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DJ-1 is an Indicator for Endogenous Reactive Oxygen Species Elicited by Endotoxin

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We previously found that DJ-1 protein of pI 5.8 (DJ-1/5.8) increased on 2D gels as DJ-1 of pI 6.2 (DJ-1/6.2) decreased, upon exposure of human cells to sublethal levels of oxidative stress, such as H_2O_2 and paraquat. Here, we show that the DJ-1/5.8 increases concomitantly with endogenous production of reactive oxygen species (ROS) under endotoxin-induced inflammatory conditions. Lipopolysaccharide (LPS) significantly increased the expression of DJ-1/5.8 in murine peritoneal macrophages (MΦ) and a murine macrophage cell line (J774). Diphenylene iodonium, a flavoenzyme inhibitor, blocked the effect of LPS on DJ-1/5.8 expression. Aminoguanidine (AG), a selective inhibitor of type II nitric oxide synthase, had no effect on the DJ-1/5.8 expression, but suppressed accumulation of nitrite in the culture medium after LPS treatment. We also examined the expression of DJ-1/5.8 in lung, since acute lung injury is seen in endotoxin shock. When female mice (6-weeks old) were intraperitoneally given LPS (10 mg/kg), myeloperoxidase (MPO) activity in lung, a marker of neutrophil infiltration, was transiently raised by 3.5 fold. The expression of DJ-1/5.8 in lung was enhanced and then reverted to the control level, in parallel with the MPO activity. These results, taken together, suggest that the DJ-1/5.8 might increase in response to endogenously produced ROS, probably due to activation of NADPH oxidase, and imply that DJ-1 may be useful as an endogenous indicator of oxidative stress status *in vivo*.

Keywords: Reactive oxygen species; Nitric oxide; DJ-1; macrophage; Lipopolysaccharide; 2D PAGE

Abbreviations: 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; AG, aminoguanidine; DCF, dichlorofluorescein; DPI, diphenylene iodonium; DTT, dithiothreitol; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Glo I, glyoxalase I; H2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HPRP, hydroperoxide responsive protein; HSP27, heat shock protein 27 kDa; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MΦ, macrophage; MPO, myeloperoxidase; NO, nitric oxide; NORP, NO responsive protein; NOS, NO synthase; ROS, reactive oxygen species; Prx, peroxiredoxin

INTRODUCTION

Oxidative stress has been implicated in the pathogenesis of a wide variety of human diseases.[1,2] To develop a better understanding

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of the mechanisms that underlie ROS-dependent disorders in biological systems, recent studies have investigated several endogenous indicators that can reflect oxidative stress status *in vivo*. For example, protein carbonyl,[3] 8-OH deoxyguanosine,[4] and isoprostanes[5] are oxidation products of protein, nucleic acid, and lipid, respectively, that are formed in injured tissue.

Reactive oxygen and nitrogen species (ROS and RNS), such as $O_2^{-\cdot}$, H_2O_2, $^{\cdot}OH$, NO and NO-derived oxidants, are produced under oxidative stress conditions. NO is established to be a signaling molecule. However, oxidant species can also modify protein structures to modulate their function through oxidation and reduction, so-called redox regulation.[6] Several lines of evidence suggest that ROS can act as second messenger-like molecules. For example, DNA binding activity of transcription factors, such as NF-κB[7,8] and AP-1,[7,9] and phosphorylation of extracellular regulated kinase[10] are enhanced upon stimulation of cell surface receptors through ROS production. Kurose *et al.* reported that G-proteins are directly modified by H_2O_2, leading to functional modulation, and they suggested that G-proteins are candidates for the target molecules.[11] However, it is still unclear whether ROS are involved in cellular signaling through redox regulation in mammalian system.

We have detected an NO responsive protein (NORP)[12] and hydroperoxide responsive proteins (HPRPs)[13,14] in human cells by 2D PAGE. The NORP, glyoxalase I (Glo I), is specifically modified by NO in cooperation with GSH.[12,15] The response is characterized by an increase of its pI from 5.0 to 5.2, decline of the enzymatic activity, and reversibility of both the pI shift and the inactivation with dithiothreitol (DTT). The HPRPs consist of four types of peroxiredoxins (Prx), heat shock protein 27 (HSP27), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and DJ-1. The HPRP response is also manifested as a shift of pI on 2D gels. Although the nature of the modification of HSP27 was identified as phosphorylation, the modified structures of the other responsive proteins are not yet clear. Although the HPRPs are responsive to sublethal levels of oxidative stress reagents, such as H_2O_2 and paraquat (PQ^{2+}), they are not responsive to NO. The response of Prxs seems to be specific to hydroperoxide, though the other HPRPs are responsive to ROS in a non-specific fashion. The pI shifts of the 4 Prxs and HSP27 are readily reversible after the removal of stress, while the change of pI of DJ-1 from 6.2 to 5.8 (DJ-1/6.2 and DJ-1/5.8) is irreversible. The response of DJ-1 is less sensitive to H_2O_2 than that of Prxs, but DJ-1/5.8 is easily detectable in PQ^{2+}-treated cells as well as Prxs. These observations raise the possibility that the oxidative stress responsive proteins may discriminate between oxidant species produced within cells even under physiological conditions. In this study, we used 2D gels to examine the response of these proteins to endotoxin-elicited oxidative stress conditions, and we suggest that DJ-1 could be useful as an indicator of oxidative stress status *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS; *E. coli* 055:B5), diphenylene iodonium chloride (DPI), *o*-methoxyphenol, a 30% solution of H_2O_2 and thioglycollate broth were obtained from Wako Chemical (Osaka, Japan). Aminoguanidine (AG) and hexadecyl trimethylammonium bromide were purchased from Sigma (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was from Molecular Probes (Eugene, Oregon, USA). Pharmalytes (preblended pH 5–8 and pH 3–10) were obtained from Amersham Pharmacia Biotech (Tokyo, Japan).

Preparation of Murine Peritoneal Macrophages

Female mice (ICR, 6 weeks) were intraperitoneally inoculated with 2 ml of 4.05% thioglycolate broth per mouse and 4 days thereafter, we

collected macrophages (MΦ) according to the method of Phaire-Washington *et al.*[16] The cells were washed with phosphate-buffered saline (PBS) twice, then plated on a 12-well plate at 1×10^6 cells/ml in RPMI1640 (Nissui Pharmaceuticals; Tokyo, Japan) with 5% heat inactivated fetal bovine serum (FBS). After 2 h, attached cells were used as macrophages. Cells were incubated in serum-free medium for stimulation with LPS.

DCF Labeling and Detection of Fluorescence

In order to monitor intracellular production of hydroperoxide, the attached MΦ were labeled with H_2DCF-DA (5 μM, 10 min), washed twice with PBS, and incubated in serum-free medium in the presence or absence of LPS. The fluorescence (ex, 480/20 nm; em, 530/25 nm) was monitored for 24 h with a plate reader (CytoFlour 4000TR; PerSeptive Biosystems, Tokyo, Japan).

Culture of Cells

J774 cells (murine macrophage cell line) were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals; Tokyo, Japan) supplemented with 10% FBS that contained 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. For stimulation with LPS, J774 cells (1×10^4 cells) were incubated in serum-free medium in a 12-well plate.

Endotoxin Shock Model

Female mice (ICR, 6 weeks) were intraperitoneally given LPS (10 mg/kg) or vehicle (saline). At the indicated times, the lungs were removed and homogenized with homogenizing buffer that contained 0.25 M sucrose, 3 mM imidazole·HCl (pH 7.2) 1 mM Na_2EDTA and 0.1% EtOH. After centrifugation at 20,000*g* for 20 min at 4°C, the supernatants were analyzed by 2D PAGE. The remaining precipitates were resuspended in 50 mM KPi buffer (pH 6.0) that contained 0.5% hexadecyl trimethylammonium bromide. The suspended pellets were sonicated for 90 s in an ultrasonic cell disrupter (Branson Sonifier 250: timer, hold, output control; 2, duty cycle; 20%; Branson, Danbury, CT, USA) and centrifuged at 20,000*g* for 10 min at 4°C. Small aliquots of the supernatants were added to 2.5 ml of myeloperoxidase (MPO) assay solution that contained 30 mM *o*-methoxyphenol and 50 μM H_2O_2 in 50 mM KPi (pH 6.0) at 25°C. Increase of absorbance at 470 nm was monitored for 3 min. MPO activity was calculated using ε470 (26,600 M^{-1} cm^{-1}) as μmol/min/mg protein and expressed as units/mg protein.[17]

Two-dimensional Polyacrylamide Gel Electrophoresis (2D PAGE)

The cells were directly lysed with lysis buffer containing 9.2 M urea, 2% (w/v) CHAPS, 2% (v/v) Pharmalytes (pH 5–8: pH 3–10, 4:1, v/v), 2 mM Na_2EDTA and 1 mM PMSF with 70 mM DTT. Otherwise, protein samples were mixed with the lysis buffer (1:1) prior to 2D PAGE analysis. 2D PAGE was performed by O'Farrell's method[18] with slight modifications.[13] In brief, for the first dimension, isoelectric focusing (IEF) gels (1.7 mm in diameter and 105 mm in length) consisted of 9.2 M urea, 2% (w/v) CHAPS, 2% Pharmalytes (pH 5–8:pH 3–10, 4:1, v/v), and 4% acrylamide (mono:bis, 30:0.8, w/w). IEF was performed at a constant voltage of 500 V for 15 h. Then the gels were equilibrated in sample buffer for SDS-PAGE for 10 min, and fixed with 1% agarose on the flat top of the second gels. Electrophoresis in the second dimension was performed as described by Laemmli.[19]

Immunoblotting

After electrophoresis, the proteins on gels were transferred onto PVDF membrane (Immobilon P; Millipore, Bedford, MA, USA), according to the method of Hirano and Watanabe.[20] The membrane was treated with a blocking reagent

overnight at 4°C, and incubated with antibodies specific to Prx II, Prx III and DJ-1[14] for 1 h at r.t. The membrane was washed with tris-buffered saline (TBS) that contained 0.1% Tween-20 twice, then incubated with peroxidase-conjugated anti-rabbit IgG (Chemicon, Temecula, CA, USA) for 1 h at r.t. It was washed with TBS plus Tween-20 twice and TBS once, and specific protein spots were visualized by an ECL kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to the manufacturer's instructions.

Image Analysis

Immunodecoration of DJ-1 was scanned with a scanner (model GT-8500; Epson, Suwa, Japan) using PHOTOSHOP 2.5J software (Adobe System, San Jose, CA, USA). Intensities of spots were quantified with NIHimage software (National Institutes for Health, Bethesda, MD, USA).

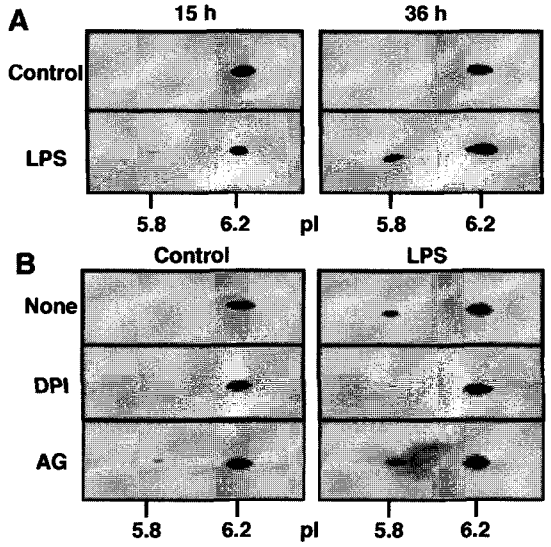

FIGURE 1 Expression of DJ-1 proteins in mouse peritoneal macrophages on 2D gels. (A) MΦ (1×10^6 cells) were stimulated with LPS (10 ng/ml). At the indicated times, cellular proteins were resolved by 2D PAGE and immunoblotting using specific antibodies against DJ-1. (B) MΦ (1×10^6 cells) were stimulated by LPS (10 ng/ml) for 24 h in the presence or absence of DPI (500 nM) or AG (500 μM). DJ-1 responses were analyzed by immunoblotting following 2D PAGE. The regions around 25 kDa and in the range between pI 5.5 and 6.5 are shown. The data are representative results from two to four independent experiments.

Protein Determination

Protein concentration was determined using the Bio-Rad protein assay kit based on the method of Bradford using bovine serum albumin as the standard.[21]

RESULTS

Detection of DJ-1 Proteins in LPS-stimulated Murine Macrophages

To detect NORP and HPRPs generated in response to endogenously produced ROS or RNS under physiological conditions, we carried

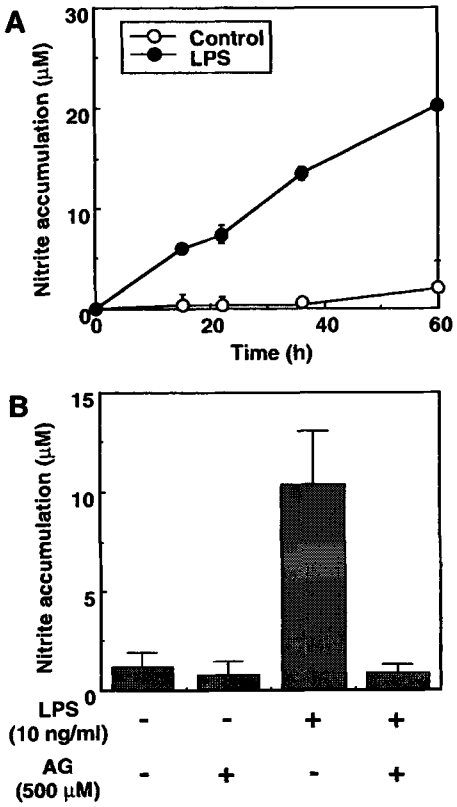

FIGURE 2 Nitrite accumulation in culture medium of mouse peritoneal macrophages stimulated with LPS. (A) MΦ (1×10^6 cells) were treated with 10 ng/ml LPS. At the indicated times, nitrite concentration in the culture medium was determined by Griess assay. The data represent means ± SD ($n = 3$). (B) Effect of AG (500 μM) on nitrite accumulation induced by LPS. The data represent means ± SD ($n = 3$).

out 2D PAGE and immunoblotting analysis of cellular proteins in murine primary MΦ treated with LPS. As shown in Fig. 1, we detected a distinct DJ-1 response in LPS-stimulated MΦ. However, we did not detect any pI shift of Prx II or Prx III by immunoblotting using specific antibodies, or any response of the other oxidative stress responsive proteins, such as Glo I, on 2D gels with silver staining (data not shown). Upon exposure of MΦ to 10 ng/ml LPS, DJ-1/5.8 was time-dependently detected after 15 h (Fig. 1A). To examine the involvement of oxidant-producing enzymes, we measured the effects of DPI, a flavoenzyme inhibitor, and AG, a selective inhibitor of type II NO synthase (NOS II), on the DJ-1/5.8 expression. Figure 1B shows that

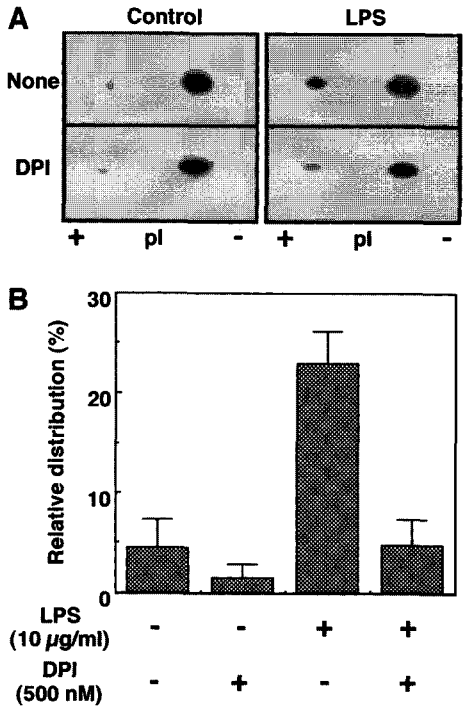

FIGURE 3 Detection of DJ-1 proteins in J774 cells on 2D gels. (A) After stimulation of J774 cells with LPS (10 μg/ml) for 16 h in the presence or absence of DPI (500 nM), DJ-1 responses were analyzed by 2D PAGE and immunoblotting. The regions around 25 kDa and in the range between pI 5.5 and 6.5 are shown. (B) The spot intensities were quantified by image analysis. Relative distribution means the ratio (%) of DJ-1/5.8 in total DJ-1 proteins (DJ-1/5.8 plus DJ-1/6.2).

DPI clearly inhibited the formation of DJ-1/5.8, while AG had no effect.

To confirm NOS II activation under this condition, we measured nitrite (NO_2^-), a stable oxidative metabolite of NO, in the culture medium after LPS treatment. As shown in Fig. 2A, NO_2^- was accumulated in response to LPS and the concentration increased continuously during 36 h stimulation. AG completely prevented this NO_2^- accumulation (Fig. 2B). These results suggest that NOS II is induced in LPS-treated MΦ. To examine the formation of other types of ROS, we used a fluorescent indicator, DCF, which can determine intracellular hydroperoxide levels. However, we failed to detect any increase of DCF fluorescence after LPS treatment, compared with the control (data not shown).

We also examined the expression pattern of DJ-1 proteins on 2D gels by using the murine macrophage cell line, J774. As shown in Fig. 3A, J774 cells slightly expressed DJ-1/5.8, besides DJ-1/6.2, without LPS stimulation. Although higher amounts of LPS (10 μg/ml) were required in the case of J774 cells compared with the primary MΦ described above, expression of DJ-1/5.8 was enhanced upon treatment with LPS (10 μg/ml). DPI (500 nM) prevented the enhancement of DJ-1/5.8 expression in J774 cells. Under this condition, no accumulation of NO_2^- was detectable in culture medium during the experimental period (data not shown). These results suggest that the enhancement of DJ-1/5.8 formation in J774 cells in response to LPS involves ROS, but not NO.

Induction of DJ-1/5.8 in Mouse Lung by Endotoxin Shock

To elicit acute endotoxin shock, female mice (6 weeks) were intraperitoneally given 10 mg/kg LPS. At this dose, although about 10% reduction of the body weight was observed after 24 h, all the mice were still alive after 3 days. At 20 mg/kg LPS, all mice died within 3 days (data not

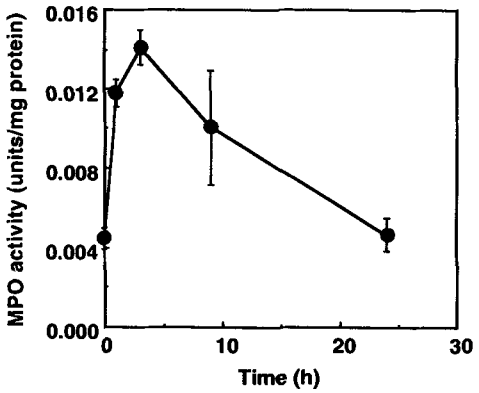

FIGURE 4 Myeloperoxidase activity in mouse lung after intraperitoneal administration of LPS. Female mice (6 weeks) were intraperitoneally given LPS at 10 mg/kg. At the indicated times, the lungs were removed and homogenized for MPO activity assay as described in Materials and Methods. The MPO activities are means ± SD ($n = 3$) from one of three independent experiments, which gave similar results.

shown). In order to examine acute lung injury after LPS administration, we examined myeloperoxidase (MPO) activity in lung as a marker of neutrophil infiltration in inflammatory response.[22] Figure 4 shows the time-course of MPO activity following LPS administration. The MPO activity transiently increased by 3.5-fold at 3 h after administration, then reverted to the control level at 24 h. This suggests neutrophil accumulation and subsequent dispersion in lungs of LPS-treated mice. The lung tissue was isolated, homogenized and subjected to 2D PAGE. As shown in Fig. 5A, both DJ-1/5.8 and DJ-1/6.2 were expressed in control lung. After LPS administration, the expression of DJ-1/5.8 transiently increased, and then reverted to the control level at 24 h. Image analysis (Fig. 5B) indicated a close correlation to the change of MPO activity in lung.

DISCUSSION

In this study, we showed that expression of DJ-1/5.8 was increased upon stimulation of macrophages with LPS and upon eliciting lung inflammation in mice by LPS administration. Previously, we observed the enhancement of DJ-1/5.8 expression upon exposure of human cells to oxidative stress, such as H_2O_2 and PQ^{2+}.[13,14] When cells were treated with sublethal levels of PQ^{2+}, DJ-1/5.8 was generated without any increase of DCF-derived fluorescence.[14] In this study, we also detected DJ-1/5.8 expression without increase of DCF-derived fluorescence. However, a flavoenzyme inhibitor, DPI, clearly

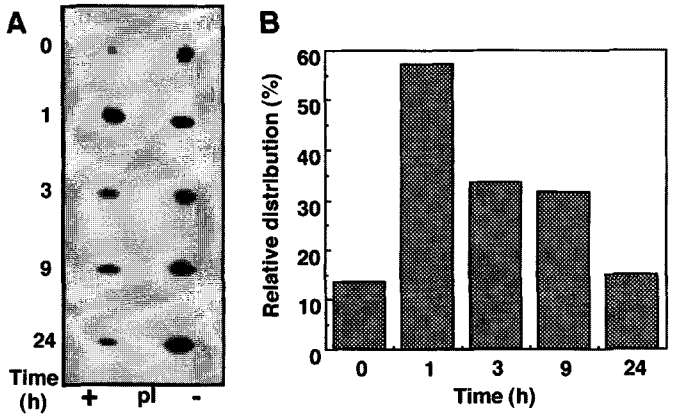

FIGURE 5 Expression of DJ-1 proteins in injured lung after endotoxin shock. At the indicated times after LPS administration as described in the legend to Fig. 4, the lungs were removed, homogenized and centrifuged as described in Materials and Methods. The supernatant was used for 2D PAGE analysis. (A) Immunodecoration, (B) quantitation by image analysis.

suppressed the formation of DJ-1/5.8 in both primary MΦ and J774 cells. It is considered that DPI can block ROS production by flavoenzymes, mainly NADPH oxidase.[23] LPS can activate NADPH oxidase in phagocytes.[24] Therefore, the expression of DJ-1/5.8 may reflect endogenous ROS production due to cell stimulation.

In LPS-treated MΦ, expression of NOS II is induced *via* activation of transcription,[25] in addition to activation of NADPH oxidase. In this system, NO_2^-, a stable oxidation product of NO, was accumulated in the culture supernatant of primary MΦ in response to LPS. The fact that AG blocked the NO_2^- accumulation supports the induction of NOS II by LPS. However, we failed to detect any pI change of Glo I, which has been detected in response to exogenous addition of NO-donating compounds,[12] even though relatively large amounts of NO are produced in MΦ in this system. It is likely that Glo I does not interact with NO under physiological conditions. In this study, AG had no effect on the DJ-1/5.8 formation induced by LPS, while DPI prevented the expression of DJ-1/5.8 in both MΦ and J744 cells. Although the nature of the structural modification of DJ-1 that is responsible for the pI shift remains unclear, our results suggest that DJ-1/5.8 is generated by endogenous ROS, independently of NO. The fact that J774 did not produce NO after LPS activation in this system supports this idea.

In this experiment using murine MΦ and J774 cells, we did not detect any pI variants of four types of Prxs, which we had previously found as HPRPs.[13] The failure to detect the pI shift of Prxs might be due to the molecular characteristics of Prxs. One of the major functions of Prxs is hydroperoxide reduction. The pI shifts of Prxs are specific to hydroperoxide, and those of Prx I and Prx II are more sensitive to extracellular hydroperoxide than that of DJ-1. The pI changes of Prxs are completely reversed after the removal of H_2O_2 from cells. In contrast, the pI shift of DJ-1 is irreversible. The lack of reversibility of the pI shift of DJ-1 in response to oxidative stress might be the reason why we could detect it under the conditions used. Moreover, DJ-1 might interact with $O_2^{-\cdot}$ or some oxidant species other than H_2O_2. The fact that DJ-1/5.8 was detectable in PQ^{2+}-treated human cells, LPS-treated MΦ and an endotoxin shock mouse lung model may support this idea.

In spite of the irreversible character of DJ-1 formation *in vitro*, we could detect the pI shift of DJ-1 in a reversible manner in mouse lung after intraperitoneal administration of LPS (Fig. 5). It is considered that the DJ-1 response, which we detected in lung might be due to neutrophils infiltrated into the lung in response to endotoxin, in addition to the DJ-1 generated in lung tissue itself, since the DJ-1/5.8 formation was closely correlated with MPO activity (Fig. 4).

DJ-1 was originally found as a gene product with cell transforming activity.[26] It is ubiquitously expressed in rodent and is especially abundant in testis.[27] Wagenfeld *et al.* found a DJ-1 homolog in epididymal fluid of drug-induced infertile male rat by 2D PAGE.[28] An RNA binding protein regulatory subunit purified from rat hepatoma cells was identical with DJ-1.[29] However, the function of DJ-1 protein is not yet clear. We previously reported that DJ-1/5.8 is detectable on 2D gels after cell culture during several days without any stress reagent.[14] In this work, DJ-1/5.8 was detectable in J774 cells and lung tissues without any treatment, and the expression of DJ-1/5.8 was enhanced in response to LPS in a DPI-inhibitable manner. These results suggest that DJ-1 could respond to small amounts of ROS produced during aerobic metabolism in cells and tissues. Our observations raise the possibility that DJ-1 might be one of the ROS target proteins, as well as Prxs. We hypothesize that DJ-1 functions as an antioxidant protein to rescue host cells from the effects of activation of ROS-producing enzymes.

In conclusion, we suggest that DJ-1 may be useful as an endogenous indicator of ROS production or oxidative stress status within cells and *in vivo*.

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