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Gene expression in rat lungs during early response to paraquat-induced oxidative stress

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Abstract. Paraquat (PQ) is a well-known pneumotoxicant and provides an established model of oxidative stress. This study focused on the transcriptional response to PQ-driven oxidative stress in rat lungs during an early phase post-injection. Rats were sacrificed at 3 h and 24 h after PQ injection (i.p., 20 mg/kg b.w.), and at 3 h after a second injection (i.p., 20 mg/kg b.w.). The left lungs were rapidly excised and used immediately for RNA preparation. The lung tissues did not show any pathological damage microscopically. Differential expression of RNAs in the lung at 3 h was investigated using a DNA array system. Fifteen genes showed a >1.7-fold change in expression level, which was confirmed by real-time PCR. Five genes related to oxidative stress, TRX, HO-1, GST-Yc, NQO-1, and RL/IF-1, and one gene, CLK3, whose function is unknown, showed a significant increase in their expression due to PQ injection. Two genes, HO-1 and NQO-1, that showed 3- and 2-fold increases at 3-h post-injection, were localized by immunohistochemistry. HO-1 was expressed in the bronchial epithelial cells, some type II cells and macrophages of control lungs, and the cells, especially the bronchial epithelial cells, were strongly stained 3 h following PQ treatment. Immunohistochemical analysis of NQO-1 also showed an increase in positive staining in the bronchial epithelial cells of PQ-treated lung sections. The expression of CYP2C6, 2C7, and 2C12, which are specific to or dominant in female liver, decreased markedly, while the male-specific CYP2C13 and 2C11 showed an increase or no effect. Further investigation is needed to clarify the role of these CYP2C family genes on the early phase of PQ toxicity. These results indicate that the acclimation to oxidative stress is already a highly complex process at the onset of PQ-induced damage and that the genes described herein may prove to be major contributors to the subsequent pulmonary fibrosis.

Introduction

Pulmonary fibrosis is a severe chronic disease with various causes and a poor prognosis. However, the etiology is obscure. In the last several decades, the main responsible agent has been thought to be reactive oxygen species (ROS) production (1,2). Extensive evidence indicates that oxidative stress regulates gene expression by modulating transcription factors such as nuclear transcription factor κB (NF-κB) and activator protein-1 (AP-1) (3,4). In addition, recent evidence suggests that the interactions between oxidation-sensitive regulatory pathways may determine cell differentiation (5), proliferation (6), cytokine production (7), and apoptosis (8). Many xenobiotics that increase the production of ROS, including paraquat (9), butyrated hydroxytoluene (10), and bleomycin (11), are also capable of producing pulmonary fibrosis. Among them, paraquat (PQ: 1,1'-dimethyl-4,4'-bipyridinium) is a well-known pneumotoxicant. Its toxic effect has been thought to be mediated by ROS produced by the enzymatic one-electron reduction of PQ, followed by a one-electron transfer to dioxygen, generating superoxide anion (12). Waddell and Marlowe (13) reported that radioactive PQ intravenously injected into mice was already localized in lungs at 3 h after administration. These results suggest that changes in RNA expression at 3-h post-injection play a crucial role in the onset of pulmonary fibrosis.

There are several methods to identify differentially expressed genes. In previous studies (14,15), we investigated the difference between gene expression in control lungs and those exposed to PQ for 3 h using differential display RT-PCR and showed that expression of the genes for TAFIIB, Lpin2, latrophilin, and plasma phospholipid transfer protein increased at 3-h post-injection. On the other hand, currently available DNA array technology allows characterization of the mRNA levels of more genes simultaneously, thus providing

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Abbreviations: PQ, paraquat, 1,1'-dimethyl-4,4'-bipyridinium; ROS, reactive oxygen species; NF-κB, nuclear transcription factor κB; AP-1, activator protein-1; TRX, thioredoxin; HO-1, heme oxygenase-1; NQO-1, NAD(P)H-oxidoreductase-1; GST-Yc, glutathione S-transferase Yc subunit; RL/IF-1, regenerating liver inhibitory factor-1; CLK3, CDC-like kinase 3; CYP, cytochrome P450; DHCR, 7-dehydrocholesterol reductase

Key words: paraquat, oxidative stress, gene expression, DNA array, real-time PCR

Table I. Primer sets used in real-time PCR.

Gene	Primer sets		Size of amplicon
CLK3 (CDC-like kinase 3)	F: AGCTGACCCACACAGACTTG	R: TTCTCCTCGCAGCTCTTGTG	94
RL/IF-1	F: TGGCCAGTGTAGCAGTCTTG	R: GCCCAGGTAGCCATGGATAG	122
Platelet-derived growth factor B subunit	F: TCTGGCCTGCAAGTGTGA	R: GGTGTCTTGGCTCGATGCT	87
Thioredoxin (TRX)	F: CATGCCGACCTTCCAGTTC	R: GGCTTCGAGCTTTTCCTTGT	79
NAD(P)H-dehydrogenase	F: GCCTTTGTTCCACAAGGATAGG	R: GCCCCTAATCTGACCTCGTT	106
Heme oxigenase-1 (HO-1)	F: ATCGACAACCCCACCAAGT	R: TTCTGTCACCCTGTGCTTGA	90
dC-stretch BP	F: AACCTTCCCTAACACCGAAA	R: ATGCGCAATTCAACCATCTC	117
Insulin-like growth factor 2 receptor	F: ATTAGAGGGACGAGCGAAAC	R: ACCTCACACCCACGATCT	177
Glutathione S-transferase Yc subunit	F: CAGGGCTGATGTTTACCTAGT	R: GAGGTTGCTGACTCTGGTT	112
CYP4502F4	F: AGCAAGGACTATGGGTCAGT	R: CTCCCCTTTGTCCACAAGAG	105
7-Dehydrocholesterol reductase	F: GCCCTTTCATGTCTGTTGGA	R: CATTGCAGGTGGGAGTGAAG	153
Protein inhibitor of nNOS	F: CACACGAGACCAAACACTTC	R: GCAAGGCTGAAGATTCAGTC	164
CYPPB1 (CYP2C6)	F: GTGCTGCATGACAGCAAAG	R: CGTTTTCCTGCTGAGAAAGG	119
Male germ cell-associated kinase	F: GGACAGACTGGGTGGCTAA	R: TGTGCGTCCTGTGGAACTC	142
CYP/phenobarbital-inducible	F: CAGGAAAGCGCATTTGTCTTG	R: AGAGAAGTTCTGGAGGATGGT	83
CYP2C7	F: GAGGAAGCACAGTGCCTAGT	R: TTGCATGGAGCACAGTTCAG	89
CYP2C11	F: CTCTGGAGACAGAGCTTTGG	R: TTGATTGGCCGATGTCCTTC	106
CYP2C12	F: ACAAGAACATCTGGCAATCC	R: GGGTACTTCATCAAGAGCAA	102
CYP2C13	F: AGGAAAACGGATGTGTTTGG	R: TTGATGTCCTTTGGGTCAAC	114

a useful tool to identify broad-spectrum changes in gene expression in tissues in response to a given stimulus (16). Satomi *et al* used cDNA arrays to analyze the subacute phase of PQ toxicity (9), but this approach has not been applied to the early stages of PQ exposure that show no functional or pathological abnormalities.

In this study, we injected PQ into rats intraperitoneally (i.p.) at a dose of 20 mg/kg b.w., and extracted RNA samples from lungs after 3 h and 24 h. Some rats received another injection at 48 h after the first, and RNA in lungs was extracted at 3 h after the second injection. First, we compared RNA expression in controls and 3-h post-injection using the array technology to identify differentially expressed genes in rat lungs. Next, the results obtained were verified by real-time PCR. In addition, the two genes that showed the biggest change at 3-h post-injection, heme oxygenase-1 (HO-1) and NAD(P)H-oxidoreductase (NQO-1), were localized by immunostaining.

Materials and methods

Materials. Six-week-old Sprague-Dawley female rats (Clea, Japan), initially weighing 120-150 g, were housed under constant temperature and humidity conditions and a 12:12-h day/night cycle with food and water *ad libitum*. The rats were randomly divided into four groups. Group 1 consisted of animals that received saline solution only and were sacrificed at 3 h after the injection (control group). Some animals received a single i.p. injection of 20 mg/kg of PQ dissolved in saline and were sacrificed at 3 h (group 2) or 24 h (group 3) after the administration. Another group of rats received two intraperitoneal injections of 20 mg/kg of PQ solution. The animals receiving a second injection 48 h after the first injection were sacrificed at 3 h after the second injection (group 4). The animals were decapitated, and left lungs were rapidly excised and used immediately for RNA preparation under ice-cold conditions. These experiments were approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the 'Guide for the Care and Use of Laboratory Animals' of Kawasaki Medical School.

Preparation of total RNA. Total RNA was prepared with Isogen (Nippon Gene, Tokyo) according to the manufacturer's instructions and then treated with RNase-free DNase I to remove any contaminating chromosomal DNA. The RNA was finally resuspended in RNase-free water and stored at -80˚C. The quality control was demonstrated by intact ribosomal RNA (28S and 18S bands) utilizing 1% denaturing agarose gel electrophoresis.

cDNA array. The RNA samples of the control group and those at 3 h after PQ injection were compared using the BD Atlas Nylon Array system (Clontech). For array hybridization, we used nylon filters with 1176 spotted cDNA (Rat Toxicology 1.2 Array, Clontech). Briefly, polyadenylated RNA was purified from each total RNA test sample prepared, reverse-transcribed, and labeled with [α-^{32}P]-dCTP in a single step using biotinylated oligo(dT) and streptoavidin-coated magnetic beads. The unincorporated [α-^{32}P]-labeled nucleotides were removed by Nucleo-Spin extraction columns (Clontech). Hybridization was carried out overnight at 68˚C according to the manufacturer's recommendation. After washing, the es were exposed to X-ray film (XAR5, Kodak) at nalysis was performed using Atlasimage 2.7 software (Clontech). Array exposures were normalized by equalizing the intensity of the signals from a set of housekeeping genes provided on the arrays.

Quantitative real-time PCR. First, we prepared cDNA using BD Advantage RT-for-PCR kit (Clontech). Briefly, total RNA (1 µg) was reverse transcribed in a 20 µl reaction mixture containing 0.5 µg of oligo(dT) as a primer and 200 U of Maloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions. Next, the cDNA was amplified with the synthetic gene-specific primer sets described in Table I. We designed the gene-specific primer sets based on sequences deposited in the NCBI GenBank database. PCR cycling conditions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 20 sec, 60˚C for 30 sec, and 72˚C for 1 min. After one convincing band showing a corresponding size was demonstrated by agarose gel electrophoresis, each amplicon was purified using phenol-chloroform and quantified by determining the optical density at 260 nm. Each amplicon was diluted serially and used as a standard for each real-time PCR. Quantitative real-time PCR was conducted by amplifying the cDNA prepared using SYBR Green Master Mix (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For amplification, 1 µl of the cDNA sample was used as a template in a PCR reaction of 25 µl including 0.2 µM each of the specific primers and 12.5 µl of 2X SYBR Green Master Mix (SYBR Green I dye, AmpliTaq Gold DNA polymerase, dNTPs, passive reference 1 and optimized buffer). Cycling conditions were 95˚C for 10 min, followed by 45 cycles of 95˚C for 20 sec, and 60˚C for 30 sec. Amplification plots were inspected to assign values for the cycle threshold (Ct), a value at which the SYBR Green fluorescence of each sample is within the exponential phase of PCR amplification. In parallel with each sample, standard curves were generated to correlate Ct values using serial dilutions of the target gene amplicon described above. The quality of the standard curve was judged from the slope and the correlation coefficient. Normalization to ß-actin provided a control for relative quantitative measurement of the transcript abundance.

Immunohistochemical staining. Lung specimens were fixed in 10% neutral formalin, embedded in paraffin, sliced at 5 µm thickness, and subjected to immunohistochemical staining using the ABC method. After deparaffinization, sections were treated with microwaves in 10 mM citrate buffer, pH 6.0, for 5 min to retrieve antigens. The sections were pretreated with hydrogen peroxide to prevent endogenous peroxidase reactions, followed with incubation with a blocking reagent (5% non-fat dry milk in PBST: PBS with 0.1% Tween-20) to minimize non-specific staining. After these procedures, the sections were incubated for at least 1 h at room temperature (r.t.) with the primary antibody diluted with a blocking reagent: anti-rat (recombinant protein) HO-1 antibody (rabbit) (SPA-895, Stressgen) diluted 200-fold or anti-human (recombinant protein) NQO-1 antibody (rabbit) (sc-25591, Santa Cruz) diluted 100-fold. After washes with PBST (3x5 min), the sections were treated for 30 min at r.t. with Envision+/HRP (Dako). After washes with PBST (3x5 min), the antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo) solution. For negative controls, we substituted a blocking reagent for each primary antibody. All slides were counterstained with hematoxylin, dehydrated, and mounted in Entellan.

Statistical analysis. Data are expressed as the mean ± SD. Differences in measured variables between experimental and control groups were assessed using Student's t-test. A p-value <0.05 was considered significant.

Results

DNA array and real-time PCR results. We employed the Clontech Atlas microarray system to quantify control (group 1) and 3 h-PQ-treated rat lung cDNAs (group 2). This array represents 1176 different rat genes. Approximately 86 out of 1176 arrayed genes (7.3%) were expressed in control lungs. However, the majority of the genes showed little change in expression levels, and there was no striking effect on RNA expression during the 3 h after PQ administration. We selected 15 genes that showed an increase or a decrease >1.7-fold for further analysis: 10 genes were up-regulated and 5 genes were down-regulated.

To confirm the data obtained by the cDNA array system, we employed real-time PCR technique for the 15 selected genes of interest (Table II). Although the extent of change detected by real-time PCR was generally low, the real-time PCR confirmed most of the data obtained using cDNA arrays, except for male germ cell-associated kinase (Mak) and cytochrome P450 b genes.

mRNA expression profile. To further characterize these 15 genes, we combined the real-time PCR data on group 3 and group 4 samples. The profiles of 8 genes showing a significant or marked change at 3-h post-injection are shown in Fig. 1. Six genes [encoding CLK3, RL/IF-1, thioredoxin (TRX), NADPH oxidoreductase (NQO-1), heme oxygenase-1 (HO-1), and glutathione S-transferase Yc subunit (GST-Yc)] were up-regulated, while 7-dehydrocholesterol reductase (DHCR) and cytochrome P450 PB1 (PB1-2 allele; CYP2C6) were down-regulated. The other 7 genes shown in Table II showed no significant change at these time points (data not shown). The expression levels of TRX, NQO-1, and GST-Yc were increased by PQ at 3-h post-injection. The former 2 genes showed a linear, time-dependent up-regulation until 24 h, while GST-Yc retained the same high level from 3- to 24-h post-injection. The level at 3 h after the second injection was the same as or higher than that at 3 h after the first injection. HO-1 expression showed an approximately 3-fold increase at 3 h after the first injection, but it had declined to the basal level by 24 h. The level was increased again by the second injection, but the level was lower than that observed after the first injection. A slight early stimulation of CLK3 and RL/IF-1 by PQ was observed. On the other hand, DHCR, which biosynthesizes cholesterol from its immediate precursor, 7-dehydrocholesterol, showed a decrease only at 3 h, whereas the PB1-2 allele (CYP2C6) exhibited a marked decrease due to PQ at all time points post-injection.

Table II. Summary of DNA microarray and real-time PCR.

No.	Gene	GenBank #	Ratio (array) Up	Ratio (array) Down	Real-time PCR Control	Real-time PCR PQ_20_3 h
1	CDC-like kinase 3 (CLK3)	X94351	3.0		4.60±0.27	5.50±2.08^{a}
2	Regenerating liver inhibitory factor-1 (RL/IF-1)	X63594	2.6		13.03±0.86	15.25±2.08^{b}
3	Platelet-derived growth factor B subunit (PDGFB)	Z14117	2.1		12.48±0.62	15.25±3.35
4	Thioredoxin (TRX)	X14878	2.0		3.58±0.30	4.33±0.49^{c}
5	NAD(P)H-dehydrogenase (DT-diaphorase)	J02608	2.0		21.52±3.33	38.85±14.36^{b}
6	Heme oxygenase-1 (HO-1)	J02722	2.0		17.44±4.48	54.70±31.49^{b}
7	dC-stretch binding protein (CSBP)	D17711	1.9		16.41±1.65	16.96±1.33
8	Insulin-like growth factor II receptor (IGF2R)	U59809	1.8		4.76±0.51	5.28±0.40
9	Glutathione S-transferase Yc subunit (GST-Yc)	K01932	1.8		9.83±1.03	16.66±5.49^{b}
10	Cytochrome P450 IIF4 (CYP2F4)	AF017393	1.7		11.45±3.44	15.21±3.53
11	7-Dehydrocholesterol reductase (DHCR)	AB016800		2.5	5.72±1.11	4.06±0.76^{b}
12	Protein inhibitor of nNOS	U66461		2.2	14.84±1.45	13.08±1.63
13	Cytochrome P450 PB1 (PB1-2 allele, CYP2C6)	K03501		2.0	13.15±13.42	4.87±6.99
14	Male germ cell-associated kinase (Mak)	M35862		1.9	13.76±2.81	20.48±9.80
15	Cytochrome P450 b (phenobarbital-inducible)	L00313		1.7	12.65±2.92	14.92±3.85

$^{a}p<0.005$, $^{b}p<0.05$, $^{c}p<0.001$.

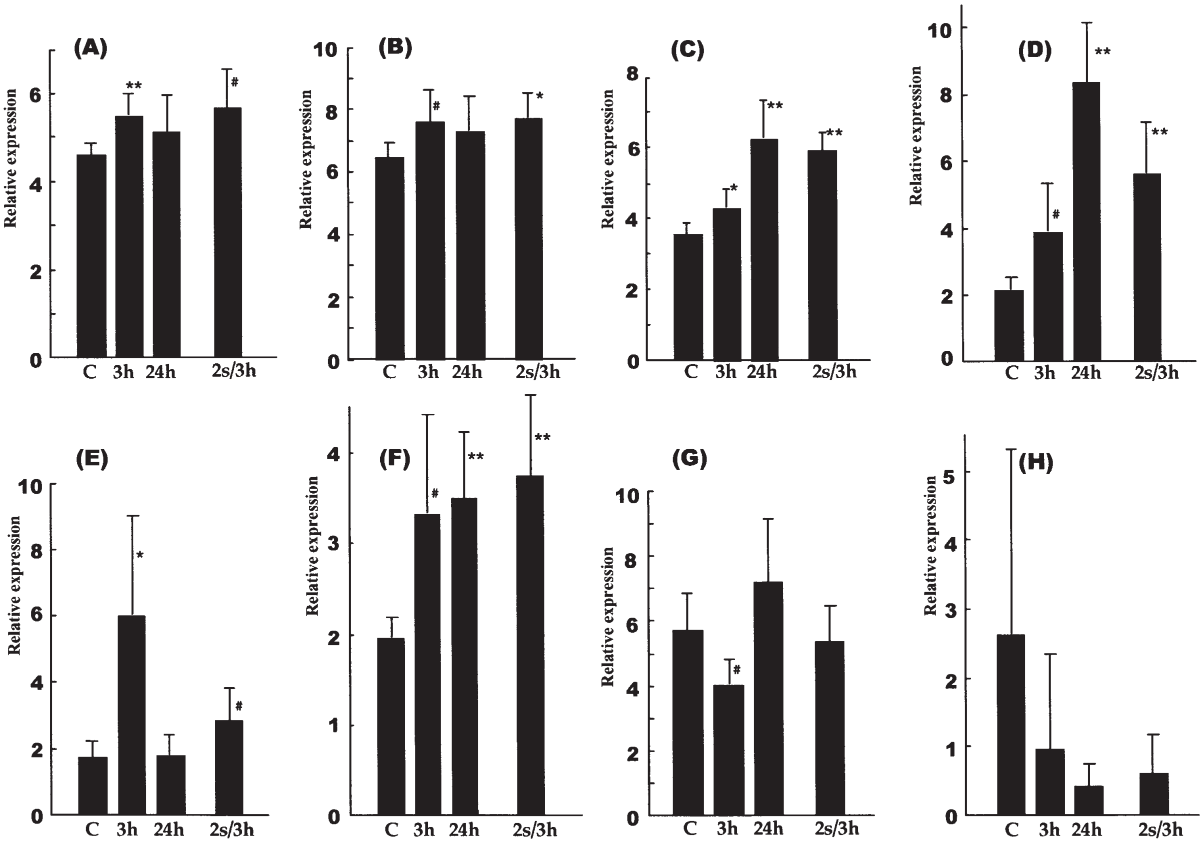

Figure 1. Quantitative real-time PCR analysis of expression of 8 genes following PQ injection. The expression levels were normalized to the value of ß-actin mRNA and represented as relative expressions. (A) CLK3, (B) RL/IF-1, (C) TRX, (D) NQO-1, (E) HO-1, (F) GST-Yc, (G) DHC, (H) CYP2C6. $^{*}p<0.05$, $^{**}p<0.005$ vs each normal value.

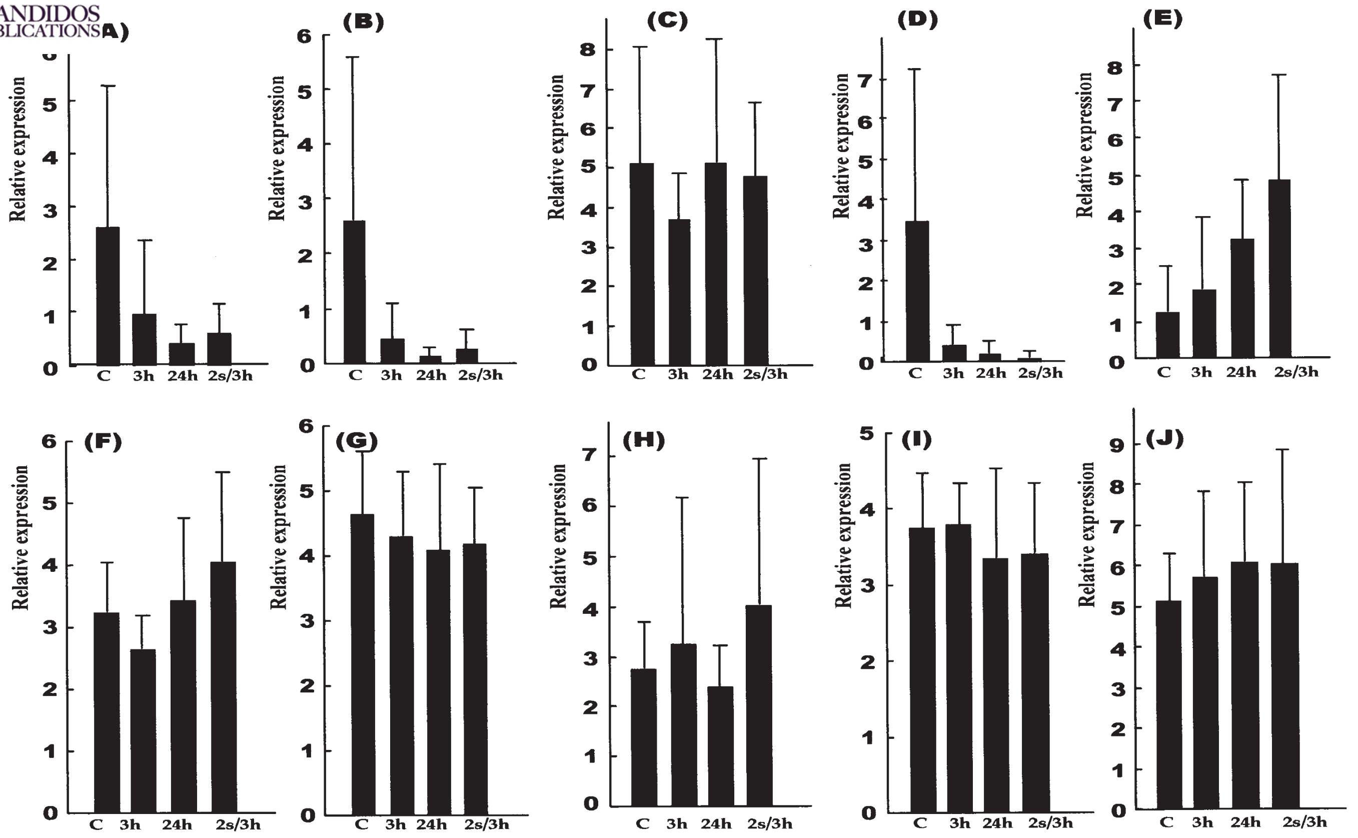

Figure 2. Quantitative real-time PCR analysis of CYP2C family members following PQ injection. The expression levels were normalized to the value of ß-actin mRNA and represented as relative expressions. (A) and (F) CYP2C6, (B) and (G) CYP2C7, (C) and (H) CYP2C11, (D) and (I) CYP2C12, (E) and (J) CYP2C13. (A-E) lung tissues, (F-J) liver tissues.

Table III. cDNA amounts estimated by the quantitative real-time PCR.

CYP	Lung	Liver
2C6^a	0.018±0.019	287.59±50.70
2C7^a	0.073±0.084	2052.61±256.42
2C11^b	0.094±0.063	0.65±0.19
2C12^a	0.484±0.528	8302.60±362.25
2C13^b	0.009±0.009	2.30±0.51

fg/µl. ^{a}Female specific or dominant; ^{b}male specific or dominant in liver.

Effect of PQ on CYP2C family. Because there was a marked decrease in CYP2C6 RNA expression, we investigated the expression of several CYP2C family members (CYP2C7, 2C11, 2C12, 2C13 as well as CYP2C6) using RNAs extracted from lung and liver. It is well-known that expression of these CYPs in liver is gender-dependent: CYP2C6, 2C7, and 2C12 are specific to or dominant in females, while CYP2C11 and 2C13 are male-specific. Our results obtained from liver were in agreement with the previous reports of gender-dependency (Table III). As shown in Fig. 2, PQ had no effect on CYP2C family members in the liver, whereas CYP2C6, 2C7, and 2C12, which are specific to or dominant in females in the liver, showed a marked decrease due to PQ injection at all time points examined. On the other hand, CYP2C13, which is specific to male liver, increased, and CYP2C11, also specific to males, showed no effect.

Immunohistochemistry of HO-1 and NQO-1. Two genes, HO-1 and NQO-1, that showed 3- and 2-fold increases, respectively, at 3-h post-injection were localized by immunostaining. Weak expression of HO-1 was observed in the bronchial epithelial cells, some type II cells, and macrophages of control rat lungs, suggesting basal expression (Fig. 3A). In contrast, in lungs treated with PQ, a positive staining was clearly visible in the same cells observed in control lungs; in particular, the bronchial epithelial cells displayed strong immunoreactivity for HO-1 (Fig. 3B). In NQO-1 immunohistochemical analysis, the staining was nearly negative in the epithelial cells in the airways of control lungs (Fig. 3C), and it was markedly enhanced in the bronchial epithelial cells in PQ-treated lungs (Fig. 3D). Immunohistochemical staining with a blocking reagent substituted for the primary antibody resulted in the absence of signals in all areas of the lung tissues (data not shown).

Discussion

We found 8 genes that showed a significant or marked change during the 3 h after injection of PQ using both a DNA array

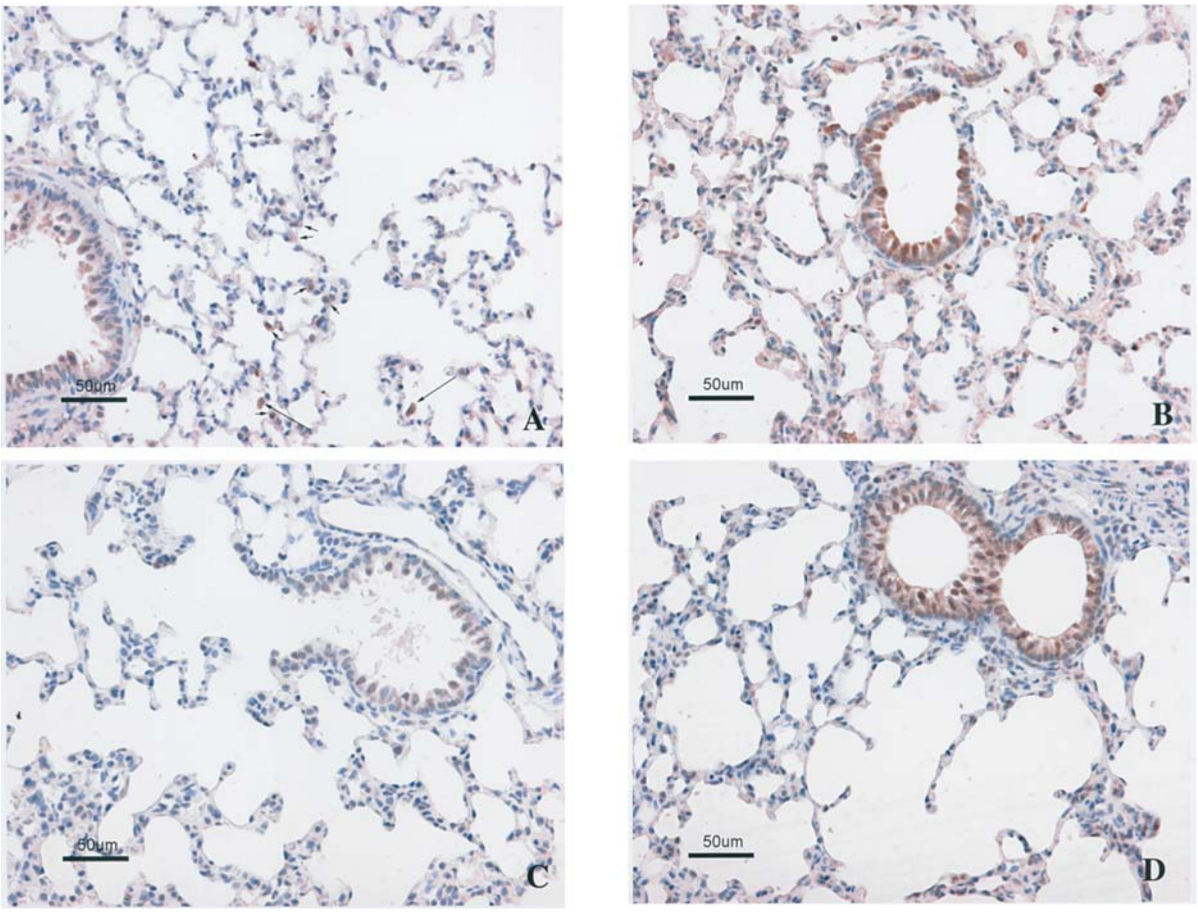

Figure 3. Immunohistochemistry of HO-1 (A and B) and NQO-1 (C and D). In normal lungs, HO-1 was weakly expressed in the bronchial epithelial cells, some type II cells (short arrows) and macrophages (long arrows) (A). In lungs treated with PQ for 3 h, HO-1 expression was more intense than it was in normal lungs (B). In normal lungs, NQO-1 was nearly negative in the epithelial cells in the airway (C), but it was markedly enhanced in PQ-treated lungs (D).

system and quantitative real-time PCR. They were CLK3, RL/IF-1, TRX, NQO-1, HO-1, GST-Yc, DHCR, and CYP2C6; the first 6 genes were up-regulated, while DHCR and CYP2C6 were down-regulated. Because the cDNAs that were spotted on the nylon filters employed did not correspond to the genes that were previously reported (14,15), we did not confirm our previous data concerning TAFIIB, Lpin2, latrophilin and plasma phospholipid transfer protein.

Under conditions of oxidative stress, such as PQ treatment, multiple antioxidant mechanisms become physiologically important. In this study, we found that 4 genes related to the antioxidant system, i.e., TRX, HO-1, GST-Yc, and NQO-1, were increased by PQ. The TRX system, like the GSH system (17), is one of the key systems of living cells to maintain their redox balance and is considered to reflect intracellular redox status (18). Up-regulation in response to oxidative stress has been reported (19). In addition, TRX has been shown to be involved in the regulation of various cellular responses, including gene expression, cell proliferation, and apoptosis (20). In our study, a significant time-dependent up-regulation due to PQ injection was observed at all points examined. Heme oxygenase is an enzyme that catalyzes the oxidation of heme to carbon monoxide, iron and biliverdin that is reduced to bilirubin (21). Among the three isoforms of heme oxygenase (HO-1, HO-2, and HO-3), HO-1 can be substantially induced and has been identified as the major 32-kDa heat shock protein HSP-32 (22). Like other stress proteins, HO-1 can be induced by a variety of stimuli, most of which are linked by their ability to provoke oxidative stress (23). As shown in Fig. 1, the expression of HO-1 RNA peaked at 3 h after PQ injection and then returned to the basal level by 24 h. Among the rat GST isoenzymes best characterized, the homodimer YcYc is one of the GSTs with the highest activity toward organic and fatty acid hydroperoxides (24). Thus, GST-Yc, a selenium-independent GST peroxidase, as well as GSHPx, might be an important component of the cellular antioxidant defense (25). The expression of GST-Yc increased significantly at all points examined, retaining a high level. The flavoprotein NAD(P)H-oxidoreductase, originally named DT-diaphorase, is an enzyme that uses NADH or NADPH to catalyze the two-electron reduction of quinone. This phase 2 enzyme has attracted attention for its protective roles against oxidative stress (26,27). Ali *et al* reported that i.p. injection of PQ into rats induced NAD(P)H-specific quinone reductase as a protective response (28). On the other hand, PQ generated superoxide anion on incubation with NADH and mitochondrial NAD(P)H-quinone oxidoreductase, resulting in the destruction of mitochondria and cell death (29). In this study, PQ stimulated the RNA expression of this enzyme, suggesting an increase in oxidative stress and the protective response due to PQ injection. The results obtained for TRX, HO-1, GST-Yc, and NQO-1 indicate that lung tissues exposed ke a strong stand against oxidative stress during the se post-injection. On the other hand, production of oxygen radicals leads to activation of oxygen-dependent transcription factors, such as AP-1 and NF-κB (30). It is well-known that these transcription factors play a central role in antioxidant response and inflammation through regulation of a large number of gene expressions. For example, the signaling mechanisms that activate the transcription of HO-1 have been investigated, one of them being NF-κB (31). Although the DNA binding activity of oxidized NF-κB is significantly diminished, the activity is restored by reducing enzymes such as TRX (32), whose expression level were increased in our experiment. In contrast, RL/IF-1 has been reported to have IκBß-like activity, which inhibits the DNA binding of NF-κB, c-Rel, and Rel B (33). In this study, the expression was significantly increased by PQ, suggesting that acclimation to oxidative stress is a highly complex process associated with broad adjustments of gene expression. A slight increase of CLK3 was observed 3-h post-injection, but its function in PQ toxicity is currently unknown.

Two genes showed a significant or marked decrease in RNA expression at 3 h after administration, DHCR and CYP2C6 (Table II). Many investigators have reported that over-generation of ROS stimulates CYP induction *in vitro* and *in vivo*. For example, ß-carotene supplementation was able to induce a number of CYP isoforms in all tissues (34). Other investigators reported that gender- and tissue-specific effects were observed in CYP induction or suppression by many xenobiotics (35). In our DNA array system, the expression of CYP2C6 decreased remarkably. Therefore, we examined the RNA expression of some members of the CYP2C family after PQ injection. As a result, the expression of CYP2C6, 2C7, and 2C12, which are specific to or dominant in females in the liver, decreased markedly, while the male-specific CYP2C13 and 2C11 showed an increase or no effect. The amounts of these CYP2C family members in lung were very small, and the individual differences were very large. Further investigation was needed to clarify the role of these CYP2C family members in the early phase of PQ toxicity.

To further evaluate the role of the antioxidant enzymes in PQ toxicity, we tried to localize two enzyme proteins, HO-1 and NQO-1, in lung tissues. We found basal expression of HO-1 in control sections, and the expression signals were increased by PQ injection, especially in the bronchial epithelial cells. The positive reaction of NQO-1 was also found in the bronchial epithelial cells in the airways. Other antioxidant proteins, TRX and its associated molecules, have been reported to be primarily expressed in the airways and in macrophages in lung tissues (36). These results suggest that the bronchial epithelial cells act strongly against oxidative stress due to PQ intake.

In conclusion, the administration of PQ affected the expression level of 8 genes as soon as 3-h post-injection. Of these 8 genes, 4 genes encoding antioxidant enzymes increased, while a transcription factor encoding IκBß-like activity also increased. Thus, a highly complex process associated with broad gene expression adjustment proceeded during the 3-h post-injection. In addition, some female-specific CYP2C family members decreased, a phenomenon that needs to be elucidated by future investigation.

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