Amino Acids (2012) 43:1547–1555
DOI 10.1007/s00726-012-1230-1

ORIGINAL ARTICLE

Induction of NAD(P)H:quinone oxidoreductase 1 expression by cysteine via Nrf2 activation in human intestinal epithelial LS180 cells

Hideo Satsu · Emi Chidachi · Yuto Hiura · Haru Ogiwara · Yusuke Gondo · Makoto Shimizu

Received: 29 August 2011 / Accepted: 20 January 2012 / Published online: 3 February 2012

Abstract In this study, we examined the effects of 20 amino acids on the expression level of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human intestinal LS180 cells. Five amino acids were associated with significant increases in NQO1 mRNA expression; the most substantial increase was induced by cysteine, which markedly increased the NQO1 mRNA level in a time- and dose-dependent manner. Cysteine also increased the protein level of NQO1 and its enzymatic activity in LS180 cells. Furthermore, cysteine significantly up-regulated NQO1 promoter activity, and this induction was completely abolished by mutation of the antioxidant response element, a binding site of the nuclear factor erythroid 2-related factor 2 (Nrf2). Knockdown experiment using siRNA against Nrf2 showed the involvement of Nrf2 on cysteine-induced increase in NQO1 mRNA expression. Further, cysteine treatment increased the amount of Nrf2 protein in the nucleus and decreased the amount of Kelch-like ECH-associated protein 1 (a suppressor protein of Nrf2) in the cytosol, suggesting that Nrf2 was activated by cysteine. Oral administration of cysteine to mice significantly increased NQO1 mRNA levels in the mouse intestinal mucosa. These findings show that cysteine induces NQO1 expression in both in vitro and in vivo systems and also suggest that Nrf2 activation is involved in this induction.

H. Satsu (✉) · E. Chidachi · Y. Hiura · H. Ogiwara · Y. Gondo · M. Shimizu
Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: asatsu@mail.ecc.u-tokyo.ac.jp

Keywords Cysteine · NAD(P)H:quinone oxidoreductase 1 (NQO1) · Nuclear factor erythroid 2-related factor 2 (Nrf2) · Intestinal epithelial cell

Abbreviations

NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
Keap1	Kelch-like ECH-associated protein 1
sMaf	Small Maf protein
ARE	Antioxidant response element
ACF	Aberrant crypt foci
AOM	Azoxymethane
GSH	Glutathione
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
NEAA	Non-essential amino acids
tBHQ	*Tert*-buthylhydroquinone

Introduction

The intestinal epithelial cells, as a barrier between an organism and its intestinal contents, are the first line of defense against frequent exposure to xenobiotics containing chemical toxicants (Carriere et al. 2001). In the biological defense process, intestinal epithelia express detoxification enzymes that play important roles in metabolism, detoxification, and exclusion of the xenobiotics (Xu et al. 2005). Among these enzymes, phase II detoxification enzymes are especially important in defense process against ingested xenobiotics; NAD(P)H quinine oxidoreductase 1 (NQO1) is a principal phase II enzyme with a major role in the complete reduction and detoxification of highly reactive quinones (Talalay 2000; Nioi and

Hayes 2004). Quinones are abundant in the environment; they are found in vehicle exhaust, cigarette smoke, and in foods. Single-electron reduction by P450 reductase produces a highly reactive semiquinone species in which an oxygen atom contains an unpaired electron. Semiquinones can react directly with cellular protein or DNA and can produce reactive oxygen species. NQO1 can protect against semiquinone-induced oxidative stress by catalyzing an obligatory single-step two-electron reduction of quinones, bypassing the reactive and toxic semiquinone intermediates (Nioi and Hayes 2004; Ross and Siegel 2004).

The expression levels of phase II detoxification enzymes, including NQO1, are regulated by the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) (Kobayashi et al. 2006). Nrf2 is located in the cytoplasm, and under basal conditions it binds to the Nrf2 suppressor protein Kelch-like ECH-associated protein 1 (Keap1). Oxidative stress and electrophiles disrupt the Nrf2–Keap1 complex, allowing Nrf2 to translocate to the nucleus. There, Nrf2 forms a heterodimer with small Maf protein (sMaf), binds to antioxidant response element (ARE) in heterodimeric combinations with other basic leucine zipper proteins, and regulates gene expressions of detoxification enzymes, including NQO1, glutathione *S*-transferase, UDP-glucuronosyltransferase 1A1, and heme oxygenase 1 (Moran et al. 2002). Therefore, it is possible that an Nrf2 inducer could increase the expression of phase II detoxification enzymes, further enhancing the defense system against xenobiotics in the intestinal epithelial cells.

It has been reported that NQO1 expression is regulated by some types of food components, including polyphenols (Tanigawa et al. 2007) and isothiocyanates (Thimmulappa et al. 2002) via activation of Nrf2. Although there are many reports of the physiological functions of amino acids, the relationship between amino acids and phase II detoxification enzymes has not been investigated. Therefore, in the present study, we used LS180 cells as a model of human intestinal epithelial cells to examine the effects of amino acids on the expression of NQO1, as well as to investigate the regulatory mechanism of these effects.

Materials and methods

Materials

The LS180 cell line, derived from human colonic cancer tissue, was obtained from DS PharmaBiomedical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Wako Pure Chemicals (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO, USA). Penicillin, streptomycin (10,000 U/ml and 10 mg/ml in 0.9% sodium chloride, respectively), and non-essential amino acids (NEAA) were purchased from Gibco (Gaithersburg, MD, USA). The lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA, USA) and the ExScript RT reagent kit and SYBR Premix Ex Taq for the real-time polymerase chain reaction were from Takara Bio (Otsu, Japan). Human Nrf2 and control siRNA were purchased from Thermo Scientific Dharmacon (Chicago, IL, USA). All the other chemicals used were of reagent grade and commercially available.

Cell culture

LS180 cells were cultured in 78.5-cm^2 plastic dishes with a culture medium consisting of DMEM, 10% FBS (v/v), 1% NEAA (v/v), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air; the culture medium was renewed on alternate days. After reaching confluence, the cells were trypsinized with 0.1% trypsin and 0.02% EDTA in PBS, and then subcultured.

Isolation of total RNA and real-time PCR

LS180 cells were cultured in 24-well plates at 1.25×10^5 cells/well for real-time PCR experiments. Cells were cultured with medium containing 10 mM of different amino acid for 24 h; 20 μM of *tert*-buthylhydroquinone (tBHQ) was used as a positive control (Keum et al. 2006). After incubation, total RNA was extracted from the cells using Isogen (Nippon gene, Japan) according to the manufacturer's instructions. The cDNA was prepared from 1 μg of the total RNA. A real-time polymerase chain reaction (PCR) was performed with SYBR Green I, as previously described (Satsu et al. 2008). After denaturing at 95°C for 15 min, PCR was performed for 40 cycles of denaturing at 95°C for 15 s, annealing for 15 s, and extension at 72°C for 10 s. The PCR primers for real-time PCR were designed by the WEB site of Roche Applied Science Universal Probe Library Assay Design Center. The sequence of PCR primers used for NQO1, Nrf2 and β-actin and each annealing temperature is shown in Table 1.

Western blot analysis

LS180 cells were cultured in 6-well plates at 5×10^5 cells/well with cysteine for 24 and 48 h prior to western blot analysis. Preparation of the cytosolic and nuclear extracts was performed as described previously (Shin et al. 2011). Volumes of extract containing 10 μg of proteins were run on 12.5% SDS-PAGE and electrophoretically transferred to PVDF membrane (Millipore). After blocking at 4°C overnight, the membrane was incubated with a specific antibody for 1 h, and further incubated for 1 h with an

Table 1 Primers for real-time PCR

Gene	Sequence (5′–3′)	Anneal temperature
Human NQO1 (NM_000903)		
Forward primer	GACCTCTATGCCATGAACTT	55°C
Reverse primer	TATAAGCCAGAACAGACTCG	
Human β-actin (NM_001101)		
Forward primer	CCAGCACAATGAAGATCAAGA	56°C
Reverse primer	AGAAAGGGTGTAACGCAACTAA	
Human Nrf-2 (NM_006164)		
Forward primer	GAGACAGGTGAATTTCTCCCAAT	60°C
Reverse primer	TTTGGGAATGTGGGCAAC	
Mouse NQO1 (NM_008706)		
Forward primer	GCAGGATTTGCCTACACAATATGC	59°C
Reverse primer	AGTGGTGATAGAAAGCAAGGTCTTC	
Mouse β-actin (NM_007393)		
Forward primer	GGCCAGGTCATCACTATTG	59°C
Reverse primer	GAGGTCTTTACGGATGTCAAC	

HRP-conjugated secondary antibody. Analyses of bound antibodies were carried out with an ECL chemiluminescent substrate (GE Healthcare) and the Lumino Image Analyzer (LAS-4000miniPR SF: Fuji Film).

Measurement of human NQO1 enzymatic activity in LS180 cells

LS180 cells were cultured in 96-well plates at 0.5×10^5 cells/well with different concentration of cysteine (0, 0.5, 1, 2.5, 5, and 10 mM) for 48 h or with 10 mM cysteine for 0, 24, and 48 h. The enzymatic activity of NQO1 in LS180 cells was measured according to a previously described method (Prochaska and Santamaria 1988).

Reporter constructs

The reporter gene plasmid was constructed by ligating the PCR-amplified human NQO1 promoter region (−797 to +17) with the pGL3-basic vector to create pGL3-NQO1. The reporter construct was sequenced with an ABI310 genetic analyzer (Applied Biosystems). Site conversion within ARE was performed (−759 GC to TA −758) with a Quick Change™ site-directed mutagenesis kit (Stratagene, CA, USA).

Transient transfection and reporter assay

The cells were seeded at 1.25×10^5 cells/well in a 24-well plate for the reporter assay. 1-day cultures of LS180 cells were transfected with the plasmid DNA (1 μg of pGL3-NQO1, and 0.1 μg of pRL-CMV) by the lipofection technique with lipofectamine and the PLUS reagent (Invitrogen). After transfection, the medium was replaced with a medium containing cysteine, and the LS180 cells were cultured for 24 h. The luciferase activity was determined by a dual-luciferase reporter assay (Promega, Tokyo, Japan) with an LB9507 Lumet luminometer (Berthold Technologies, Bad Wildbad, Germany).

Animals and in vivo experiment

Female C57BL/6 mice (7 weeks old, 17–19 g of weight; Japan SLC, Japan) were housed in an air-conditioned room (23 ± 2°C) with a 12-h light/dark cycle. They were allowed free access to food and tap water. After being acclimated to the environment for 3–4 days, the mice were divided into two groups (4 mice in each group). Every day for 16 days, one group was orally administered 400 mg cysteine/kg body weight dissolved in water, and the other group was orally administered water without cysteine. Then, the mice were killed and small intestine was removed. After washing with PBS, small intestinal tract was cut and spread. The intestinal mucosa on luminal surface was scraped with glass slide on ice and collected to Isogen solution in tube. Then intestinal mucosa in Isogen solution was homogenized with homogenizer and total RNA was extracted from homogenized intestinal mucosa, used for real-time PCR analysis. All animals received human care, and the study protocols were approved by the Committee for Care of Laboratory Animals of the Graduate School of Agricultural and Life Sciences at the University of Tokyo.

Statistical analysis

Each value is expressed as the mean ± SE. Differences among the experimental data were assessed by a one-way analysis of variance (ANOVA) followed by F-protected

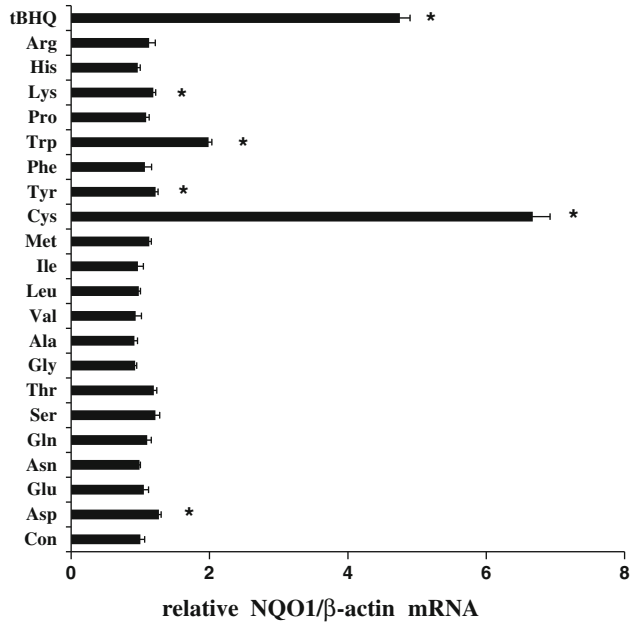

Fig. 1 Effects of amino acids on the mRNA expression level of human NQO1 in human intestinal LS180 cells. LS180 cells were incubated with 10 mM of each amino acid for 24 h, and total RNA was extracted for real-time PCR analysis of NQO1 as described in "Materials and methods". tBHQ was used as a positive control. Each value is expressed as mean ± SE ($n = 3$). *Significantly different from the control value ($p < 0.05$)

Fisher's least significant difference. All results shown are from representative runs of at least two individual experiments performed in triplicate.

Results

Effects of amino acids on the mRNA expression level of NQO1 in human intestinal LS180 cells

We examined whether several amino acids could regulate the expression level of human NQO1 mRNA in LS180 cells. The amino acid treatment had no significant effect on β-actin mRNA expression at any stage, indicating that β-actin could be used as a stable housekeeping gene throughout the experiment. We also performed cell viability test using LDH assay kit (Wako Pure Chemicals) and confirmed that the incubation of LS180 cells with 10 mM amino acids for 24 h are not cytotoxic (data not shown). Of the 20 different amino acids that were tested, five were associated with significant changes in NQO1 mRNA expression as compared to that of the control (Fig. 1). The most substantial increase was seen with cysteine treatment (>6-fold increase), followed by tryptophan (about twofold increase).

Dose- and time-dependence of cysteine-induced NQO1 mRNA expression in LS180 cells

In further studies, we focused on cysteine and further characterized its effect on the mRNA expression level of NQO1 in LS180 cells. Figure 2a shows that cysteine increased the expression level of human NQO1 mRNA in a dose-dependent manner. Further, the expression level of NQO1 mRNA increased linearly according to the incubation time, reaching about fourfold after a 24-h incubation (Fig. 2b).

Effect of cysteine on the expression level of human NQO1 protein and its enzymatic activity in LS180 cells

Western blot analysis was performed to determine whether the cysteine-induced change in NQO1 mRNA was associated with increased expression of human NQO1 protein in LS180 cells. As shown in Fig. 3, the amount of human NQO1 protein was increased in a time-dependent manner in cells cultured with 10 mM cysteine. This shows that cysteine induced the NQO1 expression at the protein level as well as the mRNA level. We further assessed the effect

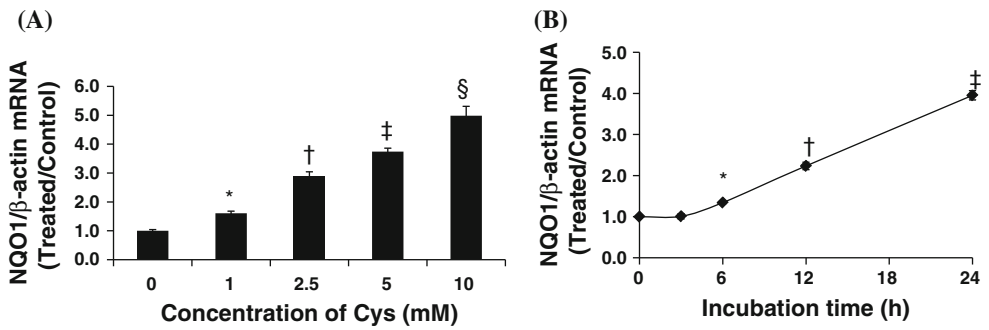

Fig. 2 Effect of cysteine on the expression level of the NQO1 mRNA in human intestinal LS180 cells. **a** LS180 cells were incubated with different concentrations of cysteine (0–10 mM) for 24 h, and real-time PCR analysis was performed as described in "Materials and methods". **b** LS180 cells were cultured with 10 mM cysteine for 0, 3, 6, 12, and 24 h. After incubation, real-time PCR was performed as described in "Materials and methods". Each value is expressed as mean ± SE. ($n = 3$). *, †, ‡, §Values with different symbols are significantly different from each other, $p < 0.05$

of cysteine on the NQO1 enzymatic activity in LS180 cells. Figure 4a indicates that the NQO1 enzymatic activity in LS180 cells cultured with 10 mM cysteine was significantly increased as compared to the control. It is also shown that the induction of NQO1 activity by cysteine was dose-dependent in LS180 cells (Fig. 4b).

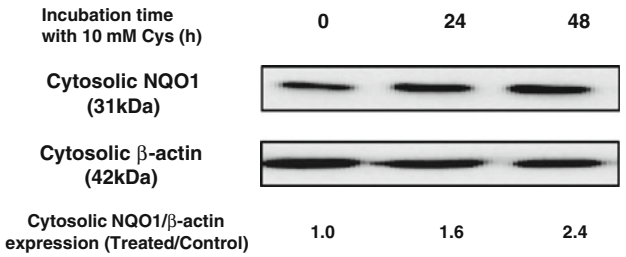

Fig. 3 Effect of cysteine on the expression level of the NQO1 protein in human intestinal LS180 cells. LS180 cells cultured in six-well plates were treated with 10 mM Cys for 0, 24, and 48 h. Then, cytosolic extracts were prepared and subjected to western blot analysis with anti-NQO1 antibody (sc-32793, Santa Cruz Biotechnology) and anti-β-actin antibody (A2228, Sigma). Anti-β-actin antibody was used as a marker for the cytosolic extracts

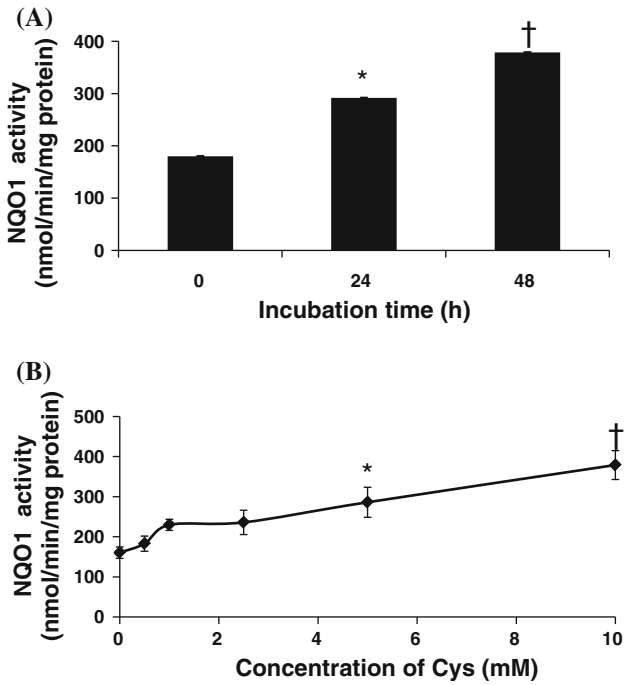

Fig. 4 Effect of cysteine on the enzymatic activity of the NQO1 in human intestinal LS180 cells. (a) LS180 cells cultured in 96-well plates were treated with various 10 mM cysteine for 0, 24, and 48 h. The enzymatic activity of NQO1 was then measured as described in "Materials and methods". (b) LS180 cells cultured in 96-well plates were incubated with 0, 0.5, 1, 2.5, 5, and 10 mM cysteine. After culturing for 48 h, the enzymatic activity of NQO1 was measured as described in "Materials and methods". Each value is expressed as mean ± SE ($n = 3$). *, †, ‡Values with *different symbols* are significantly different from each other, $p < 0.05$

The induction of human NQO1 promoter activity in LS180 cells by cysteine

To examine the regulatory mechanism behind cysteine-induced increases in NQO1 expression and activity, we constructed a reporter vector containing the promoter region (−797 to +17) of human NQO1 and assessed the effect of cysteine on the transcriptional activity of this human NQO1 promoter gene. Figure 5a shows that the promoter activity of human NQO1 was significantly and dose-dependently increased by cysteine treatment, suggesting that cysteine induced NQO1 expression at the transcriptional level.

The involvement of ARE on cysteine-induced promoter activity of NQO1

Because Nrf2 is known to play an initial role in mediating gene expression of human NQO1, we examined whether Nrf2 was responsible for the cysteine-induced up-regulation of the NQO1 promoter. We introduced two distinct mutations (−759 GC to TA −758) within the antioxidant responsive element (ARE), to which Nrf2 binds in the human NQO1 promoter to create an inactive ARE, as described previously (Nguyen et al. 1994). These ARE mutations resulted in complete suppression of the cysteine-induced luciferase activity (Fig. 5b), strongly suggesting that cysteine-induced NQO1 gene expression requires ARE and Nrf2 activation in the NQO1 promoter.

The involvement of Nrf2 on cysteine-induced increase in NQO1 mRNA expression

Having determined that cysteine induced NQO1 promoter activity and that ARE was essential for this induction, we examined the involvement of Nrf2 on NQO1 induction by cysteine using siRNA against Nrf2. We first confirmed that mRNA level of Nrf2 was suppressed by transfection with siRNA against Nrf2 (Fig. 6a). Figure 6b shows that the increase in NQO1 mRNA expression by cysteine was significantly suppressed by the transfection with siRNA against Nrf2 in comparison with control siRNA.

The effect of cysteine on nuclear Nrf2 expression and cytosolic Keap1 expression in LS180 cells

We next examined the nuclear localization of Nrf2. Since the activation of Nrf2 is generally accompanied with degradation of Keap1, we also measured the amount of Keap1 in the cytosol. The amount of Nrf2 protein in the nucleus was increased by cysteine treatment in a time-dependent manner (Fig. 7) and the amount of Keap1 protein in the cytosol was decreased by cysteine (Fig. 8). These results

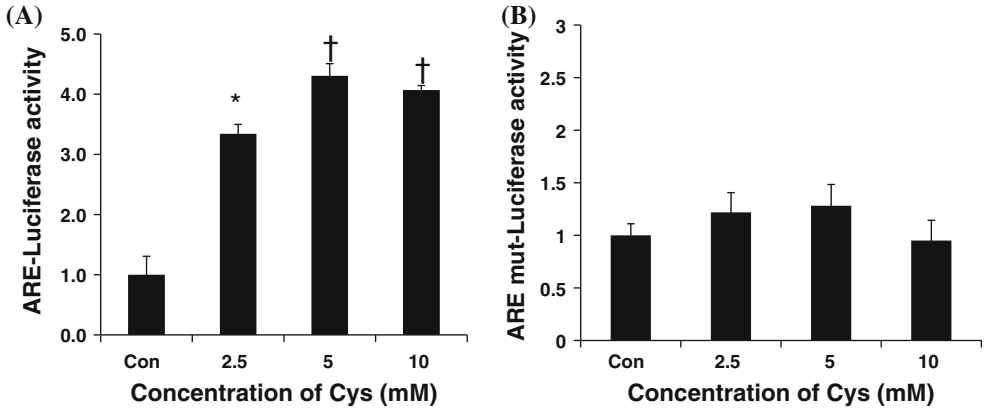

Fig. 5 Effect of cysteine on the transcriptional activity of the human NQO1 promoter in human intestinal LS180 cells. LS180 cells were cotransfected with pRL-CMV (the internal control plasmid) and pGL3-NQO1-WT (**a**) or pGL3-NQO1-AREmut (**b**) by lipofection. Transfected cells were incubated for 24 h with cysteine at 0, 2.5, 5, and 10 mM, and the reporter assay was performed as described in "Materials and methods". Each value is expressed as mean ± SE ($n = 3$). *, †, ‡, §Values with *different symbols* are significantly different from each other, $p < 0.05$

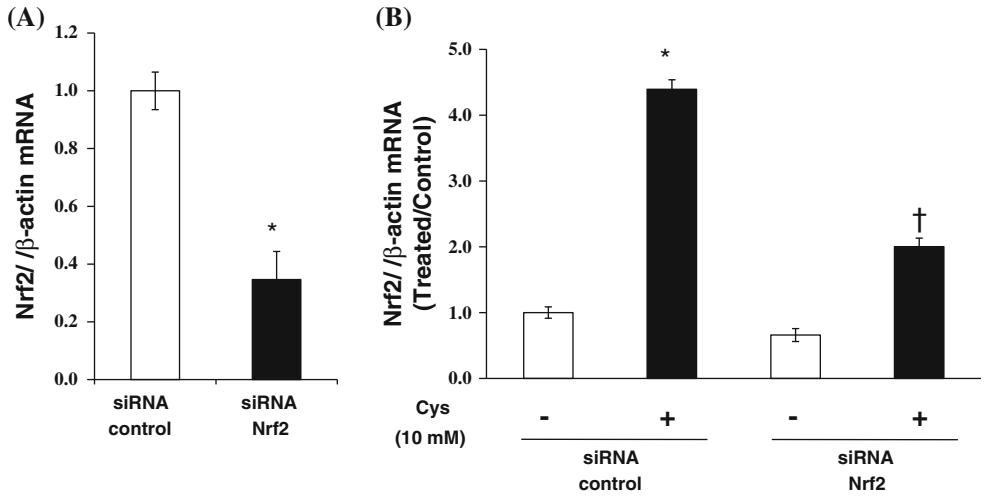

Fig. 6 Effect of Nrf2 knockdown on cysteine-induced increase in NQO1 mRNA in LS180 cells. LS180 cells were transfected with siRNAs against Nrf2 or control (scramble) using lipofectamine RNAi MAX Reagent (Invitrogen), according to the manufacturer's instructions. Then, the transfected cells were incubated with or without 10 mM Cys for 24 h, and total RNA was extracted for real-time PCR analysis. Each value is expressed as mean ± SE ($n = 3$). *, †Values with *different symbols* are significantly different from each other, $p < 0.05$

suggest that cysteine activates Nrf-2-mediated NQO1 transcription by increasing the nuclear Nrf2 protein and decreasing the cytosolic Keap1 protein.

The effect of cysteine on NQO1 mRNA expression in mouse intestinal mucosa

Administration of 400 mg cysteine/kg BW to mice for 14 days significantly increased the expression level of NQO1 mRNA in the mouse intestinal mucosa (Fig. 9). This result clearly shows that cysteine induced the NQO1 mRNA level, not only in intestinal epithelial LS180 cells in vitro, but also in mouse intestinal mucosa in vivo.

Discussion

In the present study, we examined the effects of 20 amino acids on the expression of NQO1 mRNA in human intestinal epithelial-like LS180 cells, and found that cysteine substantially up-regulated the expression and activity of NQO1. We further revealed that this cysteine-induced up-regulation of NQO1 occurred at the transcriptional level, via the Nrf2-ARE pathway. Cysteine-induced NQO1 up-regulation of was also observed in mouse intestinal mucosa in vivo.

Cysteine is one of the 20 amino acids that constitute proteins and is categorized as a non-essential amino acids.

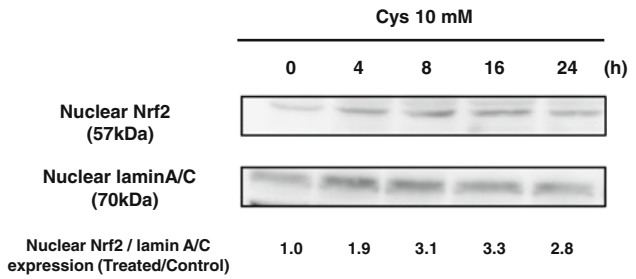

Fig. 7 Nuclear localization of Nrf2 in human intestinal LS180 cells. LS180 cells were treated with 10 mM cysteine for indicated times. The nuclear extracts were prepared and subjected to western blot analysis with anti-Nrf2 antibody (sc-722, Santa Cruz Biotechnology) and anti-lamin A/C antibody (#2032, Cell Signaling). Anti-lamin A/C antibody was used as a marker for the nuclear extracts

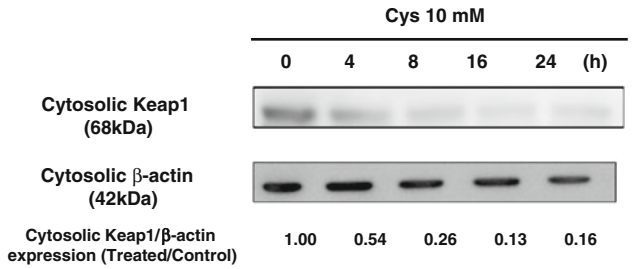

Fig. 8 Cytosolic expression of Keap1 in human intestinal LS180 cells. LS180 cells were treated with 10 mM cysteine for indicated times. The cytosolic extracts were prepared and subjected to western blot analysis with anti-Keap1 antibody (sc-15246, Santa Cruz Biotechnology) and anti-β-actin antibody. Anti-β-actin antibody was used as a marker for the cytosolic extracts

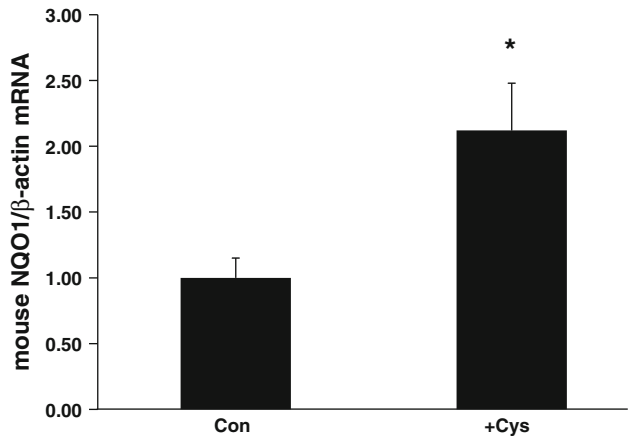

Fig. 9 Effect of cysteine on the expression level of NQO1 mRNA in mouse intestinal mucosa. Female C57BL/6 mice were orally administered water with or without cysteine for 16 days. Then, the mice were killed, intestinal mucosa was recovered, and total RNA was extracted for real-time PCR. Each value is expressed as mean ± SE ($n = 4$). *Significantly different from the control value ($p < 0.05$)

Cysteine is also known as the precursor of glutathione (GSH), which is a major antioxidant in mammalian cells, along with taurine, pyruvic acid, and sulfate (Bauchart-Thevret et al. 2009). Although most cysteine is generally metabolized in the liver (Stipanuk 2004), it is reported that 75% of ingested cysteine is absorbed in the intestine, 25% of which is utilized in the intestine (Bauchart-Thevret et al. 2009). It is therefore assumed that the intestine plays an important role in the metabolism of cysteine (Bauchart-Thevret et al. 2009; Baker and Czarnecki-Maulden 1987). There have been several reports about the physiological functions of cysteine, for example, cysteine can weaken the toxicity of trace elements, such as copper, cobalt, and selenium by chelating them (Baker and Czarnecki-Maulden 1987), and Kim et al. (2009) recently reported that oral-gastric administration of cysteine suppressed intestinal inflammation in a porcine model.

Several food-derived factors and chemical compounds have been reported to activate or induce NQO1 expression. The chemical structures of these NQO1 activators are diverse, and they have been classified into several groups (Dinkova-Kostova et al. 2004). Cysteine has a similar structure to members of the dimethyl captan group, but cysteine differs from these structures in that it has only one thiol residue. Thus, cysteine represents a new group of chemical structures that induce NQO1 expression.

It has been widely understood that phase II detoxification enzymes, including NQO1, are regulated by Nrf2 (Kobayashi et al. 2006). Nrf2 bound to the Keap1–Cul3 complex is normally ubiquitinated and digested in the cytosol. However, upon stimulation by electrophiles and oxidative stress, the thiol group of Keap1 is modulated (Kobayashi et al. 2006), allowing Nrf2 to escape and translocate to the nucleus. There, Nrf2 forms a heterodimer with sMAF and binds to ARE, leading to the induction of phase II drug metabolizing enzymes (Moran et al. 2002). In this study, we revealed that inactivation of ARE by point mutation in the human NQO1 promoter abolished cysteine-induced activation of the NQO1 promoter (Fig. 5). Further, the increase in NQO1 mRNA by cysteine was significantly suppressed by down-regulating Nrf2 expression using RNAi technique (Fig. 6). These results suggest that cysteine activates NQO1 promoter activity via ARE and Nrf2.

We also observed that the amount of Nrf2 protein in the nucleus increased in a time-dependent manner for up to 16 h of treatment with cysteine (Fig. 7), while the amount of Keap1 protein in the cytosol time-dependently decreased (Fig. 8). In human hepatic HepG2 cells, the flavonoid quercetin reportedly induces NQO1 expression via Nrf2 activation, with a time-dependent decrease of Keap1 protein in the cytosol during this treatment (Tanigawa et al. 2007). It is also reported that the Lys residue at 298 of the Keap1 protein is modulated and ubiquitinated by tBHQ treatment in HEK293 cells (Hong et al. 2005a). The Lys residue at 298 is located close to the 273 and 288 Cys residues, which are essential for inhibition of Nrf2

activation by Keap1. It is, therefore, supposed that these Cys residues are modulated by tBHQ treatment, thereby changing the structure of the Keap1–Cul3 complex, and changing the ubiquitin target molecule from Nrf2 to Keap1. If this is the case, the amount of Keap1 in the cytosol could decrease upon modulation of the Cys residues 273 and 288 by cysteine treatment. In future studies, we will examine whether the Keap1 protein is ubiquitinated by cysteine treatment.

Understanding the cysteine metabolism in intestinal cells is necessary to determine the regulatory mechanism of cysteine-induced expression of NQO1. We examined the characteristics of cysteine uptake by LS180 cells and found that cysteine uptake was performed via amino acid transporters, and was competitively inhibited by other amino acids, such as Ala, Ser, and Glu (data not shown). In the presence of these amino acids, induction of NQO1 by cysteine was clearly suppressed (data not shown), suggesting that cysteine induced NQO1 expression after being taken up by the cells via amino acid transporters. Cysteine is metabolized to various substances, such as GSH, and also modulates various proteins (Giles et al. 2003). It seems that Keap1 is regulated via modulation of its highly reactive thiol group, and the residue of the Keap1 thiol group that is involved in Nrf2 activation differs according to the modulating factor. Human Keap1 contains 27 Cys residues, and Cys residue numbers 151, 273, and 288 are involved in Nrf2 activation. Recently, mass spectrometry analysis revealed that other Cys residues are also modulated (Kobayashi et al. 2009), for example, sulforaphane modulates Cys residues 77, 226, 249, 257, 489, 513, 518, and 583 (Hong et al. 2005b); 10-shogaol, which is contained in ginger, modulates Cys residues 151, 257, and 368 of the Keap1 protein; and xanthohumol in Hop modulates Cys residues 151, 319, and 613 of the Keap1 protein (Luo et al. 2007). It will be interesting to determine whether cysteine modulates the Cys residues of the Keap1 protein directly or modulates other molecules, leading to the activation of Nrf2 indirectly, as well as whether this modulation is caused by its intact or metabolized form.

From the viewpoint of the relationship between NQO1 and gastrointestinal diseases, NQO1 is reported to suppress the carcinogen-induced formation of aberrant crypt foci (ACF), the precursor of colon cancer. An inactivating polymorphism at base 609 of the NQO1 gene is reportedly a risk factor for human colon cancer (Begleiter et al. 2006). Begleiter et al. (2003) reported that rats fed a diet including oltipraz, a NQO1 inducer, had significantly fewer ACF induced by azoxymethane (AOM) than those fed the control diet; this effect was reversed in rats treated with the NQO1 inhibitor, dicoumarol. Furthermore, rats fed the oltipraz-containing diet after AOM treatment developed 40% fewer colon adenomas and fewer colon tumors than rats fed a control diet (Begleiter et al. 2009), suggesting that NQO1 contributes not only to prevent ACF formation, but also to inhibit colon carcinogenesis at the post-initiation stage. Thus, cysteine treatment could potentially prevent ACF formation, and the following colon carcinogenesis, via NQO1 induction.

In this study, relatively high concentration of cysteine was used both in in vitro and in vivo studies. Although it may be difficult to activate NQO1 by cysteine through the daily diet because cysteine content is generally low in food proteins, cysteine supplements have recently been commercially available. Therefore, it may be achievable to activate NQO1 by having cysteine supplement. In relation to that, to reveal whether the effect of cysteine on induction of NQO1 is also observed in the physiological condition in vivo, detailed mouse experiments are necessary, changing the concentration and administered period of cysteine and assess not only NQO1 mRNA level, but also protein and activity levels. We are planning to perform detailed in vivo experiments as our next study.

In conclusion, our study demonstrates cysteine-induced increases in the expression level of NQO1, in both in vitro and in vivo systems. The up-regulation of NQO1 by cysteine occurred at the transcriptional level via the Keap1–Nrf2-mediated ARE pathway. These findings indicate novel chemopreventive properties of cysteine and suggest its potential role in protecting intestinal epithelial cells against xenobiotics.

Acknowledgments This work was partially supported by Grant-in Aid for Scientific Research (S-15108002) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by a Research Grant from Mishima Kaiun Memorial Foundation.

Conflict of interest The authors declare that they have no conflict of interest.

References

Baker DH, Czarnecki-Maulden GL (1987) Pharmacologic role of cysteine in ameliorating or exacerbating mineral toxicities. J Nutr 117:1003–1010

Bauchart-Thevret C, Stoll B, Burrin DG (2009) Intestinal metabolism of sulfur amino acids. Nutr Res Rev 22:175–187

Begleiter A, Sivananthan K, Curphey TJ, Bird RP (2003) Induction of NAD(P)H quinone: oxidoreductase1 inhibits carcinogen-induced aberrant crypt foci in colons of Sprague-Dawley rats. Cancer Epidemiol Biomarkers Prev 12:566–572

Begleiter A, Hewitt D, Maksymiuk AW, Ross DA, Bird RP (2006) A NAD(P)H:quinone oxidoreductase 1 polymorphism is a risk factor for human colon cancer. Cancer Epidemiol Biomarkers Prev 15:2422–2426

Begleiter A, Sivananthan K, Lefas GM, Maksymiuk AW, Bird RP (2009) Inhibition of colon carcinogenesis by post-initiation induction of NQO1 in Sprague-Dawley rats. Oncol Rep 21: 1559–1565

Carriere V, Chambaz J, Rousset M (2001) Intestinal responses to xenobiotics. Toxicol In Vitro 15:373–378

Dinkova-Kostova AT, Fahey JW, Talalay P (2004) Chemical structures of inducers of nicotinamide quinone oxidoreductase 1 (NQO1). Methods Enzymol 382:423–448

Giles NM, Giles GI, Jacob C (2003) Multiple roles of cysteine in biocatalysis. Biochem Biophys Res Commun 300:1–4

Hong F, Sekhar KR, Freeman ML, Liebler DC (2005a) Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. J Biol Chem 280:31768–31775

Hong F, Freeman ML, Liebler DC (2005b) Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem Res Toxicol 18:1917–1926

Keum YS, Han YH, Liew C, Kim JH, Xu C, Yuan X, Shakarjian MP, Chong S, Kong AN (2006) Induction of heme oxygenase-1 (HO-1) and NAD[P]H: quinone oxidoreductase 1 (NQO1) by a phenolic antioxidant, butylated hydroxyanisole (BHA) and its metabolite, tert-butylhydroquinone (tBHQ) in primary-cultured human and rat hepatocytes. Pharm Res 23:2586–2594

Kim CJ, Kovacs-Nolan J, Yang C, Archbold T, Fan MZ, Mine Y (2009) L-Cysteine supplementation attenuates local inflammation and restores gut homeostasis in a porcine model of colitis. Biochim Biophys Acta 1790:1161–1169

Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M (2006) Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 26:221–229

Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y, Eguchi M, Wada Y, Kumagai Y, Yamamoto M (2009) The antioxidant defense system Keap1–Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. Mol Cell Biol 29:493–502

Luo Y, Eggler AL, Liu D, Liu G, Mesecar AD, van Breemen RB (2007) Sites of alkylation of human Keap1 by natural chemoprevention agents. J Am Soc Mass Spectrom 18:2226–2232

Moran JA, Dahl EL, Mulcahy RT (2002) Differential induction of mafF, mafG and mafK expression by electrophile–response–element activators. Biochem J 361:371–377

Nguyen T, Rushmore TH, Pickett CB (1994) Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. J Biol Chem 269:13656–13662

Nioi P, Hayes JD (2004) Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat Res 555:149–171

Prochaska HJ, Santamaria AB (1988) Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 169:328–336

Ross D, Siegel D (2004) NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), functions and pharmacogenetics. Methods Enzymol 382:115–144

Satsu H, Hiura Y, Mochizuki K, Hamada M, Shimizu M (2008) Activation of the pregnane X receptor and induction of MDR1 by dietary phytochemicals. J Agric Food Chem 56:5366–5373

Shin HS, Zhao Z, Satsu H, Totsuka M, Shimizu M (2011) Synergistic effect of tumor necrosis factor-alpha and hydrogen peroxide on the induction of IL-8 production in human intestinal Caco-2 cells. Inflammation 34:440–447

Stipanuk MH (2004) Role of the liver in regulation of body cysteine and taurine levels: a brief review. Neurochem Res 29:105–110

Talalay P (2000) Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 12:5–11

Tanigawa S, Fujii M, Hou DX (2007) Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic Biol Med 42:1690–1703

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 62:5196–5203

Xu C, Li CY, Kong AN (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res 28:249–268