N-Acetyl-L-Cysteine Potentiates Interleukin-1β Induction of Nitric Oxide Synthase
Role of p44/42 Mitogen-Activated Protein Kinases

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Abstract—We have reported previously that N-acetyl-l-cysteine facilitated interleukin-1β–induced nitric oxide synthase (iNOS) expression in rat vascular smooth muscle cells. The present study compares the effect of N-acetyl-l-cysteine with other antioxidants and tested the possibility that N-acetyl-l-cysteine potentiates iNOS induction by a mechanism involving activation of p44/42 mitogen-activated protein kinases (MAPKs). The effect of N-acetyl-l-cysteine on potentiating interleukin-1β–induced nitrite production and iNOS expression was mimicked either by the enantiomers, L-cysteine and D-cysteine, or by a non–thiol-containing antioxidant, L-ascorbic acid. Interleukin-1β–activated p44/42 MAPK, and this activation was enhanced in the presence of N-acetyl-l-cysteine. Inhibition of p44/42 MAPK phosphorylation by the selective inhibitor PD98059 clearly inhibited iNOS expression induced by interleukin-1β either in the absence or in the presence of N-acetyl-l-cysteine. These observations, combined with previous results, indicate that p44/42 MAPK activation is required for interleukin-1β induction of iNOS and that N-acetyl-l-cysteine may act as a reducing agent and facilitate interleukin-1β–induced iNOS expression through a reduction/oxidation-related mechanism involving potentiation of cytokine activation of the p44/42 MAPK signaling pathway. (Hypertension. 2000;35:914-918.)

Key Words: acetylcysteine ■ interleukins ■ nitric oxide synthase ■ protein kinases ■ muscle, smooth, vascular

The inducible isofrm of nitric oxide synthase (iNOS), which is associated with immune and inflammatory responses,1 has been demonstrated to be induced by endotoxin or proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α, and interferon-γ, in many cell types. The presence of iNOS in human atherosclerotic vessels has been confirmed by using immunostaining and in situ hybridization, and the iNOS was localized to macrophages, foam cells, and vascular smooth muscle cells (VSMCs).2,3 The specific functions of iNOS in injured vessels remain speculative, but evidence from several studies indicates roles of the reaction product nitric oxide (NO) in the prevention of leukocyte and platelet adhesion,4,5 the modulation of vascular tone,6,7 the inhibition of smooth muscle cell and fibroblast proliferation,7–9 and the promotion of reendothelialization.10 These observations suggest an important functional role of cytokine-induced NO synthesis in atherosclerosis and vascular remodeling. The understanding of the mechanisms by which iNOS gene expression is regulated, therefore, is of particular clinical significance.

We reported recently11 that N-acetyl-l-cysteine (NAC), a widely used thiol-containing antioxidant that supports intracellular glutathione synthesis and can scavenge reactive oxygen intermediates, facilitates IL-1β–induced iNOS expression without obvious influence on the nuclear translocation and DNA-binding properties of nuclear factor-κB (NF-κB) in rat VSMCs. It was reported that NF-κB activation and DNA binding are required but insufficient for IL-1β–induced κB-dependent transcription.12 Mitogen-activated protein kinase (MAPK) cascades, including p44/42 MAPK, also called extracellular signal–regulated kinases (ERK1/2), the c-Jun amino-terminal kinase (JNK), and p38 MAPK, have recently been suggested to be involved in endotoxin or cytokine induction of iNOS in diverse cell types, including macrophages, mesangial cells, astrocytes, epithelial cells, pancreatic islets, cardiac myocytes, and endothelial cells.13–18 However, the reported findings are not consistent, and the mechanisms are unclear. iNOS induction in VSMCs has been documented in several studies,1–5,11 but it is not known whether MAPK cascades were involved in the mechanism for iNOS induction in this cell type. The present study was performed to test this possibility and to assess whether the potentiating effect of NAC might be related to its effects on MAPK activation.

Methods

Materials

DMEM/F-12 Ham medium (DMEM/F12), FCS, and a penicillin/streptomycin/amphotericin B mixture were purchased from Life

Received November 10, 1999; first decision November 29, 1999; revision accepted November 30, 1999.

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Technologies, Inc. Recombinant human IL-1β (specific activity \( \sim 1 \times 10^7 \) U/mg) was purchased from Genzyme. NAC, DL-cysteine, L-cysteine, d-cysteine, and L-ascorbic acid were obtained from Sigma Chemical Co. PD98059 and SB203580 were purchased from Calbiochem. Monoclonal antibody against mouse macrophage iNOS was obtained from Transduction Laboratories. Phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody, p44/p42 MAPK polyclonal antibody, phospho-p38 MAPK (Thr180/Tyr182) polyclonal antibody, and p38 MAPK polyclonal antibody were obtained from New England Biolabs. NF-κB consensus oligonucleotide was purchased from Promega. [\( \gamma \)-32P]dCTP (111 TBq/mmol) and [\( \alpha \)-32P]ATP (111 TBq/mmol) were purchased from DuPont-New England Nuclear. All other materials used were commercial products of the highest grade available.

Cell Culture

VSMCs were isolated by enzymatic digestion of the medial layer separated from the thoracic aorta of 8-week-old male Wistar rats according to the procedure of Chamley-Campbell et al. VSMCs were cultured in DMEM/F12 that was supplemented with 10% FCS, 100 U/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 0.25 mg/L amphotericin B at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells between passages 4 and 7 were used for experiments. At confluence, the cells were washed twice with serum-free medium and then maintained in DMEM/F12 with 0.1% FCS for 24 to 48 hours before use.

Determination of Nitrite

VSMCs cultured in 24-well plates were washed once with serum-free medium and cultured in phenol red–free DMEM/F12 that contained 0.1% FCS, with or without various agents, as indicated in Results, for 24 hours. The release of NO from cultures was assessed by the determination of nitrite as described previously. To adjust nitrite levels to cell protein content, cells were washed with ice-cold PBS and then incubated with 0.1 mol/L NaOH (0.5 mL per well) at 37°C for 1 hour, followed by exposure to either IL-1β or NAC or both for 24 hours in medium alone or medium that contained 3 mg/mL of IL-1 in the absence or presence of 1 mmol/L of NAC, DL-cysteine (DL-Cys), l-cysteine (l-Cys), or d-cysteine (d-Cys). The conditioned media were used for determination of nitrite concentration, and the cell lysates (blot at top) were prepared for Western blot analysis of iNOS protein. Values are mean ± SD (n = 3 for panel A and n = 4 for panel B). The Western blot shown in panel B is representative of 2 individual experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs IL-1 alone.

Statistical Analysis

The nitrite data are expressed as mean ± SD. Statistical analysis was performed by 1-way ANOVA, followed by the Scheffé F test with StatView version 4.01 (Abacus Concepts Inc). A value of P < 0.05 was considered to be significant.

Results

Cysteine and Ascorbic Acid Act Similar to NAC in Enhancing IL-1β–Induced Nitrite Production and iNOS Expression

NAC significantly enhanced IL-1β–induced nitrite production in a concentration-dependent manner. This effect was also found with either DL-cysteine, a thiol-containing amino acid, or l-ascorbic acid, an antioxidant without a thiol residue (Figure 1A). Because DL-cysteine showed potency similar to that of NAC, we further compared the effects of cysteine enantiomers. The results showed no difference between l-cysteine and d-cysteine in their ability to enhance IL-1β–induced nitrite production and iNOS expression (Figure 1B).

Electrophoretic Mobility Shift Assay

To examine whether NF-κB activation and translocation might be influenced by modulation of p44/42 MAPK activation, VSMCs were pretreated with or without PD98059 for 1 hour before the addition of IL-1β or NAC or both for 1 hour. Nuclear extracts were prepared and DNA-binding activity was assessed with an electrophoretic mobility shift assay as described previously.

Figure 1. Cysteine and ascorbic acid mimic NAC in enhancing IL-1β (IL-1)–induced nitrite production and iNOS expression. A, Cells were exposed to IL-1 (3 ng/mL) for 24 hours in the presence of various concentrations of NAC, DL-cysteine, or l-ascorbic acid. B, Cells were incubated for 24 hours in medium alone or medium that contained 3 mg/mL of IL-1 in the absence or presence of 1 mmol/L of NAC, DL-cysteine (DL-Cys), l-cysteine (l-Cys), or d-cysteine (d-Cys). The conditioned media were used for determination of nitrite concentration, and the cell lysates (blot at top) were prepared for Western blot analysis of iNOS protein. Values are mean ± SD (n = 3 for panel A and n = 4 for panel B). The Western blot shown in panel B is representative of 2 individual experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs IL-1 alone.

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Selective inhibitors were then used to test whether the activation of these 2 MAPK cascades might be involved in iNOS induction. Pretreatment of the cells for 1 hour with 20 μmol/L PD98059, a selective inhibitor of ERK1/2 activation, significantly inhibited not only IL-1β–induced ERK activation but also IL-1β–induced iNOS expression (Figure 2B). PD98059 at concentrations from 0.2 to 20 μmol/L inhibited IL-1β–induced nitrite production in a dose-dependent manner (Figure 2C). In contrast, pretreatment of the cells for 1 hour with 10 μmol/L SB203580, a selective inhibitor of p38 MAPK, showed no effect on IL-1β–induced nitrite production (Figure 2B).

As shown by Western blot analysis (Figure 3A), the levels of p-ERK1/2 in the cells stimulated with IL-1β were higher in the presence than in the absence of NAC (for phosphorylated p44 MAPK, 13.3±2.2-fold versus 4.9±2.2-fold increases, P<0.01, and for phosphorylated p42 MAPK, 3.08±0.58-fold versus 2.17±0.06-fold increases, P<0.05, respectively; on the basis of densitometric analysis of 3 separate experiments).

Neither NAC Nor PD98059 Affects IL-1β–Induced NF-κB Nuclear Translocation and DNA Binding

Treatment of the cells with IL-1β for 1 hour resulted in NF-κB nuclear translocation and DNA binding, as shown by electrophoretic mobility shift assay. This effect of IL-1β was not influenced by the addition of NAC (1 mmol/L). Pretreatment of the cells for 1 hour with PD98059 at 20 μmol/L, a concentration significantly inhibiting IL-1β–induced ERK1/2 activation and iNOS expression, showed no obvious effect on IL-1β–induced NF-κB nuclear translocation and DNA binding (Figure 4).

Discussion

To further study the mechanism by which NAC facilitated IL-1β induction of iNOS, we first compared the effect of NAC with the thiol-containing amino acids, dl-cysteine,
t-cysteine, and d-cysteine, and with a non–thiol-containing antioxidant, L-ascorbic acid. All these compounds mimicked the effect of NAC by enhancing IL-1β–dependent nitrite production and iNOS expression. NAC has been attributed with increasing intracellular glutathione synthesis (by providing the precursor, t-cysteine) and also with scavenging reactive oxygen intermediates.20,21 Because both cysteine enantiomers showed effects equipotent to the effect of NAC and because it is known that d-cysteine cannot be used by cells as a precursor for glutathione synthesis, it would appear that the NAC enhancement of IL-1β–induced iNOS expression was not solely through an increase in intracellular glutathione synthesis. This was also confirmed by using L-ascorbic acid, a non–thiol-containing antioxidant that is not a precursor for glutathione synthesis but also has an effect similar to that of NAC. It is more likely that NAC and other thiol-containing or non–thiol-containing antioxidants or reducing agents might modulate the intracellular reduction/oxidation state and produce a more reductive microenvironment that might influence reduction/oxidation-sensitive signaling pathways.

In the present study, we found that p44/42 MAPK (ERK1/2) in VSMCs was clearly activated by IL-1β stimulation and that the inhibition of p44/42 MAPK phosphorylation by PD98059, a selective inhibitor of MAPK kinase, resulted in significant reduction in nitrite production and iNOS expression induced by IL-1β, suggesting the requirement for p44/42 MAPK cascade activation to induce iNOS expression. In our experiments, p38 MAPK was only slightly activated by IL-1β stimulation. Pretreatment of the cells with SB203580, an effective inhibitor of p38 MAPK at a concentration of 10 μmol/L, showed no influence on IL-1β–induced nitrite production, indicating less importance for the p38 MAPK cascade in the IL-1β induction of iNOS in VSMCs. Both p38 MAPK and p44/42 MAPK cascades were suggested to be involved in iNOS induction, but there are discrepancies in the reported results. Silva et al15 and Chen and Wang22 reported that p38 but not p44/42 MAPK is required for iNOS induction in mouse astrocytes or in RAW 264.7 macrophages. Badger et al23 reported that SB203580, the inhibitor of p38 MAPK, inhibits IL-1–induced iNOS expression in bovine cartilage–derived chondrocytes. Ajizian et al13 and Larsen et al17 reported that both p38 and p44/42 MAPK pathways are involved in the upregulation of iNOS in murine macrophages or in rat pancreatic islets. Guan et al24 observed that p38 MAPK downregulates iNOS expression stimulated by IL-1β in rat mesangial cells. It is difficult at present to explain the discrepancies in the results produced by different cell types because there is a lack in the understanding of the mechanisms by which these 2 MAPK cascades modulate iNOS gene expression. Although p38 MAPK is reported to be activated by cytokines in several cell types,13–18,22,23 it has been shown that IL-1β activates p38 MAPK pathway in a serum-dependent manner in renal mesangial cells by an unknown mechanism.14

NAC at concentrations <10 mmol/L showed no effect on IL-1β–induced NF-κB nuclear translocation and DNA-binding activity,11 thus implicating the involvement of other pathways downstream from the IL-1 receptor. The observations that NAC enhanced IL-1β activation of p44/42 MAPK and that iNOS expression was reduced by inhibition of p44/42 MAPK activation suggest that the p44/42 MAPK cascade might be one of the potential regulatory pathways. The mechanism by which NAC enhanced IL-1β activation of p44/42 MAPK is unclear. Yan and Greene24 recently reported that NAC activated Ras and the ERK pathway, factors that were suggested to be responsible for NAC-promoted survival of PC12 cells. However, the NAC concentration used in the present study (1 mmol/L) is much lower than the 60 mmol/L level used in that study. Because 60 mmol/L NAC prevented PC12 cells from experimentally induced apoptosis, whereas 30 mmol/L NAC caused nuclear condensation and detachment in VSMCs,11 different sensitivities or responses to NAC treatment are likely in distinct cell types. Although we cannot rule out the possibility that NAC might act through other mechanisms in addition to the p44/42 MAPK cascade, our findings indicate that NAC, probably acting as a reducing agent, potentiates IL-1β activation of p44/42 MAPK and iNOS expression.

Acknowledgments

This study was supported by National Institutes of Health grants HL-53471 and HL-55001.

References


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Hypertension. 2000;35:914-918
doi: 10.1161/01.HYP.35.4.914

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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