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Oxidized Forms of Peroxiredoxins and DJ-1 on Two-dimensional Gels Increased in Response to Sublethal Levels of Paraquat

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We previously found hydroperoxide-responsive proteins (HPRPs), which are comprised of peroxiredoxin I (Prx I), Prx II, Prx III, Prx VI, HSP27, G3PDH and two unidentified proteins (HPRP-2′ and HPRP-5′), in human umbilical vein endothelial cells. It was demonstrated by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) that most HPRPs are converted into variants with lower pI upon exposure to hydroperoxides. In this study, we examined the HPRP response on 2D gels upon exposure of human endothelial cells (ECV304) to paraquat (PQ^{2+}), which generates reactive oxygen species (ROS) within cells. PQ^{2+} exerted cytotoxic effects in a dose- (10 μM–10 mM) and time- (24–168 h) dependent manner. Two-dimensional PAGE analysis revealed that HPRP-2′, and oxidized forms of Prx I, Prx II and Prx III were clearly increased upon exposure of cells to sublethal levels of PQ^{2+}. Microsequence analysis revealed that both HPRP-2 and -2′ were identical with human DJ-1. Moreover immunoblot analysis confirmed the increase of oxidized forms of Prx II, Prx III and DJ-1 in response to sublethal levels of PQ^{2+}. PQ^{2+} treatment failed to increase fluorescence intensity derived from DCF, which is believed to be an indicator for intracellular levels of hydroperoxide. Although pentachlorophenol (PCP), an uncoupler of the mitochondrial respiratory chain, clearly elevated the fluorescence, PCP had no effect on HPRP response. These observations indicated that DCF-derived fluorescence is not correlated with HPRP response. We consider that the response of Prxs and DJ-1 on 2D gels could reflect endogenous production of ROS in PQ^{2+}-treated cells, and might be a sensitive indicator of oxidative stress status.

Keywords: Hydroperoxide, peroxiredoxin, DJ-1, paraquat, 2D PAGE, endothelial cells

Abbreviations: 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; DCF, 2′,7′-dichlorofluorescein; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; HPRPs, hydroperoxide-responsive proteins; HSP27, heat shock protein 27; HUVEC, human umbilical vein endothelial cells; IEF, isoelectric focusing; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; NEPHGE, non-equilibrium pH gradient electrophoresis;

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PBS, phosphate-buffered saline; PCP, pentachlorophenol; PQ^{2+}, paraquat (1,1′-dimethyl-4,4′-dipyridinium); Prx, peroxiredoxin; ROS, reactive oxygen species; TBS, tris-buffered saline

INTRODUCTION

Oxidative stress has been implicated in various degenerative diseases, such as atherosclerosis, cancer and neurodegenerative diseases.[1] Reactive oxygen species (ROS) play a major role in oxidative stress. ROS react with various biological components, causing, for example, dysfunction of enzymes[2] and DNA damage.[3] It has been proposed that ROS, especially hydroperoxides, could regulate cellular functions by virtue of redox control of functional proteins. Activities of kinases such as JNK and MAPK are augmented by a decline of redox status owing to oxidation in the cellular environment.[4] Overexpression of antioxidant proteins such as peroxiredoxin (Prx) prevents apoptotic stimuli associated with blockade of transcription factor activation.[5] These findings support the idea that ROS could regulate the functions of cellular components. However, the molecular targets of ROS remain to be clarified, partly because of the non-selectivity and non-specificity of ROS reactivity.

Protein carbonyl,[6] 8-hydroxydeoxyguanosine[7] and 8-isoprostane[8] have been used as indicators for oxidation of proteins, nucleic acids and lipids, respectively. However, these determinants are irreversible end products, and they become detectable only after severe tissue damage has occurred. Some other indicators such as GSH[9] and ubiquinones[10,11] for oxidative stress status have also been reported. However, sensitive new indicators are still needed to evaluate oxidative stress status in diseases and pathophysiological conditions.

Previously we found hydroperoxide-responsive proteins (HPRPs) in human endothelial cells.[12] The HPRPs are comprised of 6 known proteins, Prx I, Prx II, Prx III, Prx VI, HSP27, and G3PDH, and two unidentified proteins (HPRP-2′ and HPRP-5′). Of these, four HPRPs are Prx family proteins, which catalyze hydroperoxide reduction with reducing systems such as thioredoxin and thioredoxin reductase. We found that Prxs are converted to variants with slightly lower pI in response to hydroperoxides at a nontoxic dose, and revert to their original migration positions on 2D gels after removal of hydroperoxide stress. The response of Prx II to H_2O_2 is so quick and sensitive that a 2-min exposure of cells to H_2O_2 at 100 μM causes a clear migration change on 2D gels. The nature of the structural modification of HPRPs that we can observe on 2D gels remains unknown, except in the case of HSP27, which is phosphorylated. However, the HPRP response, especially that of the Prxs, is quite selective to hydroperoxides. Overexpression of Prxs in cells could reduce the level of intracellular hydroperoxide production in response to extracellular stimuli such as growth factors[13] and environmental chemicals.[14,15] These considerations prompted us to examine whether the HPRP response on 2D gels might be useful as an endogenous indicator for intracellular formation of hydroperoxides.

Paraquat (PQ^{2+}) is a bipyridyl herbicide and is widely used as a model compound to produce ROS within cells, with a help of cellular components.[16–18] The ROS production is primarily responsible for oxidative stress and tissue damage mediated by PQ^{2+}.[19,20] In this study, we examined the HPRP response on 2D gels to endogenously produced ROS upon exposure of human endothelial cells to PQ^{2+}.

MATERIALS AND METHODS

Materials

Paraquat (PQ^{2+}, 1,1′-dimethyl-4,4′-diphenyldipyridinium) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

Pentachlorophenol (PCP) was obtained from Wako Chemical Co. (Osaka, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, Oregon, USA). Monoclonal antibodies specific to HSP27 were from Funakoshi (Tokyo, Japan). Medium 199 (M199) was purchased from GIBCO BRL (Grand Island, NY, USA). Synthetic peptides were from Sawady Technology (Tokyo, Japan).

Culture of ECV304 Cells and Treatment with Reagents

Human endothelial cell lines (ECV304) were cultured in M199 supplemented with 5% heat-inactivated fetal bovine serum containing 100 U/ml penicillin and 100μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Chemicals were dissolved to form appropriate solutions, which were filtered before addition to the culture medium. Cell viability was assessed by MTT assay.[21] In brief, ECV304 cells in 96-well plates (1×10^4 cells/well) were treated with PQ^{2+} at the indicated conditions. After incubation for several days, MTT solution was added and the cells were cultured for another 4 h. Colored MTT was dissolved and monitored at 540 nm by a plate reader (Reader 510, Organon Teknika, Tokyo, Japan).

DCF Labeling and Detection of Fluorescence

DCF labeling was performed according to the method of Rothe and Valet.[22] ECV304 cells in 12-well plates (1×10^5 cells/well) were incubated with 5 μM H_2DCF-DA for 10 min at 37 °C. Then, the medium was aspirated and the cells were washed once with phosphate-buffered saline (PBS). Freshly prepared media that contain drugs or vehicle were added and fluorescence (ex, 480/20 nm; em, 530/25 nm) was monitored by a plate reader (CytoFlour 4000TR, PerSeptive Biosystems, Tokyo, Japan).

2D PAGE Analysis, Preparative 2D PAGE and Microsequencing

Cells after treatments were lysed in a lysis buffer that contained 9.2 M urea, 2% CHAPS, 2% ampholines (pH 5–8:pH 3–10, 4:1, v/v), 2 mM EDTA·2Na and 1 mM PMSF, with 70 mM DTT. After centrifugation at $2{,}000 \times g$ for 10 min, supernatants were analyzed by 2D PAGE.[23,24] Proteins on 2D gels were visualized by silver staining. Preparative 2D PAGE and microsequnce analysis were performed as described previously.[12,25]

Preparation of Polyclonal Antibodies

Dodecapeptide [(C)VDDSKEYFSKHN], dodecapeptide [(C)AASKEYFQKVNQ] and hexadecapeptide [(C)GKEVAAVQVKAPLVLKD] corresponding to carboxyl terminal sequences of human Prx II, Prx III and DJ-1, respectively, were conjugated to keyhole limpet hemocyanin with a linkage between cysteine at the carboxyl terminal of the peptides and a bifunctional spacer. The respective conjugates were used to immunize rabbit by subcutaneous injection. After two booster injections at 2-week intervals, anti-Prx II (α-Prx II), α-Prx III and α-DJ-1 polyclonal antibodies were purified from rabbit serum through a peptide ligand affinity column.

Immuno-blot Analysis

Proteins separated by 2D PAGE were blotted onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA) according to the method of Hirano and Watanabe.[26] The membrane was treated with a blocking reagent overnight at 4 °C, and incubated with α-Prx I, α-Prx III or α-DJ-1 prepared as described above for 1 h at r.t. It was washed with tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) twice, and incubated with peroxidase-conjugated second antibodies for 1 h at r.t. After further washing with TBS-Tween twice and TBS once, the specific

protein spots were visualized with an ECL kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to the manufacturer's instructions.

RESULTS

We previously found HPRPs in primary cultured endothelial cells (HUVEC) derived from human umbilical vein.[12] When we substituted a human endothelial cell line (ECV304) for HUVEC, all the HPRPs except for HPRP-5′, were similarly detected upon exposure of ECV304 to extracellular H_2O_2 (Figure 1, Table I), although ECV304 was slightly more resistant to the oxidant than HUVEC. In this study we examined HPRP response to endogenously produced ROS in PQ^{2+}-treated ECV304 cells.

First, cell viability was assessed by MTT assay after exposure of ECV304 cells to PQ^{2+}. As shown in Figure 2A, 24-h exposure to PQ^{2+} at 1 mM had no effect on cell viability. After a 7-day exposure of ECV304 cells, a dose-dependent toxic effect was observed. The LD_{50} value of PQ^{2+} at the 7th day was about 300 μM. The toxic effect of PQ^{2+} on the cells was also dependent on the duration of exposure (Figure 2B).

We analyzed the pattern of protein expression in PQ^{2+}-treated cells for 7 days by 2D PAGE to ascertain whether or not HPRPs respond to endogenously produced ROS. Silver-stained gels revealed that some of the proteins responded to PQ^{2+} on 2D gels, as compared with control gels (Figure 3(A–C)). HPRP-2′ was significantly intensified after 7-day exposure to 30 μM PQ^{2+}, whereas HPRP-2 was slightly diminished (Figure 3(A,B)). Variants of Prx II and Prx III were also detectable upon exposure of cells to at least 100 μM PQ^{2+} for 7 days (Figure 3C). When the first dimension of 2D PAGE was carried out using non-equilibrium pH gradient electrophoresis (NEPHGE), it became clear that Prx I responded to PQ^{2+} as well as did Prx II and Prx III (data not shown). However, no response of Prx VI, HSP27 and G3PDH were observed under these conditions, though these HPRPs were detectable upon exposure of cells to H_2O_2.

HPRP-2 and HPRP-2′ had the same molecular weight, and the increase of HPRP-2′ in response to PQ^{2+} and H_2O_2 corresponded to the decrease

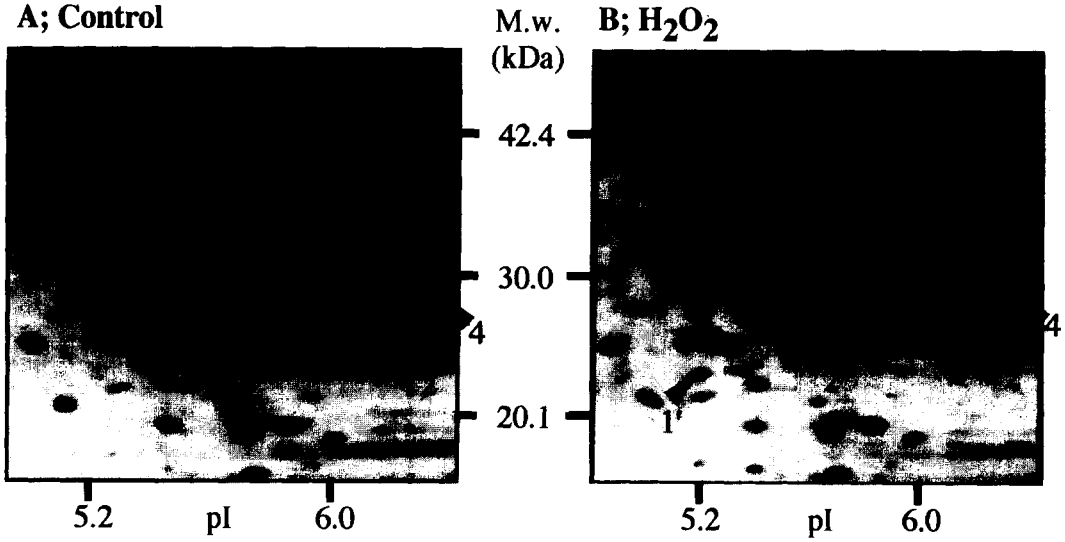
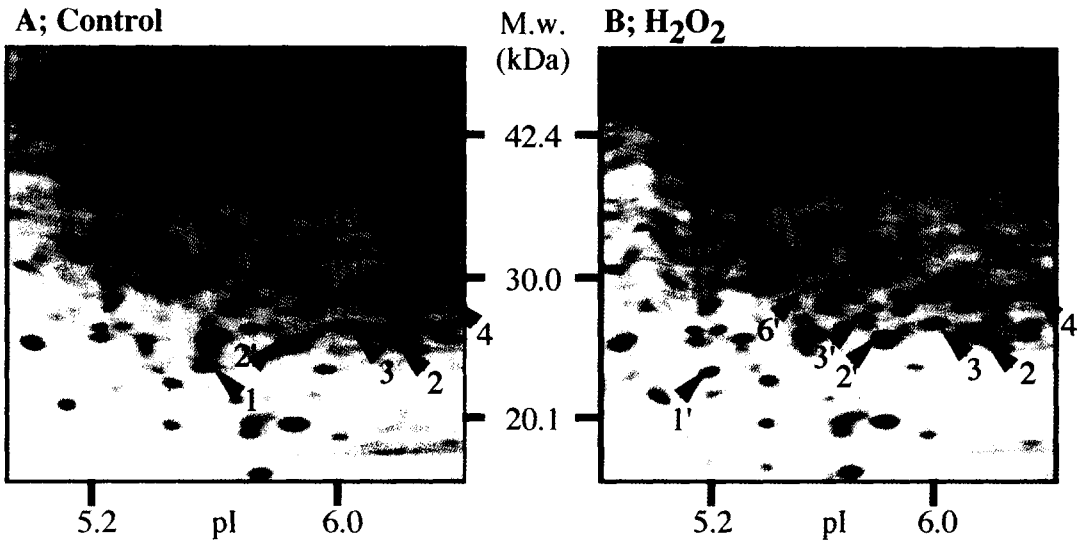


FIGURE 1 Protein Expression Patterns in H_2O_2-treated ECV304 Cells. Confluent ECV304 cells were treated with 300 μM H_2O_2 for 30 min, and protein expression patterns were analyzed by 2D PAGE. Proteins on gels were visualized with silver staining. (A) Control cells; (B) H_2O_2-treated cells. Protein spots that correspond to hydroperoxide-responsive proteins are marked by arrowheads with numbers according to the previous paper (12). (See Color plate I at the end of this issue.)



Color Plate I (see page 304, figure 1) Protein Expression Patterns in H_2O_2-treated ECV304 Cells. Confluent ECV304 cells were treated with 300 μM H_2O_2 for 30 min, and protein expression patterns were analyzed by 2D PAGE. Proteins on gels were visualized with silver staining. (A) Control cells; (B) H_2O_2-treated cells. Protein spots that correspond to hydroperoxide-responsive proteins are marked by arrowheads with numbers according to the previous paper (12).

TABLE I Hydroperoxide-responsive proteins

HPRP		Sequence obtained in this study	Protein identified
Control	H_2O_2-treated		
HPRP-1 (23.5, 5.5)[a]	HPRP-1′ (23.5, 5.2)		Prx II[b,c]
HPRP-2 (25.1, 6.2)	–	(K)DPVQCSRDVVIC	DJ-1
–	HPRP-2′ (25.1, 5.8)	(K)APLVLK (K)DPVQCSRDVV (K)GAEEMETVI (K)EGPYDVVVLPGGNL (K)VILHPLAK	DJ-1
HPRP-3 (26.4, 6.2)	HPRP-3′ (26.6, 5.7)		Prx III
HPRP-4 (27.6, 6.4)	HPRP-4′ (27.6, 6.0)		Prx VI
HPRP-6 (28.4, 6.0)	HPRP-6′ (28.4, 5.6)		HSP27
HPRP-7 (24.0, >8)	HPRP-7′ (24.0, >8)		Prx I
HPRP-8 (40.0, >9)	HPRP-8′ (40.0, >9)		G3PDH

[a] In parenthesis, the molecular mass in kDa and the pI are shown.
[b] Prx I, peroxiredoxin I; Prx II, peroxiredoxin II; Prx III, peroxiredoxin III; Prx VI, peroxiredoxin VI; HSP27, heat shock protein 27; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
[c] Ref. [12].

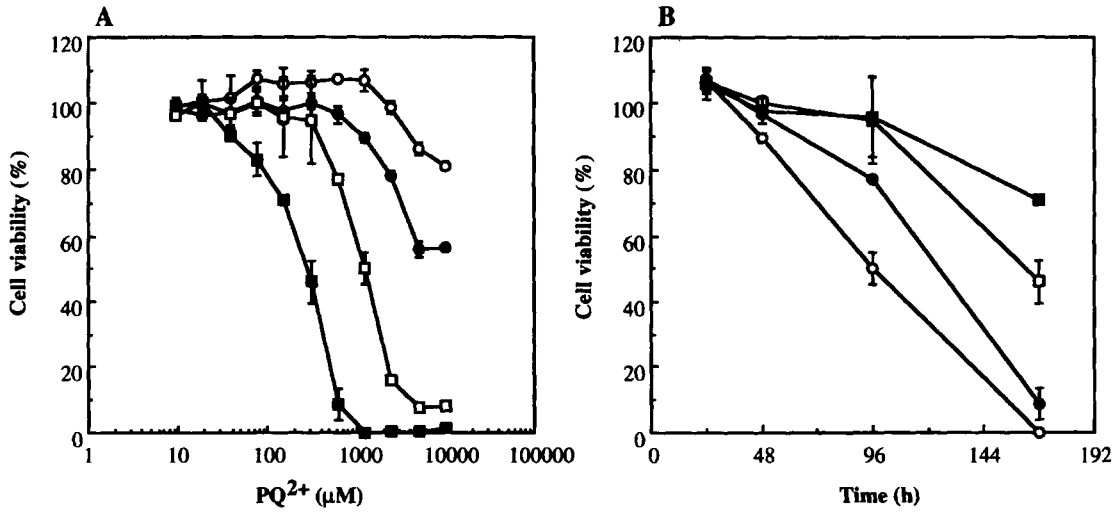


FIGURE 2 Effect of PQ^{2+} on ECV304 Cell Viability. ECV304 cells (1×10^4 cells / well) were cultured in medium that contained PQ^{2+} at the indicated concentrations. After exposure for appropriate times, cell viability was assessed by MTT assay as described in Materials and Methods. (A) Dependency on exposure concentration. Exposure time, 24 h (○), 48 h (●), 96 h (■), 168 h (■); (B) Dependency on exposure time. Exposure concentration, 1250 μM (○), 625 μM (●), 313 μM (□), 156 μM (■). Data represent means ± SD ($n = 3$).

of HPRP-2.[12] Therefore, we assumed that these 2 HPRPs were the same protein, with a post-translational modification in HPRP-2′. To verify the identity of HPRP-2 and HPRP-2′, we analyzed their internal amino acid sequences. The results showed that both proteins are human DJ-1 (Table I), which was originally identified as a gene product with oncogenic activity. These results indicated that human DJ-1 is converted to a variant having lower pI in response to PQ^{2+} and hydroperoxides.

We prepared polyclonal antibodies (pAbs) to carboxyl terminal peptide fragments of Prx II, Prx III and DJ-1, to examine the response of these HPRPs in detail. Immuno-blot analysis using these pAbs demonstrated that Prx II, Prx III and

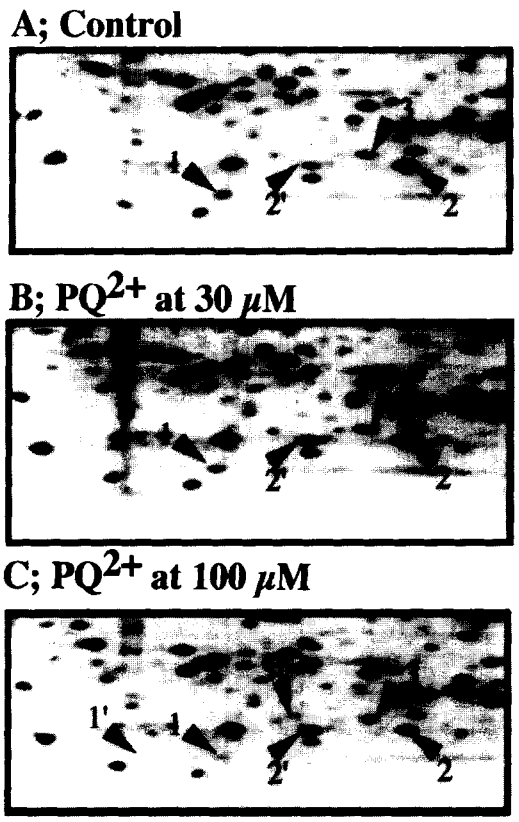
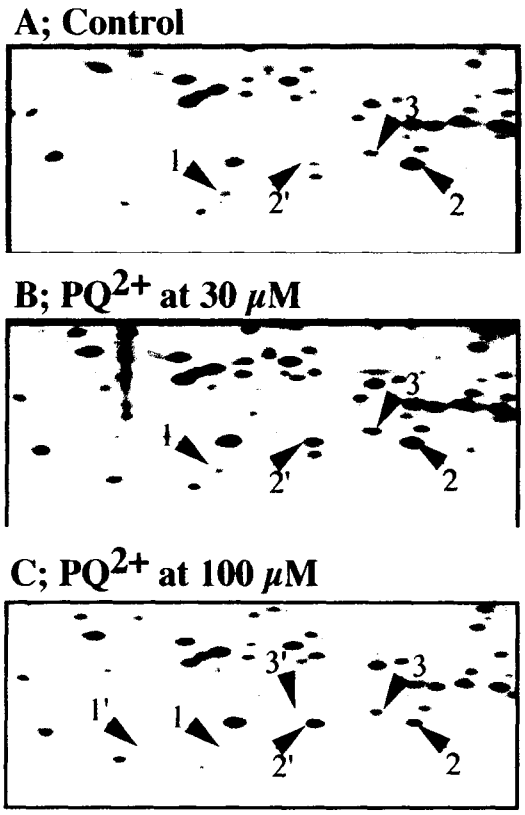


FIGURE 3 2D PAGE Analysis of Proteins in PQ^{2+}-treated ECV304 Cells. ECV304 cells were incubated with PQ^{2+} at the indicated concentration for 7 days. Proteins analyzed by 2D PAGE were visualized with silver staining. (A) Control; (B) 30 μM; (C) 100 μM. The gels range from 20 to 30 kDa along the perpendicular axis and from pI 5.0 (left) to pI 6.5 (right) along the horizontal axis. HPRPs are marked by arrowheads with numbers as described in the legend to Figure 1. (See Color plate II at the end of this issue.)

DJ-1 were transformed to the respective variant upon exposure of cells to PQ^{2+} (Figure 4). Immunodecoration showed that Prx II was converted into its variant after PQ^{2+} treatment at 30 μM for 24 h, although the variant form of Prx II was detectable only after 7-day treatment with PQ^{2+} at 100 μM on silver-stained gels. As for Prx III, immuno-blot analysis revealed that the response of Prx III was less sensitive to PQ^{2+} than that of Prx II, though some minor spots were detectable in addition to Prx III on silver-stained gels. DJ-1 was also converted to its variant after PQ^{2+} treatment at 30 μM for 24 h. In contrast to Prx II and Prx III, immunodecoration of DJ-1 was well correlated with the expression patterns obtained from silver-stained gels. The order of sensitivity in the response of HPRPs to PQ^{2+} was DJ-1 > Prx II > Prx III. No enhancement of HSP27 phosphorylation was observed by immuno-blot analysis under these conditions (Figure 5), while extracellularly added H_2O_2 clearly enhanced HSP27 phosphorylation. It is noteworthy that the variant of DJ-1, as well as Prx II, increased during the culture of cells in medium without PQ^{2+} (Figure 4(A,C)), suggesting that these proteins might respond to ROS produced by physiological metabolism, such as mitochondrial respiration and cellular senescence. Our results indicate that Prxs and DJ-1 can respond to endogenously produced ROS, as well as extracellularly added hydroperoxides such as H_2O_2.

To assess intracellular formation of ROS mediated by PQ^{2+}, we measured fluorescence in PQ^{2+}-treated ECV304 cells that had been pre-labeled with dihydrodichlorofluorescein diacetate (H_2DCF-DA). Dichlorofluorescein (DCF) is widely used as an indicator for intracellular production of hydroperoxides.[22] Since DCF-derived fluorescence is intense, we can sensitively monitor change of intracellular hydroperoxide levels elicited by receptor-mediated signal transduction.[27] As shown in Figure 6, PQ^{2+} treatment at 1 mM failed to increase the DCF-derived fluorescence. On the other hand, PCP, a herbicide that uncouples the mitochondrial respiratory chain,[28,29] significantly enhanced DCF-derived fluorescence in a dose-dependent manner. The fluorescence intensity induced by PCP at 200 μM corresponded to that of extracellularly added H_2O_2 at about 3 μM (data not shown). Although PCP clearly increased the cellular fluorescence derived from DCF, no response of HPRPs was detected on 2D gels (data not shown). Furthermore, when ECV304 cells were post-labeled with H_2DCF-DA after 7-day exposure to PQ^{2+} at 100–1,000 μM and the fluorescence was monitored for a further 4 h, no change of fluorescence from the control levels was seen (data not shown).



Color Plate II (see page 306, figure 3) 2D PAGE Analysis of Proteins in PQ^{2+}-treated ECV304 Cells. ECV304 cells were incubated with PQ^{2+} at the indicated concentration for 7 days. Proteins analyzed by 2D PAGE were visualized with silver staining. (A) Control; (B) 30 μM; (C) 100 μM. The gels range from 20 to 30 kDa along the perpendicular axis and from pI 5.0 (left) to pI 6.5 (right) along the horizontal axis. HPRPs are marked by arrowheads with numbers as described in the legend to Figure 1.

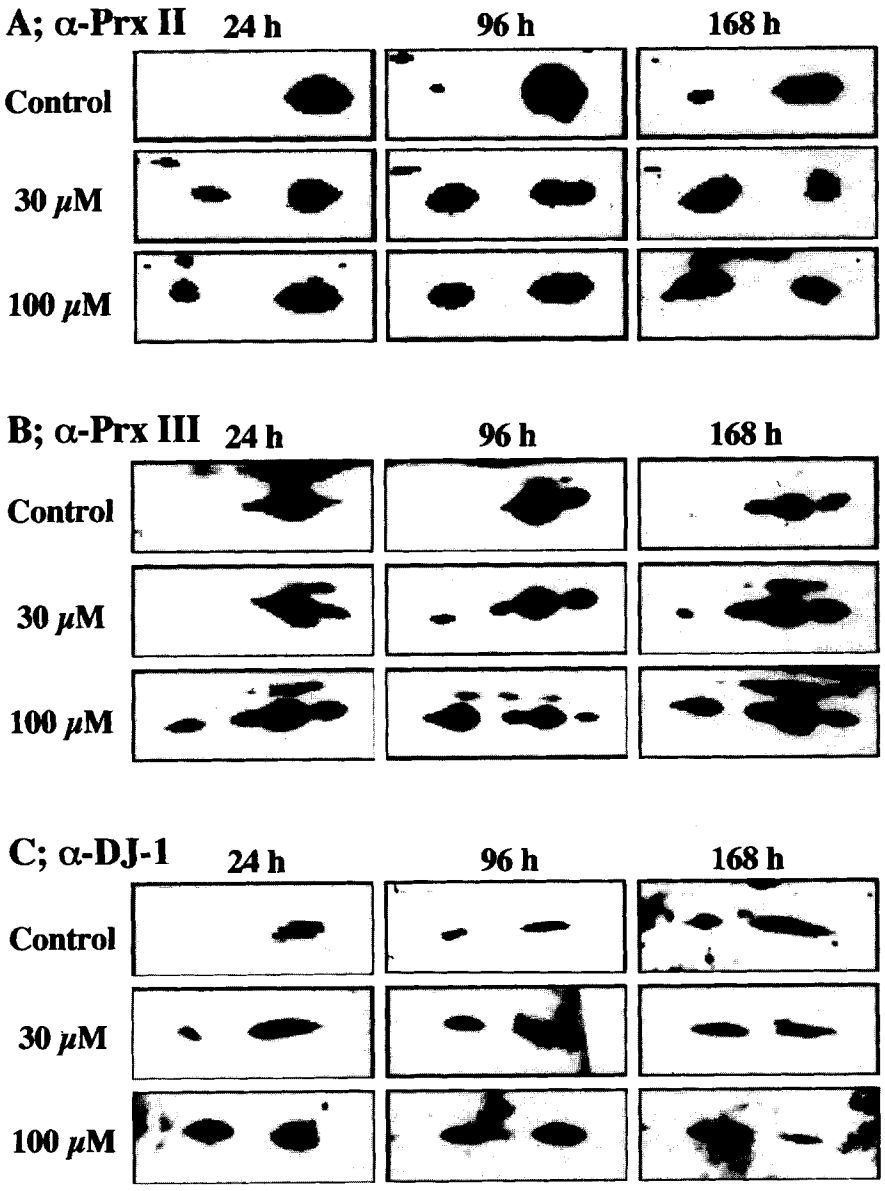
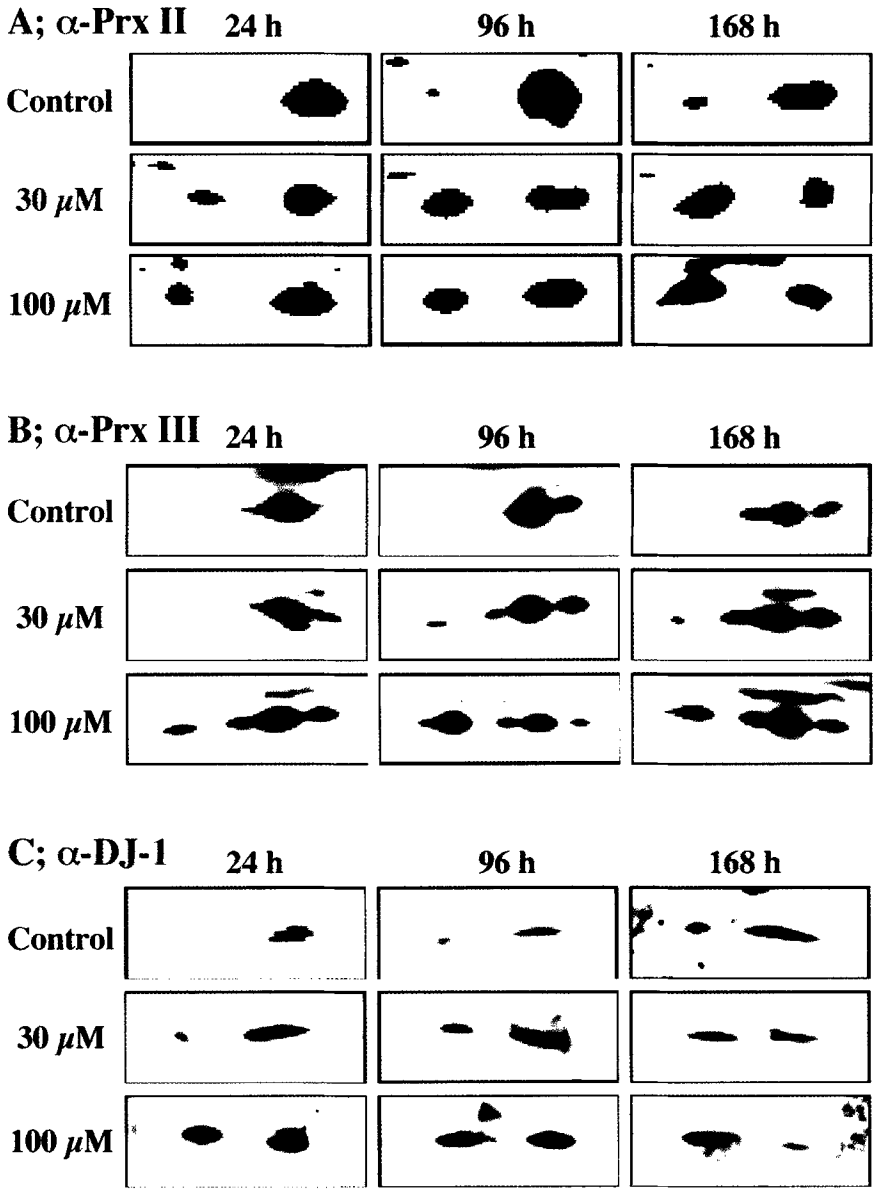


FIGURE 4 Immuno-blot Analysis of Proteins in PQ^{2+}-treated ECV304 Cells. ECV304 cells were incubated with PQ^{2+} at the indicated concentration. At the indicated times after PQ^{2+} treatment, cells were harvested and proteins were separated by 2D PAGE. Prx II, Prx III and DJ-1 protein were detected by immuno-blot analysis with specific antibodies. (A) α-Prx II; (B) α-Prx III; (C) α-DJ-1 protein. The areas corresponding to the respective proteins are enlarged. Horizontal axis; left (+), right (−). (See Color plate III at the end of this issue.)

DISUCUSSION

In the present study, we demonstrated that Prx I, Prx II, Prx III and DJ-1 are converted into variants detectable on 2D gels upon exposure of human endothelial cells to sublethal levels of PQ^{2+}. We previously found that these HPRPs responded to extracellularly added hydroperoxides, but not to other oxidants such as arsenite and nitric oxide.[12] Prxs are well-known proteins that catalyze hydroperoxide reduction.[30] PQ^{2+} produces ROS within cells with the help of



Color Plate III (see page 307, figure 4) Immuno-blot Analysis of Proteins in PQ^{2+}-treated ECV304 Cells. ECV304 cells were incubated with PQ^{2+} at the indicated concentration. At the indicated times after PQ^{2+} treatment, cells were harvested and proteins were separated by 2D PAGE. Prx II, Prx III and DJ-1 protein were detected by immuno-blot analysis with specific antibodies. (A) α-Prx II; (B) α-Prx III; (C) α-DJ-1 protein. The areas corresponding to the respective proteins are enlarged. Horizontal axis; left (+), right (−).

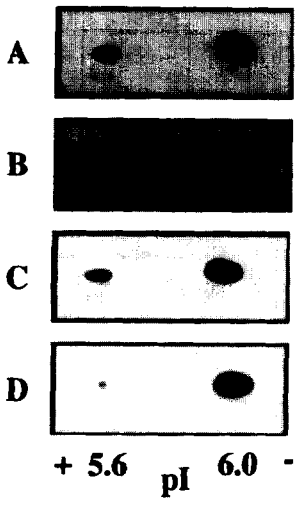


FIGURE 5 Enhancement of HSP27 Phosphorylation by H_2O_2, but not by PQ^{2+}. ECV304 cells were treated with H_2O_2 at 300 μM for 30 min or PQ^{2+} at 1 mM for 24 h. The phosphorylation state of HSP27 in ECV304 cells were analyzed by immuno-blotting. (A) Control cells at 30 min; (B) H_2O_2-treated cells; (C) Control cells at 24 h; (D) PQ^{2+}-treated cells. Left spot, monophosphorylated HSP27, right spot, non-phosphorylated HSP27. (See Color plate IV at the end of this issue.)

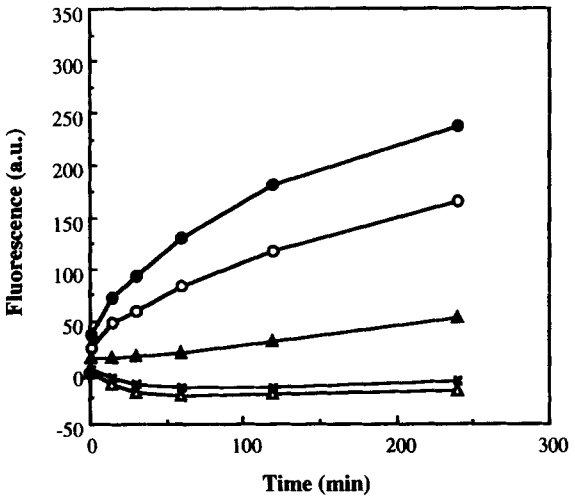
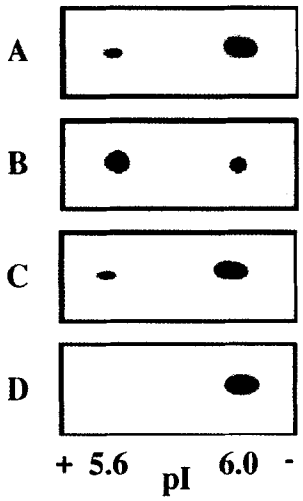


FIGURE 6 Detection of Intracellular Hydroperoxide Production by DCF-derived Fluorescence. ECV304 cells (1×10^5 cells/well) were labeled with 5 μM H_2DCF-DA for 10 min. After that, the cells were incubated with fresh media containing drugs or vehicle. Fluorescence was monitored at the indicated times. Control (×); PCP at 200 μM (●); PCP at 60 μM (○); PCP at 20 μM (▲); PQ^{2+} at 1 mM (△). Data represent a typical result in two independent experiments.

cellular redox enzymes.[16,17] Cellular hydroperoxides were undetectable by DCF in this system. Therefore, we suggest that Prxs and DJ-1 responded to intracellular production of ROS, and these HPRPs might be useful as endogenous indicators of oxidative stress status.

PQ^{2+}-induced HPRP response was quite different from that induced by extracellularly added H_2O_2. For example, DJ-1 was the most sensitive to PQ^{2+} in this study, while higher amounts of extracellular H_2O_2 were required for detection of HPRP-2′ in the previous work. No enhancement of HSP27 phosphorylation was observed even after severe cellular damage induced by PQ^{2+}, whereas phosphorylation of HSP27 was enhanced by extracellular H_2O_2. In addition, PQ^{2+} failed to increase DCF-derived fluorescence under conditions where Prxs and DJ-1 clearly responded. H_2O_2 at about 3 μM and PCP at 200 μM intensified DCF-derived fluorescence, but did not induce HPRP response. These differences in HPRP response suggest that cellular components might be able to differentiate the mode or site of production of ROS.

In this study, human DJ-1 was identified as a novel responsive protein to oxidative stress. Like other HPRPs, DJ-1 is converted into a variant having a slightly more acidic pI on 2D gels. In the previous study, we detected DJ-1 and its variant as HPRP-2 and -2′ upon exposure of HUVEC to hydroperoxides such as H_2O_2.[12] Compared with other HPRPs, the sensitivity of DJ-1 to H_2O_2 was very low. Further, HPRP-2′ was detectable on 2D gels even after a further 24-h incubation without H_2O_2 stress, whereas other HPRPs had mostly remigrated to their original locations.[12] These results indicate that DJ-1 was unable to recover its native form even after the removal of the stress, while the changes in Prxs were completely reversible. Interestingly, DJ-1 was the most sensitive protein to PQ^{2+} in this study. Since ROS induction by PQ^{2+} is small but continuous, the above finding might be explained by the irreversibility of the DJ-1 response.



Color Plate IV (see page 308, figure 5) Enhancement of HSP27 Phosphorylation by H_2O_2, but not by PQ^{2+}. ECV304 cells were treated with H_2O_2 at 300 µM for 30 min or PQ^{2+} at 1 mM for 24 h. The phosphorylation state of HSP27 in ECV304 cells were analyzed by immuno-blotting. (A) Control cells at 30 min; (B) H_2O_2-treated cells; (C) Control cells at 24 h; (D) PQ^{2+}-treated cells. Left spot, monophosphorylated HSP27, right spot, non-phosphorylated HSP27.

Although Prxs were originally recognized as thiol-specific antioxidant proteins, one of their major biological functions is to eliminate hydroperoxides.[31] Thus, it is reasonable that Prxs are detected as HPRPs. One the other hand, the biological role of DJ-1 remains unclear. Nagakubo *et al.* isolated human DJ-1 cDNA[32] and showed that DJ-1 had a cooperative transforming activity with H-Ras. Wagenfeld *et al.*[33] detected a DJ-1 homolog in epididymal fluid of drug-induced infertile male rat by 2D PAGE. They found that rat DJ-1 mRNA is expressed at a high level in the testis.[34] Hod *et al.*[35] revealed an RNA binding protein regulatory subunit from rat hepatoma cells to be identical with DJ-1. These reports support the idea that DJ-1 is an important protein with broad biological functions. However, no information is available to suggest the nature of the relationship between DJ-1 and oxidative stress.

Wagenfeld *et al.*[33] gave the isoelectric point of rat DJ-1 detected on 2D gels as 5.8, though the computer-calculated isoelectric point from the encoded amino acid sequence is 6.3. They suggested that DJ-1 might be post-translationally modified to acquire the slightly lower pI. Our experimental data indicated that the isoelectric points of the variant and parent form of DJ-1 are 5.8 and 6.2, respectively. These values correspond well to Wagenfeld's data. Structural modifications of HPRPs remain unclarified, except for the phosphorylation of HSP27. Oxidative conversion of a sulfhydryl group(s) at a Cys residue(s) to a cysteine sulfinic acid (Cys-SO_2H) is the most plausible candidate to be responsible for the mobility shifts of HPRPs on 2D gels, since the modification of HPRPs is relatively specific to hydroperoxide or ROS-producing drugs.[12] The mechanism of toxicity of ornidazole, an ROS-producing nitroaromatic compound[36] that induces infertility, is reported to be based on inactivation of glycolytic enzymes such as triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase.[37] These enzymes are known to be inactivated by oxidation.[38,39] It is also known that sperm is labile to oxidative stress.[40] Thus, we speculate that the DJ-1 variant might be shed from sperm upon exposure to oxidative stress induced by infertility-inducing drugs. Although the biological roles of DJ-1 are still obscure, this is the first time that DJ-1 has been found to be an oxidative stress-responsive protein at the level of protein modification.

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