.tokushima-u.ac.jp

dichroism spectrometry. Phospho-ERK 1/2 antibody (Thr202/Tyr204), phospho-p38 MAPK antibody (Thr180/Tyr182), and the SAPK/JNK assay kit were purchased from New England Biolabs, Inc. (Beverly, MA, USA). All other chemicals were of reagent grade, were obtained from commercial sources, and were used without further purification.

Rat aortic smooth muscle cells (RASMC) were taken from the thoracic aortae of male Sprague-Dawley rats by the explant method as previously described (6). RASMC were seeded ($1-2 \times 10^4$ cells \cdot cm $^{-2}$) and grown to subconfluence in 60 mm culture dishes (IWAKI, Tokyo) in DMEM-10% serum.

Cells were made quiescent for 48 h in serum-free DMEM and preincubated with L-NAC or D-NAC in the presence or absence of BSO for 30 min. After treatment, cells were washed with oxygenated, prewarmed (37°C) Krebs-Ringer Hepes buffer (pH 7.4), then harvested, and centrifuged at $1000 \times g$ for 5 min at 4°C to obtain the cell pellets. The pellets were mixed with 100 μ l ice-cold phosphate buffer containing 5 mM diethylenetriaminopentaacetic acid (DTPA) and 0.1 M potassium phosphate (KPi) buffer, pH 6.8. Homogenate was prepared and a small aliquot was taken for protein measurement. After that, 100 μ l of ice-cold acid buffer containing 40 mM HCl, 10 mM DTPA, 20 mM ascorbic acid, and 10% trichloroacetic acid (TCA) was added to the remaining volume of homogenate to make a suspension, which was then centrifuged at $14,000 \times g$, and the resulting supernatant solution was stored at -80°C until GSH measurement within 4 weeks. The GSH concentration was determined by the method previously described by Senft et al. (9).

For immunoblot analysis and JNK assay, cells were made quiescent for 48 h in serum-free DMEM and preincubated with or without L-NAC or D-NAC or BSO for 30 min followed by Ang II stimulation. After treatment, cells were lysed and sonicated (Handy Sonic UR-20 P; Tomy Seiko Co., Ltd., Tokyo) on ice for 1 min, transferred to microcentrifuge tubes, and then centrifuged at $16,000 \times g$ for 20 min at 4°C. The protein concentrations of the supernatants were measured with a protein assay kit (PIERCE, Rockford, IL, USA) and stored at -80°C until MAP kinase assay.

ERK1/2 and p38 MAPK assays were performed with Western blotting using anti-phosphospecific ERK1/2 or p38 MAPK antibodies (New England Biolabs) as described previously (10). JNK activity was measured with a commercially available kit based on phosphorylation of recombinant c-Jun (New England Biolabs) (10). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and were quantified by densito-

metry in the linear range of film exposure using a UMAX Astra 2200 scanner (UMAX Technologies, Fremont, CA, USA) and NIH image 1.60 software.

For the [^3H]-thymidine incorporation experiment, subconfluent RASMC in 12-well culture plates were made quiescent by placing them in serum-free DMEM for 2 days, then stimulated with Ang II (100 nM) in the presence or absence of L-NAC or D-NAC or BSO for 24 h. The stimulated cells were pulsed with $1 \mu\text{Ci} \cdot \text{ml}^{-1}$ [^3H]-thymidine during the last 8 h of culture. Cells were washed once with PBS and twice with ice-cold 5% trichloroacetic acid (TCA) to remove the unincorporated [^3H]-thymidine; then they were solubilized in 100 μ l 0.25 N NaOH in 0.1% SDS and neutralized. Aliquots of samples were added to 10 ml of scintillation fluid and counted (Aloka 703, Tokyo).

Values are reported as the mean \pm S.D. from experiments done in quintuplicate. Two-way analysis of variance was used to determine the significance among groups, after which a modified *t*-test with Bonferroni's post hoc test was used for comparison between individual groups. A value of $P < 0.05$ was considered to be statistically significant.

We investigated the effects of L-NAC and D-NAC on cellular GSH concentration in RASMC. The cells were treated with L-NAC (10 mM) or D-NAC (10 mM) in the presence or absence of BSO (100 μM) for 30 min and were lysed for GSH measurement. Results showed that both L-NAC and D-NAC similarly increased cellular GSH concentration in RASMC, which was significantly suppressed by BSO, an inhibitor of γ -glutamyl-cysteine synthetase (Fig. 1). These findings suggest that NAC increases the cellular GSH level in VSMC. Our results

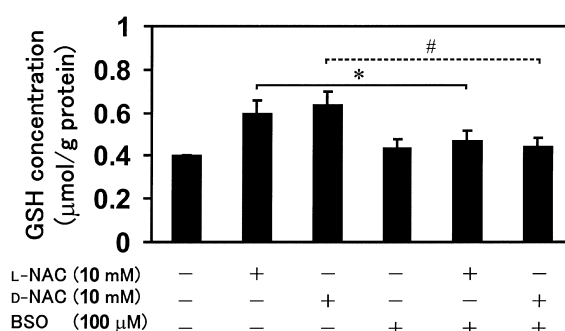


Fig. 1. Effects of L-NAC and D-NAC on cellular GSH concentration in RASMC. Cells were treated with L-NAC (10 mM) or D-NAC (10 mM) in the presence or absence of BSO (100 μM) for 30 min and were lysed for GSH measurement. GSH concentration was measured as described in the text. Values are expressed as GSH concentration in $\mu\text{mol/g}$ protein of sample (values are the mean \pm S.D., $n = 5$). The asterisks represent significant differences compared with the values of the effects of L-NAC and D-NAC on GSH concentration (* $^{\#}P < 0.05$).

are consistent with the findings that GSH synthesis is dependent on NAC in GSH-depleted human umbilical vein smooth muscle cells (11). However, it was reported that administration of L-NAC to mice apparently increased the rate of glutathione synthesis, whereas the unnatural D-isomer of NAC failed to do so (12). If D-NAC cannot be enzymatically converted into GSH, it is difficult to explain our findings that D-NAC could up-regulate GSH and that D-NAC-increased GSH levels were suppressed by BSO in VSMC. Although we have no direct evidence for an explanation, D-NAC may have had regulatory effects on cellular GSH through unknown converting mechanisms or unknown interactions with other molecules in our experimental environment. Further studies are needed to elucidate the exact mechanism of D-NAC to increase GSH level in VSMC in our experimental conditions.

Next, we examined the effects of various concentrations of L-NAC and D-NAC on Ang II-induced ERK1/2, JNK and p38 MAPK activation in RASMC. We found that both L-NAC and D-NAC similarly inhibited Ang II-induced JNK and p38 MAPK activation in a concentration-dependent manner (from 1 to 100 mM), whereas they had effects on ERK1/2 activation only at the highest concentration (100 mM) (data not shown). Thus, we further studied the effects of L-NAC, D-NAC, and

BSO on Ang II-induced JNK and p38 MAPK activation in RASMC. The cells were pretreated with L-NAC (10 mM), D-NAC (10 mM), and BSO (100 μ M) for 30 min before the addition of Ang II (100 nM) for 10 min. Both L-NAC and D-NAC significantly inhibited Ang II (100 nM)-induced JNK and p38 MAPK activation and BSO pretreatment significantly recovered these effects (Fig. 2). These findings indicate that both L-NAC and D-NAC can similarly reduce Ang II-stimulated JNK and p38 MAPK activation through their modulation of cellular GSH in VSMC. In a previous study, we also demonstrated that various antioxidants inhibited Ang II-induced activation of JNK and p38 MAPK in VSMC (6). In addition, intracellular GSH has been reported to be a key regulator for alkylating agent-induced p38 MAPK and JNK activation (13).

Furthermore, we determined the effects of L-NAC and D-NAC on Ang II-induced increases in [3 H]-thymidine incorporation into RASMC. Cells were growth-arrested and stimulated with Ang II (100 nM) in the presence or absence of L-NAC (10 mM), D-NAC (10 mM), and BSO (100 μ M) for a 24-h incubation period. Ang II-induced increases in [3 H]-thymidine incorporation was consistent with the findings that Ang II caused VSMC proliferation of 1.62 ± 0.08 -fold the control, which was evaluated by cell number counting. We found that both stereoisomers

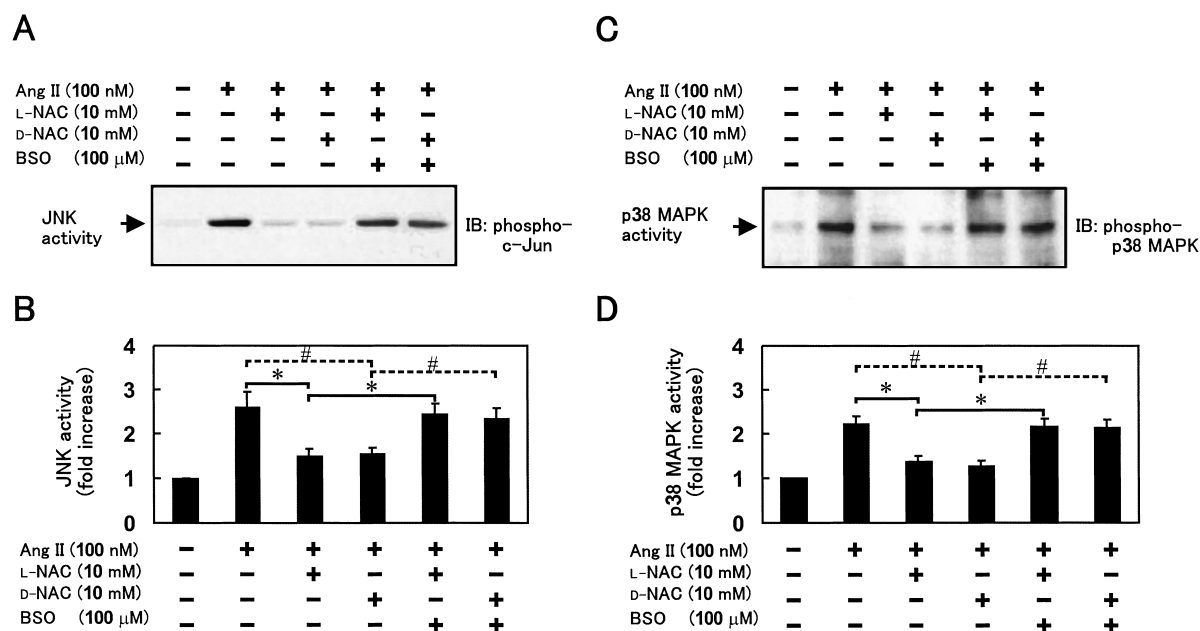


Fig. 2. Effects of L-NAC and D-NAC on Ang II-induced JNK and p38 MAPK activation in RASMC. Cells were pretreated with or without L-NAC (10 mM) or D-NAC (10 mM) or BSO (100 μ M) for 30 min before the addition of Ang II (100 nM) for 10 min. Cells were then harvested, lysed, and used for subsequent analysis. The activities of JNK and p38 MAPK were measured as described in the text. A and C: Representative blots are shown. B and D: Densitometric analysis of JNK and p38 MAPK activation. Values were normalized by arbitrarily setting the densitometry of control cells (without agonists) to 1.0 (values are the mean \pm S.D., $n = 5$). The asterisks represent significant differences compared with the values of Ang II-induced MAP kinase activation with or without L-NAC and D-NAC as well as BSO-induced recovery effects (* $^{\#}P < 0.05$).

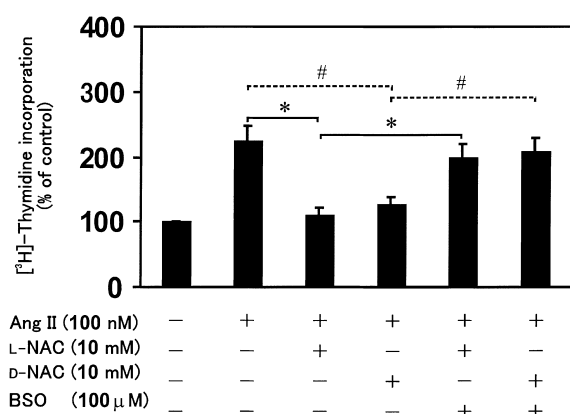


Fig. 3. Effects of L-NAC and D-NAC on Ang II-induced increases in [³H]-Thymidine incorporation in RSMC. [³H]-Thymidine incorporation was measured as described in the text. Values are expressed as percentages of the control (without agonists), which was taken as 100% (values are the mean \pm S.D., $n = 5$). The asterisks represent significant differences compared with the values of Ang II-induced increases in [³H]-thymidine incorporation with or without L-NAC and D-NAC as well as BSO-induced recovery effects (*, # $P < 0.05$).

of NAC markedly attenuated Ang II-induced [³H]-thymidine incorporation and that BSO preincubation significantly reversed their inhibitory effects (Fig. 3). These findings also suggest that both stereoisomers of NAC have similar inhibitory effects on ROS-sensitive VSMC proliferation through regulation of cellular GSH. We previously showed that L-NAC significantly reduced endothelin-induced increases in DNA synthesis in RSMC (7). A recent study also reported that L-NAC prevented DNA damage induced by ultraviolet A and visible radiation in human fibroblasts (14). However, D-NAC, which is known to be incapable of participating in GSH synthesis, was only half as effective as L-NAC in inhibiting topoisomerase-II α activity, an enzyme involved in DNA synthesis as well as perturbing cell cycle progression through G2 (15). In the other report, both L-NAC and D-NAC suppressed proliferation and DNA synthesis by PC12 cells and prevented apoptotic death (16). Although MAPK-mediated cellular phenotypic modulations, such as proliferation or apoptosis, may be agonist and cell type specific, inhibition by NAC stereoisomers of JNK and p38 MAPK activation in RSMC may imply their atheroprotective effects because these MAPKs activation have been reported to be implicated in VSMC hypertrophy and proliferation (1, 7).

Thus, our present findings indicate the comparable effects of NAC stereoisomers in regulating intracellular GSH and the redox-dependent intracellular signaling mechanisms in VSMC.

References

- Berk BC. Redox signals that regulate the vascular response to injury. *Thromb Haemost.* 1999;82:810–817.
- Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol.* 1999;39:67–101.
- Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 1983;52:711–760.
- Neuhauser M, Grotz KA, Wandira JA, Bassler KH, Langer K. Utilization of methionine and N-acetyl-L-cysteine during long-term parenteral nutrition in the growing rat. *Metabolism.* 1986;35:869–873.
- Rao GN, Katki KA, Madamanchi NR, Wu Y, Birrer MJ. JunB forms the majority of the AP-1 complex and is a target for redox regulation by receptor tyrosine kinase and G protein-coupled receptor agonists in smooth muscle cells. *J Biol Chem.* 1999;274:6003–6010.
- Kyaw M, Yoshizumi M, Tsuchiya K, Kirima K, Tamaki T. Antioxidants inhibit JNK and p38 MAPK activation but not ERK 1/2 activation by angiotensin II in rat aortic smooth muscle cells. *Hypertens Res.* 2001;24:251–261.
- Kyaw M, Yoshizumi M, Tsuchiya K, Kirima K, Suzuki Y, Abe S, et al. Antioxidants inhibit endothelin-1 (1–31)-induced proliferation of vascular smooth muscle cells via the inhibition of mitogen-activated protein (MAP) kinase and activator protein-1 (AP-1). *Biochem Pharmacol.* 2002;64:1521–1531.
- Sheffner AL, Medler EM, Bailey KR, Gallo DG, Mueller AJ, Sarett HP. Metabolic studies with acetylcysteine. *Biochem Pharmacol.* 1966;15:1523–1535.
- Senft AP, Dalton TP, Shertzer HG. Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. *Anal Biochem.* 2000;280:80–86.
- Yoshizumi M, Abe J, Haendeler J, Huang Q, Berk BC. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J Biol Chem.* 2000;275:11706–11712.
- Voskoboinik I, Soderholm K, Cotgreave IA. Ascorbate and glutathione homeostasis in vascular smooth muscle cells: cooperation with endothelial cells. *Am J Physiol.* 1998;275:C1031–C1039.
- Wong BK, Chan HC, Corcoran GB. Selective effects of N-acetylcysteine stereoisomers on hepatic glutathione and plasma sulfate in mice. *Toxicol Appl Pharmacol.* 1986;86:421–429.
- Wilhelm D, Bender K, Knebel A, Angel P. The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. *Mol Cell Biol.* 1997;17:4792–4800.
- Morley N, Curnow A, Salter L, Campbell S, Gould D. N-Acetyl-L-cysteine prevents DNA damage induced by UVA, UVB and visible radiation in human fibroblasts. *J Photochem Photobiol B.* 2003;72:55–60.
- Grdina DJ, Murley JS, Roberts JC. Effects of thiols on topoisomerase-II alpha activity and cell cycle progression. *Cell Prolif.* 1998;31:217–229.
- Ferrari G, Yan CY, Greene LA. N-Acetylcysteine (D- and L-stereoisomers) prevents apoptotic death of neuronal cells. *J Neurosci.* 1995;15:2857–2866.