Arch Toxicol (2002) 76: 530–537
DOI 10.1007/s00204-002-0379-x

MOLECULAR TOXICOLOGY

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Paraquat-induced gene expression in rat lung tissues using a differential display reverse transcription-polymerase chain reaction

Received: 13 May 2002 / Accepted: 10 June 2002 / Published online: 25 July 2002

Abstract Increased formation of reactive oxygen species is a cause of paraquat (PQ)-induced injury and also provides a link between the signaling pathways and transcriptional events that regulate the expression of a large number of genes. However, the molecular mechanisms involved in PQ-induced injury remain unclear. To investigate the changes in gene expression at the onset of PQ injury, we used the differential display-polymerase chain reaction (PCR) method. Rats were treated intraperitoneally with 20 mg/kg PQ, and after 3 h the lungs were immediately excised. Samples of mRNA from normal and treated rats were used to prepare radiolabeled cDNAs, which were electrophoresed. Then the transcription levels were compared. We isolated 26 fragments of cDNA that were potentially affected by PQ, and determined their nucleotide sequences. Six clones of interest were selected and analyzed further. The reverse transcript-PCR based on their sequence information confirmed the differential expression for five clones: four clones were up-regulated and one was down-regulated. We were particularly interested in two genes that had homology with the known gene: TATA box-binding protein-associated factor, RNA polymerase II, B, 150 kDa (TAFIIB), and a candidate gene for lipodystrophy, Lpin2. Both genes were significantly up-regulated within 3 h of PQ intake and the stimulation continued during our 24-h observation period. In addition, up-regulation of Lpin2 was observed in the lungs, but not in the liver and kidneys. In situ hybridization using lung sections showed that the expression of both genes was strongly visualized in Clara cells and in alveolar macrophages. These findings suggest a stimulation of transcription levels and changes in lipid metabolism in Clara cells and in macrophages in the lungs, which result in their playing a crucial role at the onset of PQ-driven pulmonary injury.

Keywords Paraquat · Gene expression · Differential display · Clara cell · Rats

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Introduction

The differential display-polymerase chain reaction (DD-PCR) allows visual comparison of transcription levels between two biological situations (Liang and Pardee 1992; Wang et al. 1996). The resultant cDNAs are separated on large polyacrylamide gels and visualized by autoradiography, providing a partial RNA population fingerprint. The differentially displayed cDNAs can then be eluted from the gel, re-amplified and cloned, so allowing them to be further characterized. Once a transcript of interest is identified following sequencing, the reverse transcript (RT)-PCR and/or Northern blotting can be used to confirm its expression in tissues. We can also obtain clear evidence for the localization of the transcript expression in specific cells within the tissue samples using specific riboprobes. Thus, the protocols can be used to identify mRNA transcripts that are expressed at different levels in the tissue samples.

The herbicide, paraquat (1,1'-dimethyl-4,4'-bipyridinium; PQ), is a well-known pneumotoxicant that may exert its toxic effect by elevating the intracellular levels of reactive oxygen species (ROS) (Tampo et al. 1999; Mitsumoto et al. 2001). The injury induced by PQ is characterized by initial acute damage, followed by

fibroblast proliferation and exaggerated collagen production. Extensive evidence indicates that ROS regulate gene expression by modulating a large number of transcription factors, including the nuclear transcription factor κB and the activator protein-1 (Marshall et al. 2000; Zhou et al. 2001). It has been increasingly recognized that cell differentiation, proliferation, cytokine production and apoptosis are determined by the interactions between oxidation-sensitive regulatory pathways (Napoli et al. 2000; Wang et al. 2001). However, the precise molecular events driven by PQ exposure are still unknown.

In this study, we attempted to identify by the DD-PCR method the gene transcripts involved at the onset of PQ-induced injury. The DD-PCR was performed with RNA samples from the lungs of rats extracted 3 h after 20 mg/kg PQ intake, at which time the animals showed no apparent abnormalities. A number of differentially expressed RNAs were detected, cloned and sequenced. PQ-driven changes in six clones of interest were confirmed using the RT-PCR: five clones showed the same effect as that obtained by the DD-PCR. We selected two clones that had homology to the known gene, and examined localization of the expression in lung tissues by in situ hybridization.

Materials and methods

Animals

Six-week-old Wistar rats weighing 120–150 g were used. The rats received a single intraperitoneal injection of 20 mg/kg PQ dissolved in 1.0 ml saline. Control animals received 1.0 ml saline solution only. The rats were decapitated and tissues were rapidly excised and immediately used for RNA preparation. Rat lungs for in situ hybridization were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in increasing concentrations of ethanol, transferred to xylene, and then embedded in paraffin. 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

Tissue samples (up to 200 mg) were homogenized in Isogen (Nippongene, Tokyo, Japan) using a Polytron homogenizer, and then mixed with chloroform. The clear upper phase obtained after centrifugation (12,000 rpm for 15 min) was transferred to another microtube, precipitated with isopropanol and recentrifuged for 10 min. The resultant RNA pellet was washed with 70% ethanol, air-dried and dissolved in water treated with diethylpyrocarbonate (DEPC). The total RNAs obtained were treated with RNase-free DNase I to remove any contaminating chromosomal DNA before use as a template for reverse transcription to cDNA. The RNA content, finally resuspended in DEPC-treated water, was determined spectrophotometrically at 260 nm on a Beckman DU650 spectrophotometer.

Differential display-polymerase chain reaction (DD-PCR)

The total RNA from each lung of the 3 h-exposed or non-treated rats was pooled, and an aliquot of the total RNA was transcribed to cDNA with reverse transcriptase using each anchor primer: $GT_{15}X$ (X = A, C, G). Each 20 μl reaction mixture contained 1.0 μg total RNA, 2.5 μM primer, 0.5 mM of each dNTP, 0.5 U RNase inhibitor and 200 U MMLV-RT in 1× first-strand buffer (Clontech, Palo Alto, Calif., USA). The mixture was incubated for 60 min at 42°C, followed by heating at 94°C for 5 min. The reactions were diluted with sterile distilled water to a final volume of 100 μl and stored at –20°C for further use in the PCR.

PCR was performed with each anchor primer used in the cDNA synthesis in combination with each of 26 arbitrary primers (10 mer). The arbitrary primers were obtained from a commercially available kit (Gene Taq FP Set A; Nippongene). Each 20 μl reaction mixture contained 1.5 μl of prepared cDNA solution, 0.5 μM anchor primer, 0.5 μM arbitrary primer, 0.2 mM of each dNTP, 1 mCi [α-^{33}P] dCTP (Amersham, Buckinghamshire, UK) and 2 U Taq DNA polymerase (Takara, Shiga, Japan) in 1× PCR buffer. Amplifications were performed for 35 cycles at 94°C for 25 s, 40°C for 2 min, 72°C for 1 min, and 72°C for 7 min. The cDNA fragments amplified by the PCR were separated on 7% non-denaturing polyacrylamide gels in Tris-borate buffer. The gels were blotted onto 3MM Whatman papers without fixing, dried, and exposed to autoradiography.

Recovery of bands and reamplification of cDNAs

The dried gel was aligned with the autoradiogram, and cDNA bands of interest were excised from the gel. The gel slices (along with the Whatman 3MM paper) were soaked in a 100 μl solution of 0.1% sodium dodecyl sulfate (SDS), 0.5 M ammonium acetate and 10 mM magnesium acetate. The cDNA extracted was treated with phenol/chloroform, followed by ethanol, and redissolved in 10 μl Tris-HCl/EDTA buffer. For reamplification, 2 μl of the cDNA extraction solution was used as a template in a PCR reaction of 20 μl including the same primer set as that employed in the DD-PCR. The PCR conditions were as described above, except that no radioisotope was used. Amplified products were analyzed by electrophoresis on 1% agarose gel.

Cloning and sequencing of the reamplified cDNAs

Amplified cDNA fragments were ligated to a pZero2 vector (Invitrogen, San Diego, Calif., USA) and then transformed to competent *E. coli* TOP10 cells (Invitrogen). After screening of positive colonies, plasmids were prepared and purified using Qiagen plasmid Mini kits (Qiagen, Tokyo, Japan). Sequencing analysis was performed using the ABI Prism Dye Terminator cycle sequencing ready reaction kit protocol with an automated DNA sequencer (Perkin Elmer ABI, Shelton, Conn., USA). Rapid amplification of cDNA end (RACE) PCR was performed to amplify the 5'-end of the candidate cDNAs according to the protocol of the Gibco BRL 5'-RACE system kit (Invitrogen). The sequences obtained were compared for homology with the sequences present in the current GenBank database through the National Center for Biotechnology Information (NCBI) with the BLAST program.

Semiquantitative RT-PCR

The reverse transcription (RT) reaction for the first-strand cDNA synthesis with total RNAs was carried out using the Advantage RT-for-PCR Kit (Clontech) according to the manufacturer's manual. The PCR was done with FastStart Taq DNA polymerase (Roche, Japan) and the gene-specific primers that were synthesized on the basis of the sequence information obtained. The first step of template denaturation and enzyme activation was 5 min at 95°C and the last extension step was 5 min at 72°C. The PCR cycle consisted of denaturation at 95°C for 30 s, annealing at each temperature for 30 s, and extension at 72°C for 30 s. The PCR was initially performed with different cycle numbers to find the optimal number for quantitative amplification of the target gene. Table 1 shows the primers and conditions for the PCR. Aliquots of the

Table 1. Primers and conditions for comparative RT-PCR

Clones	Primers		Annealing temperature (°C)	No. of cycles
B8C4	Sense	5'-GCATTTCTTCCAGCAAAGAAGTGC-3'	63	28
	Antisense	5'-CGTTGTTTCTTGCAACTAACTC-3'		
B14C2	Sense	5'-TCAGCCTTGGCAGCCAGAGAAG-3'	65	27
	Antisense	5'-GACTTCCAGCTGACTCTCTAGC-3'		
B15C2	Sense	5'-GGTAGATCAGCTCTGGAAGCTG-3'	56	30
	Antisense	5'-TAAGAACAAGCCCGAGCTCTGG-3'		
B20C2	Sense	5'-CCATCGTTATCGCTTAAAGGG-3'	67	39
	Antisense	5'-TGAGCTCACTCTCTCAGGATC-3'		
B22C7	Sense	5'-CACGAGTTTGCAGAGAACCCT-3'	67	31
	Antisense	5'-CCAGATTTCTTCGGCATCTTG-3'		
B31C2	Sense	5'-GGTCTACAAAGAGAAACAGC-3'	60	26
	Antisense	5'-GAAATTTGAAGTCTTTATTGAACC-3'		

PCR-amplified products were electrophoresed on 2% agarose gels, stained with ethidium bromide and then measured under UV illumination (Gel Print 1000i Plus; Genomic Solutions, Tokyo, Japan). The amounts of amplified products were determined by the densities of the corresponding bands using computer software (Basic Quantifier v. 1; Genomic Solutions). Normalization to β-actin provided a control for semi-quantitative measurement of transcript abundance.

Preparation of digoxigenin-labeled riboprobes

Plasmids containing the insert of interest were linearized with the restriction enzymes, and antisense and sense riboprobes were transcribed from the T7 or Sp6 RNA polymerase promoter. We used the digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Indianapolis, Ind., USA) according to the manufacturer's protocol. Briefly, each in vitro transcription reaction contained 1× transcription buffer, 1× DIG-RNA labeling mixture, 20 U T7 or Sp6 RNA polymerase, 20 U RNase inhibitor, 0.5 μg template and up to 10 μl DEPC-treated distilled water. This reaction mixture was incubated at 37°C for 2 h and then 1 μl RNase-free DNase I was added and incubation continued for a further 15 min. The RNA was precipitated overnight at –20°C in a solution containing sodium acetate, ethanol, and glycogen. The precipitated RNA, after washing in 70% ethanol, was resuspended in 15 μl DEPC-treated water containing 1 mM dithiothreitol and 100 U RNase inhibitor.

In situ hybridization

Tissue paraffin sections (5 μm) were de-waxed in xylene and rehydrated in a graded alcohol solution. Sections were then incubated at 37°C for 20 min in proteinase K (1 μg/ml prewarmed at 37°C), rinsed in phosphate-buffered saline/0.1% Tween 20 (PBT), fixed for 20 min in 4% paraformaldehyde, and rinsed in PBT. Sections were acetylated with 0.25% acetic anhydride in 100 mM triethanolamine (pH 8) for 15 min, followed by washing in PBT. Next, they were incubated with a prehybridization mixture containing 50% deionized formamide and 5× SSC (1× SSC: 150 mM sodium chloride, 15 mM sodium citrate) at 55°C for 2 h. Then they were incubated with a hybridization mixture containing 50% deionized formamide, 5× SSC, 50 μg/ml tRNA, 1% SDS, 5× Denhardt's solution, 50 μg/ml heparin and 1 ng riboprobe (sense or antisense) at 55°C overnight. Following hybridization, the unbound probe was removed by washing sequentially in 2× SSC containing 50% deionized formamide for 30 min at 55°C, 2× SSC for 10 min at 55°C, and 0.2× SSC twice for 20 min at 55°C. Sections were washed in 100 mM Tris-HCl buffer (pH 7.5) in 150 mM NaCl/0.1% Tween 20 (TBST), blocked in TBST containing 1% blocking reagent (Boehringer Mannheim) for 30 min at room temperature (RT) and incubated for 2 h at RT in alkaline phosphatase-conjugated sheep anti-DIG diluted (1:250) in a blocking buffer. After three washings in TBST for 5 min at RT, sections were incubated with a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt to visualize the tissues that hybridized with the riboprobe. Development was stopped by rinsing the sections thoroughly in PBT. Following all these steps, sections were counterstained with 0.1% nuclear fast red (Kernechtrot) for 2 min at RT to stain the nuclei.

Immunostaining of Clara cells

Immunostaining was performed on consecutive sections with polyclonal anti-urine protein 1 (UP1) (Dako, Glöstrup, Denmark) diluted (1:250) in a blocking buffer at RT for 2 h, followed by incubation with the LSAB2 kit (Dako) to detect Clara cells in terminal bronchioles. To observe the nuclei histologically, the sections were counterstained with hematoxylin.

Results

DD-PCR and sequencing analysis

To detect mRNAs whose expression is up- or down-regulated by PQ exposure, the DD-PCR was used to compare each RNA level in the lungs from the treated and untreated rats, 3 h after administration of 20 mg/kg PQ. Twenty-six differentially displayed bands were isolated from the gels and cloned to enable further analysis, including sequencing. We selected six clones of interest (designated as B8C4, B14C2, B15C2, B20C2, B22C7 and B31C2); five clones were up-regulated and one was down-regulated. Additional 5'-end cDNA sequences of these six clones were obtained using the RACE PCR. Next, the sequencing results obtained were compared with the database held at NCBI. Table 2 summarizes these results. The B14C2 clone, over 83% of its length (410 bp), had 82% homology with the mRNA for human KIAA1262 protein (AB033088). The B15C2 clone, over almost its entire length (361 bp), showed 89% homology with the mRNA for human TATA box-binding protein (TBP)-associated factor, RNA polymerase II, B, 150 kDa (TAFIIB: NM 003184). The B22C7 clone, over its entire length (358 bp), showed 93% homology with the mRNA for mouse lipin 2 (Lpin2: NM 022882), which is a candidate gene for lipodystrophy and is required for normal adipose tissue. The other clones, B8C4, B20C2 and B31C2, were unknown ones. We

Table 2. Summary of paraquat-regulated clones in rat lungs by differential display-polymerase chain reaction (DD-PCR)

Clones	Result by DD-PCR	Length of fragments (bp)	Percent homology (accession number)
B8C4	Up	514	Unknown
B14C2	Up	495	82% Human KIAA1262 (AB033088)
B15C2	Up	363	89% TBP-associated factor (NM 003184)
B20C2	Down	496	Unknown
B22C7	Up	358	93% Lpin2, mRNA (NM 022882)
B31C2	Up	486	Unknown

submitted the sequence of the B15C2 clone as a partial codon of the rattus TAFIIB to the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB072351.

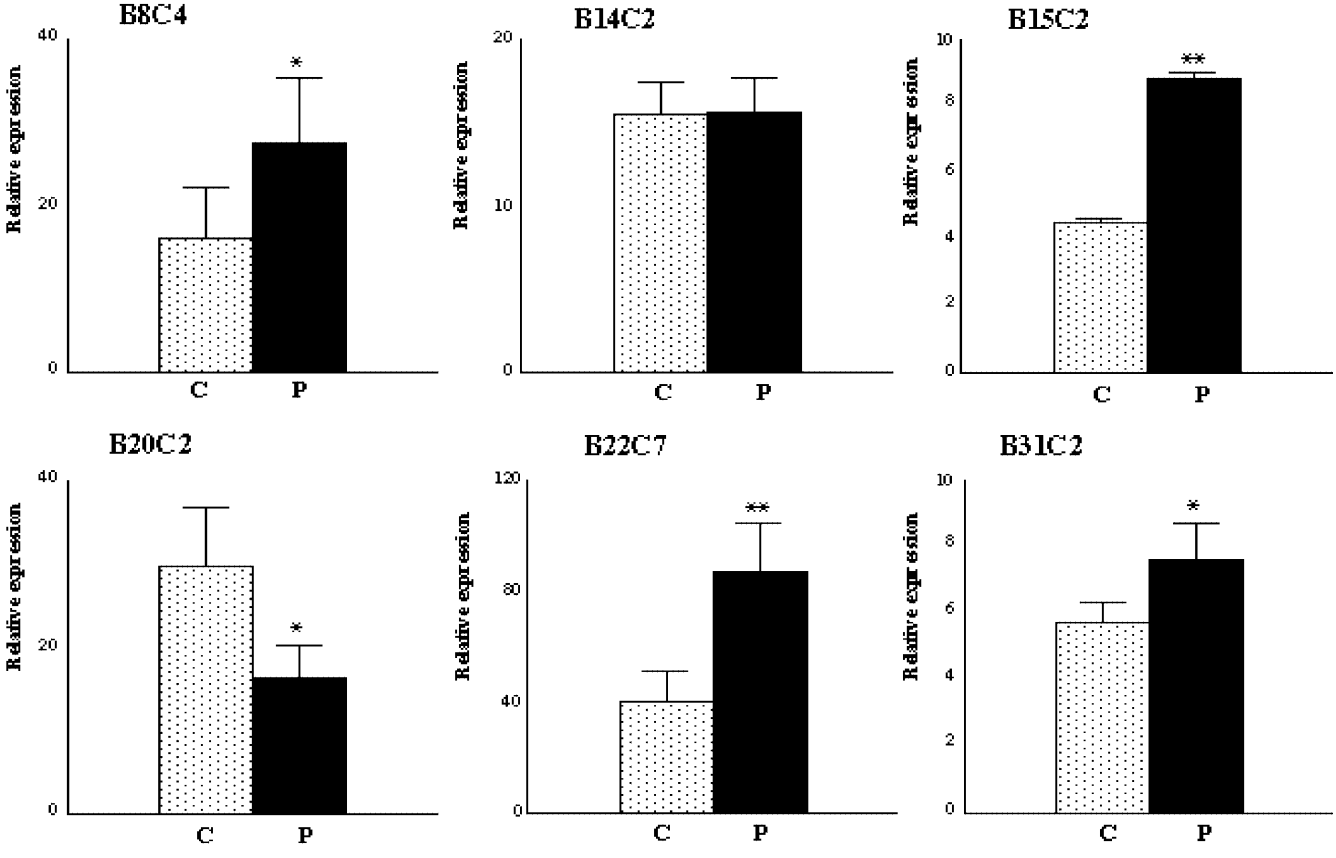
Confirmation of differential expression using RT-PCR

To confirm the differential expression of the candidate genes detected by the DD-PCR, we performed a semiquantitative RT-PCR using specific primers as described in the Materials and methods section. As shown in Fig. 1, significant changes in their expression levels were similarly observed at the B8C4 ($P < 0.05$), B15C2 ($P < 0.01$), B20C2 ($P < 0.05$), B22C7 ($P < 0.01$) and B31C2 ($P < 0.05$) clones. In contrast, there was no effect on the B14C2 clone. We were especially interested in two clones that showed homology to the known gene: B15C2 and B22C7. At 3 h after the PQ injection, the TAFIIB (B15C2) and Lpin2 (B22C7) mRNA expressions respectively increased significantly about two-fold. This stimulation of both genes was observed during 24 h with the same expression level (Fig. 2). In addition, the increase in expression of Lpin2 3 h after the PQ injection was seen only in the lungs and not in the liver and kidneys. Increased expression of TAFIIB, on the other hand, was also observed in the liver and kidneys (Table 3).

Fig. 1. Verification of the changes in gene expression by paraquat (PQ) in rat lungs using semiquantitative RT-PCR as described in the Materials and methods section. The mRNA expression levels in control rats (*C*) and PQ-treated rats (*P*) were normalized to the value of β-actin mRNA and represented as relative ratios (means + SD, $n = 6$–7). $^{*}P < 0.05$, $^{**}P < 0.01$, for control versus PQ-treated rats

In situ hybridization and immunostaining

To determine which cell types were expressing each RNA of TAFIIB and the Lpin2, rat lung sections were



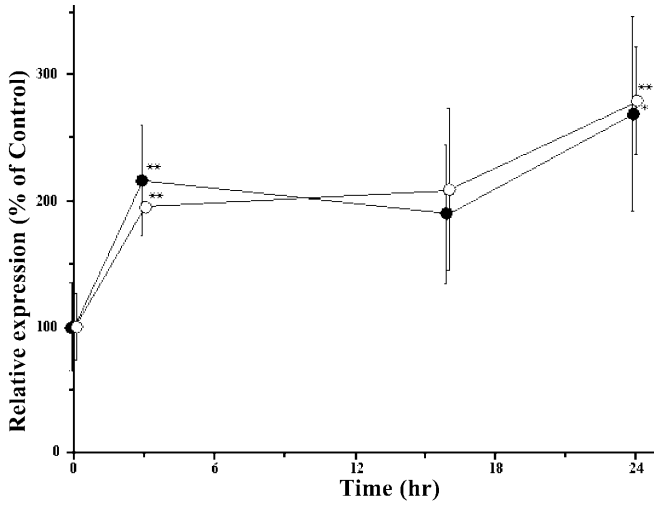


Fig. 2. Time-course of gene expression of TAFIIB (○) and Lpin2 (●) by paraquat (PQ) in rat lungs using semiquantitative RT-PCR. Data are expressed as percentage of control (means ± SD, $n = 6$–7). $^{*}P < 0.05$, $^{**}P < 0.01$, for time 0 versus each time observed

Table 3. Effect of paraquat (PQ) on the expression of TAFIIB and Lpin2 genes in the lungs, liver and kidneys of rats. Data are expressed as means ± SD ($n = 6$–7)

Organ	TAFIIB		Lpin2	
	Control	PQ (3 h)	Control	PQ (3 h)
Lung	4.5 ± 0.1	8.8 ± 0.2**	40.1 ± 10.9	87.0 ± 17.6**
Liver	3.9 ± 0.8	10.9 ± 1.7**	13.7 ± 5.0	10.2 ± 3.2
Kidney	2.5 ± 0.4	5.5 ± 1.8	84.0 ± 2.0	79.5 ± 14.6

$^{**}P < 0.01$

analyzed by in situ hybridization, using each of the riboprobes, which were labeled with digoxigenin 11-UTP. The sections were also counterstained with nuclear fast red (Kernechtrot). Figure 3 shows the in situ hybridization analysis of the TAFIIB and Lpin2 genes. TAFIIB was expressed in all the cells in the sections according to expectations, and especially strong positive signals were detected in some bronchiolar cells as well as in alveolar macrophages (Fig. 3A). Similarly, as shown in Fig. 3C, the expression of Lpin2 was also expressed in all cells and strongly positive signals were detected in bronchiolar cells as well as in scattered alveolar macrophages. No signal was detected using the sense probes (Fig. 3B, D). Next, we stained consecutive sections of the lung tissues using an antisense-labeled riboprobe or polyclonal anti-urine protein 1 (UP1), which is known to stain Clara cells specifically. Figure 4 shows two consecutive sections; one was stained by in situ hybridization for expression of the Lpin2 gene and the other was stained by anti-UP1. These data are highly suggestive that Clara cells are consistent with cells strongly stained by in situ hybridization and also suggest that they could play a crucial role during the onset of pulmonary injury by PQ.

Discussion

PQ undergoes a redox-cycling reaction following increased oxidative stress. There is convincing evidence that oxidative stress plays an important role in the cellular dysfunction resulting in clinical manifestations of PQ poisoning, i.e., pulmonary edema, atelectasis and fibrosis. Despite extensive research, however, questions remain regarding the pathophysiological mechanisms and their relationship to therapeutic strategies. Several growth factors that promote fibrogenesis have been suggested to play a role in the pathogenesis of interstitial lung fibrosis (Antoniades et al. 1990; Piguet et al. 1990; Khalil et al. 1996). Fibroblast growth factor (FGF)-1 and the FGF receptor have been shown to be actively synthesized during the development of pulmonary fibrosis induced in rats with PQ plus hyperoxia (Barrios et al. 1997). In addition to pro-inflammatory cytokines such as interleukin-1 and tumor necrosis factor (Erroi et al. 1992), chemotactic cytokine interleukin-8 might be involved in the pulmonary injury caused by PQ (Bianchi et al. 1993). However, the significance of these PQ-driven events depends on whether they are initiating factors or secondary complications of the progression of PQ injury. In the present study, we applied a differential display method to search for the genes involved in the onset of PQ-induced injury. The rats received one injection of 20 mg/kg PQ. Although two injections of this dose caused pulmonary injury 3 days thereafter (Ogata and Manabe 1990), one injection had no apparent clinical and toxic effect by 3 h. We searched for genes whose expression was up- or down-regulated using the DD-PCR method. This method makes it possible to throw light on the effect of PQ on the expression of unexpected genes, including those directly involved in its metabolism. We isolated 26 fragments of cDNA from lungs which were potentially regulated by PQ, and six clones of interest were selected. Sequence comparison of three of these clones revealed similarities to the known genes, whereas the others showed no similarity. RT-PCR of five clones confirmed the same effect of PQ on the expression as that obtained by DD-PCR, whereas it was not confirmed in the remaining one. We were especially interested in the B15C2 and B22C7 clones, TAFIIB and Lpin2, respectively. We demonstrated that the expression of TAFIIB and Lpin2 was significantly stimulated by PQ during the 3 h after its intake and the stimulation continued during our 24 h observation period. The TATA-binding protein-associated factors (TAFs) within the transcription factor IID, including TAFIIB, control differential gene transcription through interactions with activators and core promoter elements (Kaufmann et al. 1998; Martinez et al. 1998). In our experiments, the stimulation of TAFIIB expression was also seen in the liver and kidneys as well as the lungs. These facts suggest that the living cells in these organs are under conditions in which the activity of transcription of a lot of genes is stimulated.

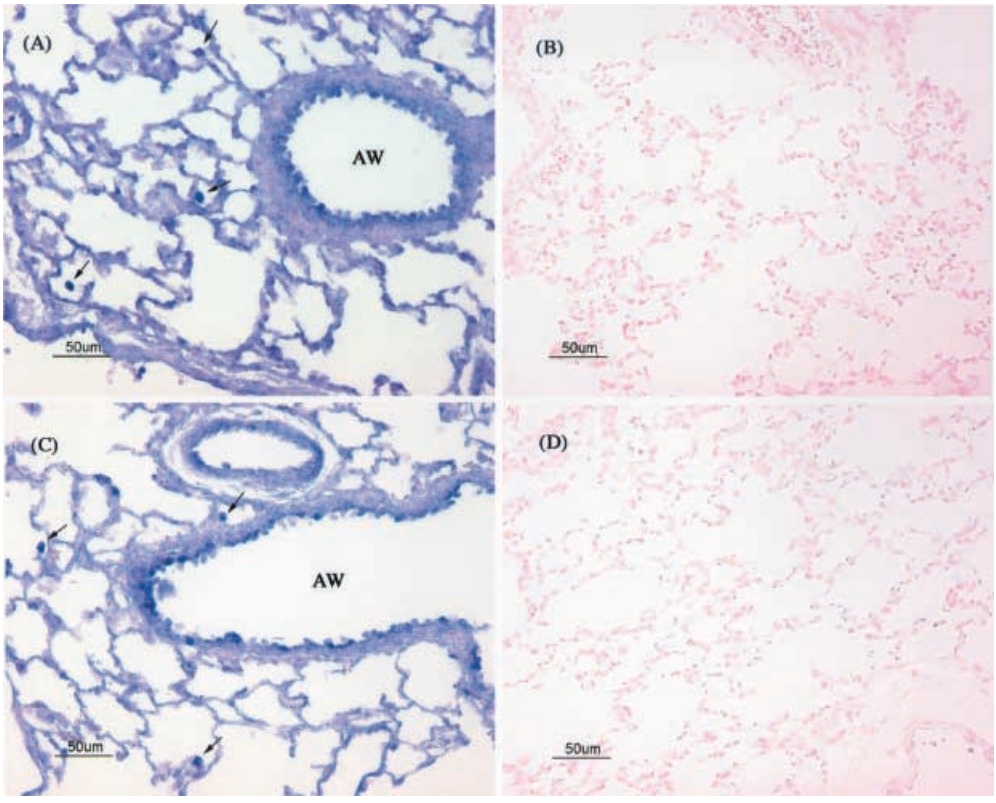


Fig. 3A–D. In situ hybridization analysis of TAFIIB (**A**, **B**) and Lpin2 (**C**, **D**) expression in rat lungs. **A**, **C** Sections labeled by antisense digoxigenin-cRNAs for TAFIIB (**A**) or Lpin2 (**C**), followed by alkaline phosphatase immunostaining. **B**, **D** Sections labeled by sense digoxigenin-cRNAs for TAFIIB (**B**) or Lpin2 (**D**), followed by alkaline phosphatase immunostaining. The sections were couterstained with nuclear fast red; *AW* an airway (bronchiole), *arrows* indicate alveolar macrophages

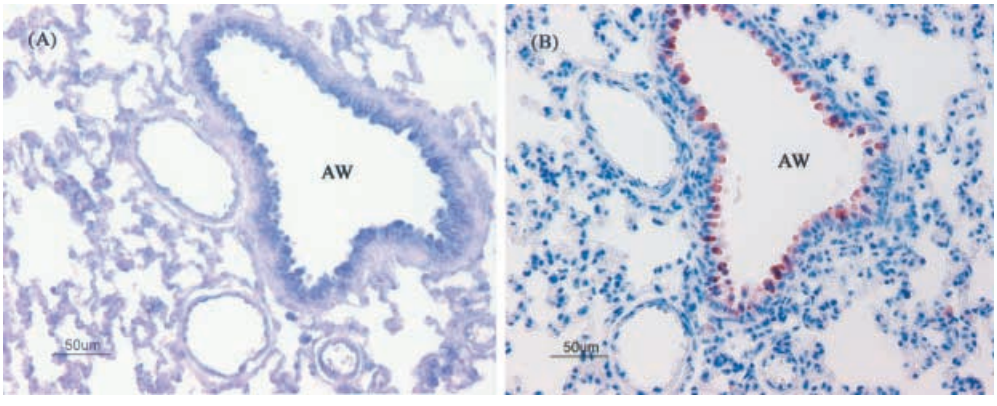


Fig. 4A,B. In situ hybridization analysis of Lpin 2 by an antisense probe and immunohistochemical detection of Clara cells. **A** Lpin2 mRNA detected by in situ hybridization. **B** Clara cells detected by immunohistochemical staining. The sections were couterstained with nuclear fast red (**A**) or hematoxylin (**B**); *AW* airway (bronchiole)

On the other hand, it must be noted that the stimulation of Lpin2 expression was seen only in the lungs and not in the liver and kidneys. Oxidation of lipids in lipoproteins and cell membranes is functionally important for the cells. The pathogenic effects of the pneumotoxicant have been identified as changes in the lipids of membranes (Bagchi et al. 1993; Tomita and Okuyama 1994; Adachi et al. 2000). The gene Lpin encodes a novel family of nuclear proteins, lipin, composed of at least three members. Previous reports stress that Lpin is required for normal adipose tissue development and is a candidate gene for lipodystrophy (Peterfy et al. 2001; Huffman et al. 2002). Although the molecular function of Lpin2 is unclear, the lipids in lipoproteins and membranes might be affected through a stimulation of the transcription level of lipin. On the other hand, alteration or inactivation of pulmonary surfactant plays an important role in acute lung injury (Chen et al. 2001; Gunther et al. 2001; Taeusch and Keough 2001). The stimulation of Lpin2 suggests that synthesis or secretion of lipids in surfactants may be decreased at the onset of injury caused by PQ. Leikauf et al. (2002) reported that the susceptibility to acute lung injury by mediators including oxidants is heritable and they have approached the candidate genes. Our results indicate that Lpin2 is one of the candidate genes associated with PQ-driven lung injury. Which cell types express these genes in the lungs? In situ hybridization of TAFIIB and Lpin2 mRNAs in rat lungs revealed widespread distribution of the transcripts with dense signals in Clara cells as well as in alveolar macrophages. Since bronchiolar Clara cells are major loci of cytochrome P-450 monooxygenase in

the lung, it is beyond doubt that the effect of xenobiotics, including PQ, is greatest on the cells among mammalian respiratory tract cells (Masek and Richards 1990; Smith and Brian 1991; Elia et al. 2000). In addition, Clara cells produce and secrete pulmonary surfactant. Leikauf et al. (2002) noted that the expression levels of surfactant proteins and Clara cell secretary protein decreased markedly during the progression of acute lung injury. Many investigations have focused on the effect on protein synthesis in Clara cells (Royce and Plopper 1997; Harrod and Jaramillo 2002; Wang et al. 2002). By contrast, studies on another component of surfactant, phospholipids, have been scant. Our data suggest that PQ-induced injury starts with functional abnormalities of the lipid in Clara cell surfactant. Although it is known that alveolar type II cells also synthesize and secrete pulmonary surfactant, we obtained no data showing any remarkable staining of type II cells. This suggests that an abnormality in Clara cell surfactant is crucial at the onset of the injury. Quintero et al. (2002) reported that an increase in the number of cells, including macrophages, caused by acute lung injury altered surfactant levels through enhanced clearance and degradation of lipids. The stimulation of Lpin2 expression observed in alveolar macrophages seems to provide a challenge to further understanding of PQ-induced injury and biological responses to PQ exposure.

In conclusion, during the initial stage of PQ-induced injury, 26 differentially expressed genes were cloned and sequenced by means of the differential display technique and six clones were selected. Of these, two clones corresponding to TAFIIB and Lpin2 were especially interesting. The expression of both genes was significantly up-regulated by PQ. In addition, the up-regulation of Lpin2 was seen in the lungs but not in the liver and kidneys. In situ hybridization in lung sections showed that the expression of TAFIIB and Lpin2 was especially remarkable in Clara cells and in alveolar macrophages. These results suggest that Clara cells and macrophages play an important role during the initial stage of PQ-induced injury pursuing a chronic pathosis.

Acknowledgements This study was supported, in part, by Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant Nos. 10670405 and 13670439) and by a project grant (No. 13-404) from Kawasaki Medical School. The authors thank Ms Y. Yoshida and Ms K. Miyake for their excellent technical assistance in preparing tissue sections.

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