JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 475–481

Received July 28, 2004
Accepted December 17, 2004
DOI 10.1211/0022357055731
ISSN 0022-3573

Hydroquinone modulates reactivity of peroxynitrite and nitric oxide production

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Abstract

Peroxynitrite ($ONOO^-$), a potent cytotoxic oxidant formed by the reaction of nitric oxide (•NO) and superoxide radical ($•O_2^-$), may be rapidly lethal in a cellular milieu due to oxidization and nitration processes. In the present study, hydroquinone displayed strong $ONOO^-$ scavenging activity and inhibitory effect on NO production in murine macrophage RAW264.7 cells. Hydroquinone strongly scavenged $ONOO^-$ induced dihydrorhodamine 123 oxidation in a dose-dependent manner compared with other reactive species such as $•O_2^-$ and •NO. Hydroquinone also decreased levels of $ONOO^-$ induced nitrotyrosine of glutathione reductase and consequently prevented the enzyme from $ONOO^-$ induced damage. Furthermore, hydroquinone suppressed NO production, a cellular pathway for $ONOO^-$ formation, in lipopolysaccharide-activated RAW264.7 cells via inhibition of inducible NO synthase expression. The inhibitory effect by hydroquinone seems to be mediated by interruption of lipopolysaccharide-induced signalling such as activation of nuclear factor-κB and extracellular signal-related kinases 1 and 2. The results suggest that hydroquinone may potently modulate reactivity of $ONOO^-$ and may therefore be a useful agent against $ONOO^-$ mediated diseases.

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Funding: This work was supported by a grant (PF002201-07/-08) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Ministry of Science and Technology.

Introduction

Nitric oxide (NO) is produced in mammalian cells through the oxidation of L-arginine by a family of enzymes known as nitric oxide synthase (Stuehr 1999). NO has been reported to have antimicrobial and antitumour activity, but NO overproduction by inducible nitric oxide synthase (iNOS) has been reported to be relevant in the pathophysiology of several diseases such as rheumatoid arthritis, atherosclerosis, acute respiratory distress syndrome and pancreatic cancer (Deliconstantinos et al 1996; Geller & Billia 1998; Kroncke et al 1998).

One of the reactive components linked to NO-mediated diseases has been identified to be peroxynitrite ($ONOO^-$). This toxic component is generated by a reaction of NO and superoxide ($•O_2^-$) from several cells such as endothelial cells, neutrophils and macrophages (Huie & Padmaja 1993). Compared with unstable free radicals, $ONOO^-$ is a relatively stable toxic component and therefore it can stably induce oxidation of thiol (-SH) groups from some proteins, nitration of tyrosine, nitrosation (e.g. formation of *S*-nitrosoglutathione), and lipid peroxidation, resulting in inducing cellular toxicity by alteration of cell metabolism and signalling pathway (Deliconstantinos et al 1996; Kroncke et al 1998). There have been extensive efforts to develop drugs with powerful $ONOO^-$ scavenging effects and/or NO inhibition from various natural sources such as plants and vegetables (Chung et al 2001; Goss et al 1999).

In the process of screening potent antioxidative agents, we found that a simple phenolic compound, hydroquinone (Figure 1), exhibited potent scavenging effects against the generation of $ONOO^-$ (Kim, A. R. et al 2002). The aim of the present study was to further define the effectiveness of hydroquinone. The modulatory effect of hydroquinone on $ONOO^-$ reactivity and cellular $ONOO^-$ generation pathway (NO production) was investigated using dihydrorhodamine 123 (DHR 123) and lipopolysaccharide (LPS)-activated RAW264.7 cells.

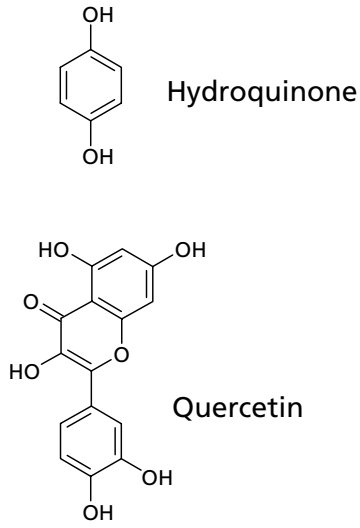

Figure 1 Structure of hydroquinone and quercetin.

Materials and Methods

Materials

Oxidized glutathione (GSSG), NADPH, glutathione reductase (GSH reductase) type II from wheat germ, bovine serum albumin type V, DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), diethylenetriaminepentaacetic acid, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), LPS, hydroquinone, quercetin (Figure 1), and nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fluorescent 4,5-diaminofluoroscein diacetate (DAF-2) was from Calbiochem (San Diego, CA, USA). DHR 123 and $ONOO^-$ were from Molecular Probes (Eugene, OR, USA) and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively. Antibody to nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies to iNOS and phospho-extracellular signal-related kinases 1 and 2 (ERK1/2) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nuclear factor-κB (NF-κB) (5′-GAGAGGCAAGGGGATTCCCTTAGTTA GGA-3′) consensus oligonucleotides and polymerase chain reaction primers were from Bioneer (Daejeon, Korea). All other chemicals were of the highest purity available from either Sigma Chemical Co. or Junsei Chemical Co. (Tokyo, Japan).

Measurement of $ONOO^-$, $\cdot O_2^-$ and •NO scavenging activity

$ONOO^-$, $\cdot O_2^-$ and •NO scavenging activity by hydroquinone and other standard compounds was measured by monitoring the oxidation of DHR 123, H_2DCFDA and DAF-2 induced by the generating agents for $ONOO^-$, $\cdot O_2^-$ and •NO (10 μM $ONOO^-$, kidney homogenate (1 mg protein mL^{-1}) and 2 mM sodium nitroprusside) as reported previously (Kooy et al 1994; Whiteman et al 1996; Nagata et al 1999; Chung et al 2001). The oxidation of DHR 123, H_2DCFDA and DAF-2 was measured using a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively, at room temperature.

Measurement of GSH reductase activity

Enzymatic activity of GSH reductase was determined by the NADPH reduction method as reported previously (Mavis & Stellwagen 1968). GSH reductase (0.6 U mL^{-1}) in the presence or absence of hydroquinone or quercetin was incubated with shaking at 37°C for 5 min. After the addition of $ONOO^-$, the mixture was further incubated for 10 min. Enzyme activity was measured by a spectrophotometer at 340 nm after adding substrate solution containing 1 mM GSSG, 0.09 mM β-NADPH, 0.13% (w/v) bovine serum albumin in 75 mM potassium phosphate buffer with 2.6 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.4).

Sample preparation for detection of tyrosine-nitrated GSH reductase

Hydroquinone or quercetin was pre-incubated in the presence or absence of 25 μL GSH reductase (25 mg mL^{-1}) with shaking for 5 min at 37°C. After the addition of $ONOO^-$ in 0.3 M NaOH, the mixtures were further incubated for 10 min at 37°C.

Cell culture

RAW264.7 cells obtained from American Type Culture Collection (Rockville, MD, USA) were cultured with Dulbecco's modified Eagle's medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), glutamine (233.6 mg mL^{-1}) and antibiotics (0.25 μg mL^{-1} amphotericin B and 72 μg mL^{-1} gentamicin) at 37°C with 5% CO_2.

Quantification of NOx level (nitrite and nitrate)

RAW264.7 cells were pre-incubated with or without testing compounds (hydroquinone, quercetin and L-NAME) for 3 h and continuously activated with 100 ng mL^{-1} LPS for 24 h. NO metabolites (NOx) were measured from culture supernatant after deproteinizing with an equal volume of methanol. The assay was carried out using an NO-analysing system (ENO-20; Eicom Corp., Kyoto, Japan) (Kimura et al 1999). The absorbance of the product dye was measured at 540 nm using a flow-through spectrophotometer.

Preparation of cytosolic fraction and nuclear extracts

The cytosolic fraction and nuclear extracts were prepared from the cells as reported previously (Kim, H. J. et al 2002). Hydroquinone or quercetin-treated cells in the presence or absence of LPS were washed with ice-cold phosphate-buffered saline, and harvested to get the cell pellet by centrifugation 12 000 *g*, 5 min) at 4°C. Nuclear extracts were obtained from the pellets according to the method reported previously (Kim, H. J. et al 2002). Protein concentrations

were determined by the bicinchoninic acid method (Smith et al 1985).

Western blot analysis

Total protein equivalents were separated on 8–12% sodium dodecyl sulfate-polyacrylamide mini-gel using a Laemmli buffer system at 100 V, and were transferred to PVDF membrane at 100 V for 1.5 h. The membrane was immediately placed in a blocking solution (5–10% non-fat dry milk in TBS-T buffer (pH 7.5) containing 10 mM Tris, 100 mM NaCl and 0.1% Tween 20) at 4°C overnight. The membrane was then washed in TBS-T buffer for 30 min and further incubated with antibodies to nitrotyrosine (1:2000), iNOS (1:1000) and phospho-ERK1/2 (1:500) at room temperature for 1–2 h. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence system.

Extraction of total RNA and reverse transcription polymerase chain reaction

Total RNA from LPS-treated RAW264.7 cells was prepared by adding TRIzol reagent (Gibco), according to the manufacturer's protocol. Semiquantitative reverse transcription polymerase chain reaction amplification was carried out as reported previously (Hong et al 2003). The primers (Bioneer, Seoul, Korea) used in this experiment were as follows: iNOS: forward 5′-CCCTTCCGAAGTT TCTGGCAGCAGC-3′ and reverse 5′-GGCTGTCAGA GCCTCGTGGCTTTGG-3′; β-actin: forward 5′-GTGG GCCGCCCTAGGCACCAG-3′ and reverse 5′-GGAGG AAGAGGATGCGGCAGT-3′.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed as reported previously (Kim, H. J. et al 2002). Briefly, 15 μg of nuclear protein extract was incubated in binding buffer containing 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 1% Nonidet P-40, 10 mM Tris (pH 7.5), and 1 μg of poly(dI-dC)• poly(dI-dC) for 20 min at 4°C. Radiolabelled NF-κB consensus oligonucleotide (20 000 cpm of ^{32}P) was then added and further incubated for 20 min at room temperature. The DNA-binding complexes were separated by 7% native polyacrylamide gel in 0.5 × TBE buffer (0.045 M Tris-borate/0.001 M EDTA). Specific binding was confirmed by competition reaction with a 50-fold excess of unlabelled, identical oligonucleotides.

Statistical analysis

The Kruskal–Wallis test and the Mann–Whitney *U*-test were used to determine the statistical significance of differences between values for various experimental and control groups. Data are expressed as means ± s.e.m. of at least three independent experiments performed with triplicates.

Results

Scavenging effect of hydroquinone on the reactivity of toxic radical species ($ONOO^-$, •NO and $•O_2^-$)

Since hydroquinone showed strong scavenging activity at 1 μg mL^{-1} in preliminary studies, dose-dependent $ONOO^-$ scavenging activity was examined. Table 1 shows that hydroquinone potently suppressed the oxidation of DHR 123 to fluorescent rhodamine 123 mediated by authentic $ONOO^-$ (inhibitory concentration 50% (IC50) = 1.4 ± 3.1 μM), which was comparable with that of penicillamine (4.5 ± 0.1 μM) and quercetin (0.9 ± 0.2 μM). To compare the scavenging effect of hydroquinone on $ONOO^-$ reactivity, the neutralizing effect on the reactivity of other toxic species (•NO and $•O_2^-$) was also examined. As shown in Table 1 and Figure 2, hydroquinone weakly scavenged the reactivity of •NO and $•O_2^-$ (IC50 values of 8.5 and 190 μM, respectively) compared with $ONOO^-$. Quercetin showed similar scavenging activity. Other standard compounds, trolox and carboxy-PTIO, also displayed inhibitory activity, with IC50 values of 92.7 (against $•O_2^-$) and 1.5 (against •NO) μM, respectively.

Effects of hydroquinone on the formation of nitrotyrosine and enzyme damage of GSH reductase induced by $ONOO^-$ exposure

$ONOO^-$ modifies the activity of several enzymes with thiol groups, which are essential for their catalytic function (Minetti et al 2002). The representative enzyme is GSH reductase, a rate limiting enzyme for maintaining the intracellular GSH level. $ONOO^-$ induced nitrotyrosine

Table 1 Inhibitory concentration 50% (IC50) of hydroquinone on scavenging activity of $ONOO^-$, $•O_2^-$, •NO and NOx (nitrate and nitrite).

Compound	IC50 (μM)			
	$ONOO^{-a}$	$•O_2^{-a}$	•NO[a]	NOx[b]
Hydroquinone	1.4 ± 3.1	188.8 ± 41.9	8.5 ± 0.1	5.6 ± 0.8
Quercetin	0.9 ± 0.2	53.1 ± 10.4	5.9 ± 0.5	13.9 ± 1.1
Penicillamine	4.5 ± 0.1			
Trolox		92.7 ± 13.0		
Carboxy-PTIO			1.5 ± 0.2	
L-NAME				191.3 ± 21.4

[a]$ONOO^-$, $•O_2^-$ and •NO scavenging activity of the tested compounds was measured by monitoring the oxidation of dihydrorhodamine 123, H_2DCFDA and DAF-2 by authentic $ONOO^-$ (10 μM), kidney homogenate (1 mg protein mL^{-1}) and sodium nitroprusside (2 mM). [b]RAW264.7 cells (0.5×10^6) were pre-treated with different concentrations of hydroquinone, quercetin or L-NAME for 3 h and further incubated in the presence of absence of lipopolysaccharide (100 ng mL^{-1}) for 24 h. The NOx level was determined by a NO analysing system. Data are mean ± s.e. of three independent measurements.

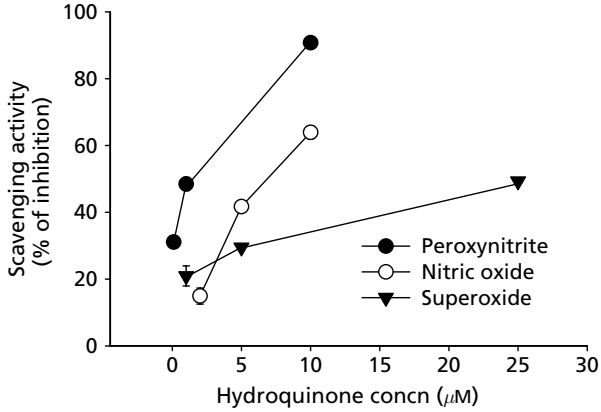

Figure 2 Scavenging effect of hydroquinone on toxic radical species. Peroxynitrite ($ONOO^-$), nitric oxide and superoxide scavenging activity of hydroquinone was measured by monitoring the oxidation of dihydrorhodamine 123, H_2DCFDA and DAF-2 by authentic $ONOO^-$ (10 μM), kidney homogenate (1 mg protein mL^{-1}) and sodium nitroprusside (2 mM). Data are mean ± s.e. of three independent measurements.

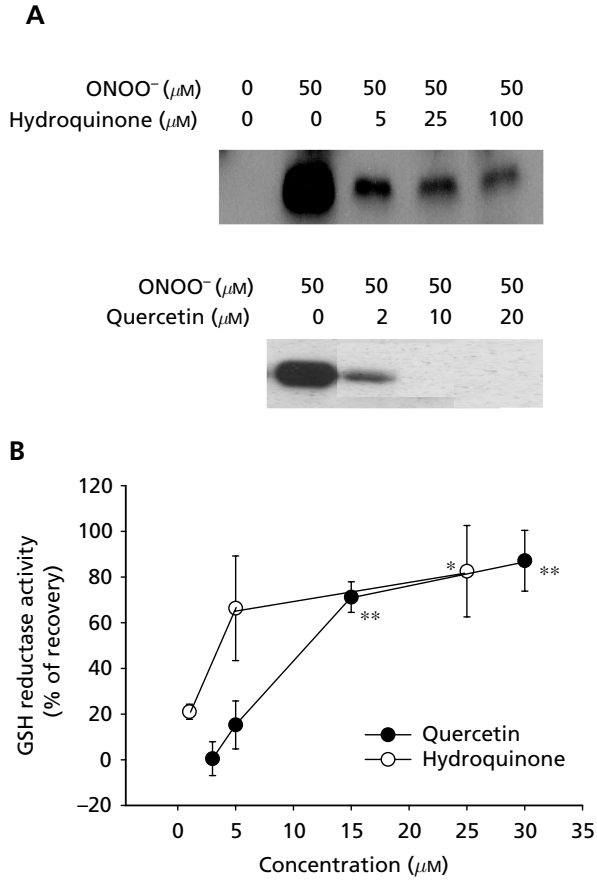

Figure 3 Protective effects of hydroquinone on nitrotyrosine formation (A) and damage of glutathione (GSH) reductase (B) induced by peroxynitrite ($ONOO^-$) exposure. A. The nitrotyrosine level of GSH reductase induced by $ONOO^-$ in the absence or presence of hydroquinone or quercetin was analysed by Western blot with antibody to nitrotyrosine. The data are representative of three different experiments with similar results. B. The alteration of GSH reductase activity induced by $ONOO^-$ was determined by a NADPH reduction method. The protective effect of hydroquinone and quercetin against $ONOO^-$ (100 μM) mediated inhibition of GSH reductase activity was evaluated under the same conditions. Data are mean ± s.e. of three independent measurements. *$P < 0.05$ and **$P < 0.01$, significantly different compared with normal or control.

formation (determined by antibody to nitrotyrosine) at a concentration of 50 μM (Figure 3A; Kim, A. R. et al 2002), and directly damaged the enzymatic activity of GSH reductase (Figure 3B; Kim, A. R. et al 2002). As shown in Figure 3A, hydroquinone (from 5 μM) prevented nitrotyrosine formation mediated by $ONOO^-$. At 100 μM hydroquinone, a faint nitrotyrosine band was still seen but it was inhibited by up to 90% when determined quantitatively. Quercetin (2 μM) strongly suppressed nitrotyrosine formation (Figure 3A). Hydroquinone also prevented $ONOO^-$ (100 μM) induced GSH reductase damage in a dose-dependent manner (Figure 3B). Thus, hydroquinone (25 μM) recovered enzyme activity decreased by $ONOO^-$ treatment by up to 80%. Furthermore, at 5 μM, hydroquinone was more effective than quercetin at recovering enzyme activity (up to 60%).

Effect of hydroquinone on NOx production from LPS-activated RAW264.7 cells

NO is one of the components required to generate cellular $ONOO^-$ (Huie & Padmaja 1993). Therefore, we investigated if hydroquinone is capable of modulating NO production from activated macrophages by determining the NOx level. The cytotoxicity of hydroquinone and quercetin to murine macrophage RAW264.7 cells was first examined. Figure 4A shows that hydroquinone did not affect the cell viability of RAW264.7 cells even at concentrations up to 50 μM, whereas quercetin did. Therefore, we evaluated the inhibitory effect of hydroquinone on NO production. Figure 4B shows that hydroquinone strongly suppressed the production of NOx in LPS-activated RAW264.7 cells, with an IC50 of 5.6 μM. Quercetin also diminished NOx production, with an IC50 of 13.9 μM. To confirm the inhibitory effect of hydroquinone and quercetin on NO production, iNOS expression was examined from LPS-stimulated macrophages. As shown in Figure 4C, LPS greatly induced iNOS expression and hydroquinone suppressed its expression in a dose-dependent manner, as did quercetin, at 5 and 25 μM. Indeed, a non-cytotoxic concentration (5 μM) of hydroquinone inhibited iNOS expression up to 50% when determined quantitatively. To determine if the hydroquinone inhibition is evident at the transcriptional level, the amount of iNOS mRNA expression was examined by semi-quantitative reverse transcription polymerase chain reaction. As shown in Figure 4D, hydroquinone (at 50 μM) strongly suppressed the mRNA expression, suggesting that this compound may regulate the transcription pathway for iNOS synthesis.

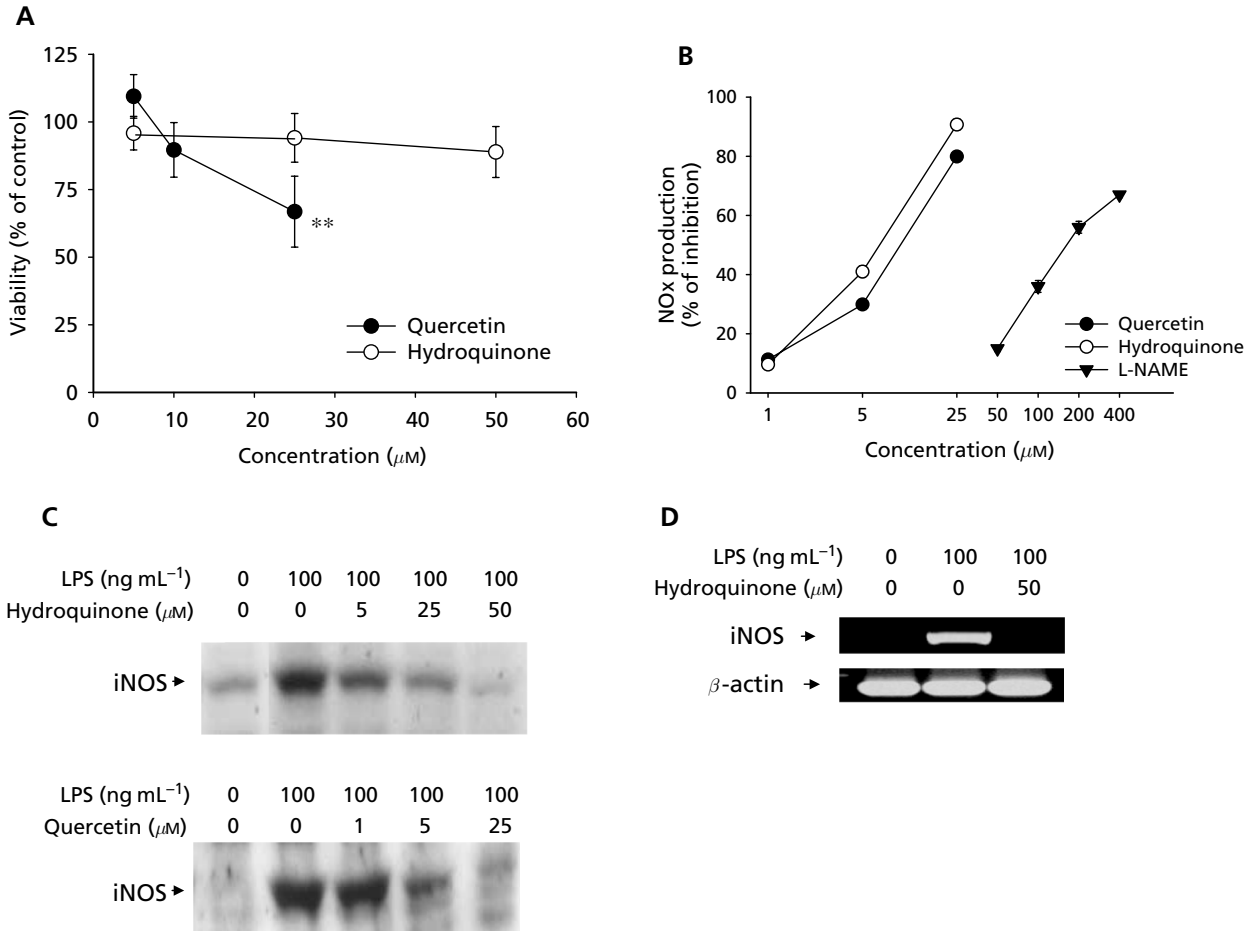

Figure 4 Effect of hydroquinone on the cell viability of RAW264.7 cells (A), NOx (nitrate and nitrite) production (B), inducible nitric oxide synthase (iNOS) expression (C) and iNOS mRNA level (D) in lipopolysaccharide (LPS)-induced RAW264.7 cells. A. The cell viability of hydroquinone- or quercetin-treated RAW264.7 cells in the absence of LPS (100 ng mL^{-1}) was determined by conventional MTT assay. Data are mean ± s.e. of three independent measurements. $^{**}P < 0.01$, significantly different compared with normal. B. RAW264.7 cells (0.5×10^6) were pre-treated with different concentrations of hydroquinone, quercetin or L-NAME for 3 h and further incubated in the presence of absence of LPS (100 ng mL^{-1}) for 24 h. The NOx level was determined by a NO analysing system. Data are mean ± s.e. of three independent measurements. C. RAW264.7 cells (5×10^6) were treated with various concentrations of hydroquinone or quercetin in the presence or absence of LPS (100 ng mL^{-1}) for 24 h. Total cellular protein was separated on sodium dodecyl sulfate-polyacrylamide gels and blotted with antibody to iNOS. D. RAW264.7 cells (5×10^6) were treated with hydroquinone (50 μM) in the presence or absence of LPS (100 ng mL^{-1}) for 24 h. The mRNA level of iNOS was analysed by reverse transcription-polymerase chain reaction. The data are representative of three different experiments with similar results.

Effect of hydroquinone on the activation of NF-κB and ERK 1/2

NO production is critically mediated by LPS-induced intracellular signalling, such as activation of transcription factors (NF-κB and AP-1) and various kinases (protein tyrosine kinases and mitogen activated protein kinases), to induce iNOS expression (Guha & Mackman 2001). Therefore, the molecular mechanism of hydroquinone inhibition of LPS-induced iNOS expression and NO production was investigated in terms of these signalling pathways. Figure 5 shows that DNA-binding activity of NF-κB, greatly increased at 30 and 60 min, and ERK activation (judged by phosphorylation) upon LPS treatment were strongly blocked by hydroquinone treatment, suggesting that hydroquinone may interrupt LPS-induced signalling pathways for iNOS expression. In contrast, quercetin inhibited ERK activation but not NF-κB binding activity (Figure 5).

Discussion

$ONOO^-$ is a potent oxidant generated by the reaction between $\bullet O_2^-$ and •NO, especially in disease states such as chronic or acute inflammation (Deliconstantinos et al 1996; Chung et al 2001). The ability of $ONOO^-$ to oxidize or nitrate tyrosine residues from a variety of proteins contributes to $ONOO^-$ mediated cellular dysfunction (Stamler 1994; Kooy et al 1997; Mondoro et al 1997). Because of the lack of endogenous enzymes responsible

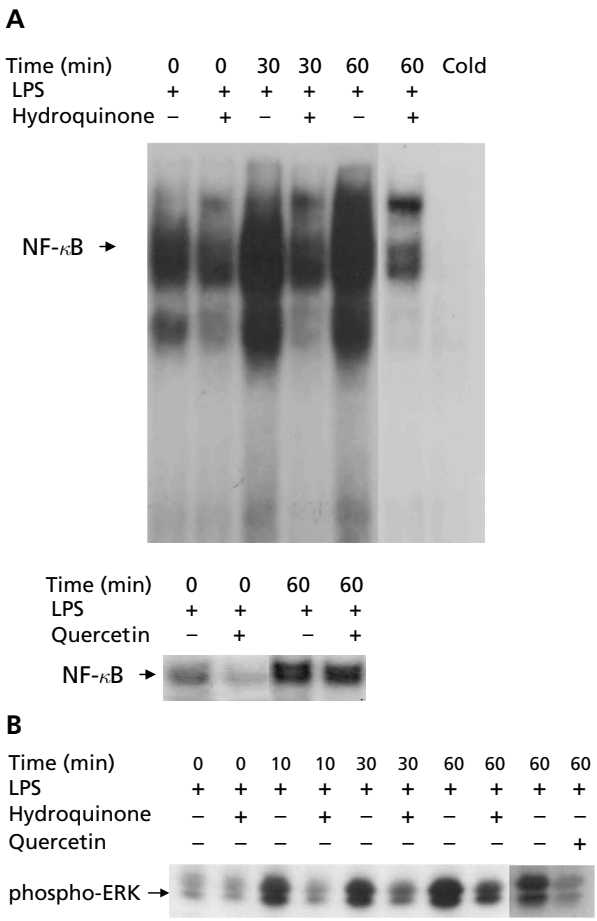

Figure 5 Effects of hydroquinone on nuclear factor-κB (NF-κB) activation (A) and extracellular signal-related kinases 1 and 2 (ERK1/2) phosphorylation (B). A. The cells were treated with hydroquinone (50 μM) or quercetin (25 μM) in the presence or absence of lipopolysaccharide (LPS) (100 ng mL^{-1}) for the indicated times. Nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay. B. Total lysates prepared from the hydroquinone (50 μM) or quercetin (25 μM) treated cells stimulated by LPS (100 ng mL^{-1}) were separated on sodium dodecyl sulfate-polyacrylamide gels and immunoblotted with antibodies to phospho-ERK1/2. The data are representative of three different experiments with similar results.

for $ONOO^-$ inactivation, the development of specific $ONOO^-$ scavengers is an important therapeutic strategy. In the course of screening anti-$ONOO^-$ agents, we found that hydroquinone strongly neutralized $ONOO^-$ reactivity (Kim, A. R. et al 2002). In the present study, we investigated the mechanism of the scavenging activity of hydroquinone, its protective effect of GSH reductase, and effect on NO production, an upstream pathway for cellular $ONOO^-$ generation, and compared hydroquinone with the potent antioxidant quercetin and other standard compounds.

The results suggest that hydroquinone is a selective $ONOO^-$ scavenger. It scavenged the reactivity of $ONOO^-$ by 130-fold or 8-fold greater than the neutralizing activity against $\bullet O_2^-$ and •NO (Figure 2; Table 1). The selectivity of hydroquinone was more striking (50-fold) than the standard compound quercetin. Since the phenolic antioxidant compound itself is known to chemically modify via nitration or electron donation when it reacts with $ONOO^-$ (Pannala et al 1997, 1998), additional experiments are necessary to clarify its chemical conversion. We next examined if hydroquinone is capable of protecting the cellular redox system. To do this, GSH reductase, which enzymatically restores the level of GSH from GSSG, was selected because it is the main antioxidant defence system (Francescutti et al 1996). $ONOO^-$ treatment generated both nitrotyrosine formation and decreased the activity of GSH reductase (Figure 3; Kim, A. R. et al 2002). Hydroquinone and quercetin both prevented nitrotyrosine formation. However, unlike quercetin treatment, a low level of nitrotyrosine was still evident after hydroquinone treatment (Figure 3B). This may be due to a hydroquinone-insensitive nitrotyrosine reaction or it is possible that the antibody may recognize nitrohydroquinone, adducted with GSH reductase, which displays structural similarity to nitrotyrosine (Figure 1). Nonetheless, in terms of enzyme activity, the protective effect of hydroquinone was more effective than quercetin. Thus, at low concentrations (5 μM), hydroquinone, but not quercetin, recovered $ONOO^-$ induced damage of the enzyme by up to 60% (Figure 3B). The reason why hydroquinone was more effective than quercetin at lower concentrations is not clearly understood but it may be due to different mechanisms of scavenging action. Taken together, the results suggest that hydroquinone can prevent $ONOO^-$ induced damage of GSH reductase (and other important proteins) and maintain an appropriate GSH level. Whether these in-vitro effects also occur in-vivo needs to be evaluated.

Cellular $ONOO^-$ is generated by chemical reaction between $\bullet O_2^-$ and •NO, a strong pro-inflammatory mediator (Huie & Padmaja 1993; Kroncke et al 1998). To determine if hydroquinone can modulate cellular $ONOO^-$ formation, we tested its inhibitory effect on NO production in LPS-activated macrophage RAW264.7 cells. Thus, the cytotoxicity of hydroquinone as well as quercetin was first explored. Figure 4A shows that hydroquinone did not have any cytotoxic effect on cell viability at concentrations up to 50 μM, whereas quercetin significantly affected cell viability at 25 μM. Furthermore, cell viability was not affected even when hydroquinone was co-treated with LPS, suggesting that the hydroquinone metabolite, nitrohydroquinone, potentially generated by reactive oxygen species (ROS) and reactive nitrogen species, had no harmful effect on the cells (data not shown). At non-cytotoxic concentrations, hydroquinone suppressed NOx production in a dose-dependent manner (Figure 4B). The inhibition of NO production by hydroquinone seems to be exerted at both transcriptional and translational levels, because hydroquinone suppressed both mRNA and protein levels of iNOS (Figure 4C, D), as also shown in the case of quercetin and some naturally occurring antioxidants (Lee et al 2000). This indicates that hydroquinone may interrupt the activation of the upstream pathway for iNOS expression such as mitogen activated protein kinases and transcription factors. Indeed, hydroquinone inhibited the activation of NF-κB and ERK phosphorylation, a hallmark of ERK activation (Figure 5). Although

the inhibitory mode of action of hydroquinone on NO production was not clearly demonstrated in this study, the inhibition of LPS-induced ROS generation or a signalling enzyme responsible for NO production could be an explanation. This is because NF-κB and ERK are activated by LPS-induced ROS generation and there are a series of signalling proteins that activate these two proteins (Chung et al 2002; Rahman 2003). However, as it displayed lower scavenging activity against $\bullet O_2^-$ reactivity than the inhibitory effect of NO production (Table 1), a better way of understanding the mechanism of action would be to look for the target enzyme(s), which may be different from that of quercetin according to the patterns of cytotoxicity (Figure 4A) and NF-κB inhibition (Figure 5A).

In conclusion, hydroquinone, which is present in vegetables, fruits, coffee, tea, beer and wine, may be regarded as a potent regulator of $ONOO^-$ mediated pathological diseases by directly scavenging and indirectly blocking cellular $ONOO^-$ production pathways such as NO synthesis. The main mechanism of scavenging activity and NO inhibition by hydroquinone may be due to nitration of the compound itself and interruption of the intracellular signalling pathway. Our results also suggest that hydroquinone may be a potent $ONOO^-$ scavenger and this needs to be evaluated in in-vivo disease models.

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