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Peroxiredoxin III-deficiency Sensitizes Macrophages to Oxidative Stress

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As a mitochondrial scavenger of reactive oxygen species (ROS), peroxiredoxin III (PrxIII) plays an important role in regulating intracellular ROS level. We previously found that PrxIII knockout (PrxIII$^{-/-}$) mice were more sensitive than wild-type (PrxIII$^{+/+}$) controls to intratracheal inoculation of lipopolysaccharide (LPS), but the precise mechanism remained to be obscure. In the present study, we detected the levels of ROS and tumour necrosis factor alpha (TNF-α) in mouse bone-marrow-derived macrophages. LPS stimulation induced transient increase of ROS production and augmentation of TNF-α accumulation in PrxIII$^{-/-}$ macrophages. In addition, we observed reduced viability and increased apoptosis in PrxIII$^{-/-}$ macrophages exposed to LPS. Our results provide direct evidence that PrxIII is necessary for macrophages to protect against LPS-induced oxidative stress.

Key words: lipopolysaccharide, macrophage, mouse, peroxiredoxin III, reactive oxygen species.

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PrxIII, peroxiredoxin III; ROS, reactive oxygen species; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tumor necrosis factor alpha.

Since the first report that MER5, now called peroxiredoxin III (PrxIII), is a new-type antioxidant gene possessing active cysteines (*1*), PrxIII has been extensively investigated for its antioxidant function. PrxIII is ubiquitously expressed in mammalian tissues and is mainly located in mitochondria (*2*, *3*). Since mitochondria are the main source of reactive oxygen species (ROS) production and are sensitive to oxidative damage, abundance of PrxIII in mitochondria implies that PrxIII plays an important role in regulating intracellular ROS level.

According to our previous study, PrxIII knockout (PrxIII$^{-/-}$) mice could grow mature without histological abnormalities in organs but were more susceptible to lipopolysaccharide (LPS)-induced oxidative stress than wild-type controls (PrxIII$^{+/+}$), which was supposed to be the result of excessive response of PrxIII-deficient macrophages (*4*). To provide insight into the underlying mechanism, we detected ROS production in macrophages derived from mouse bone marrow.

After flushing bone marrow from femurs of 6-week-old mice, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin (100 μg/ml)/streptomycin (50 μg/ml) supplemented with 10% L-929 cell-conditioned medium as a source of macrophage colony-stimulating factor. Seven days later, macrophages were harvested using phosphate-buffered saline (PBS, pH 7.2) containing 10 mM EDTA and 20% FBS. Harvested cells were re-cultured at 1.25×10^6 cells/ml density in FBS- and phenol red-free medium. Twenty-four hours later, 2 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes) was added to each well and ROS production in macrophages was estimated at indicated times through oxidation of DCFH-DA which was monitored by a fluorescent measurement system (Cytofluor 2350, Millipore) at 504 nm excitation and 524 nm emission (*4*).

Under basal conditions, there was no significant difference in ROS production between PrxIII$^{-/-}$ macrophages and wild-type controls (data not shown). We then detected ROS production in macrophages stimulated with 100 ng/ml LPS (Sigma). As shown in Fig. 1, the ROS level was higher in PrxIII$^{-/-}$ macrophages than in wild-type controls at 1- and 3-h points (PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.05$). Interestingly, significant decrease of ROS was observed in PrxIII$^{-/-}$ macrophages at 12- and 24-h points as compared with wild-type controls (PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$).

Since we previously observed inflammatory infiltration in lungs of PrxIII$^{-/-}$ mice exposed to LPS (*4*), the release of tumour necrosis factor alpha (TNF-α), a pro-inflammatory mediator, from macrophages was measured in the present study. Harvested macrophages were re-cultured overnight in six-well plates (1.25×10^6 cells/ml) with 4 ml of medium per well in a humidified incubator with 5% CO_2 at 37°C. After addition of 100 ng/ml LPS, culture supernatants were collected at indicated times and TNF-α level was assessed using Mouse TNF-α BD OptEIA ELISA Set (BD

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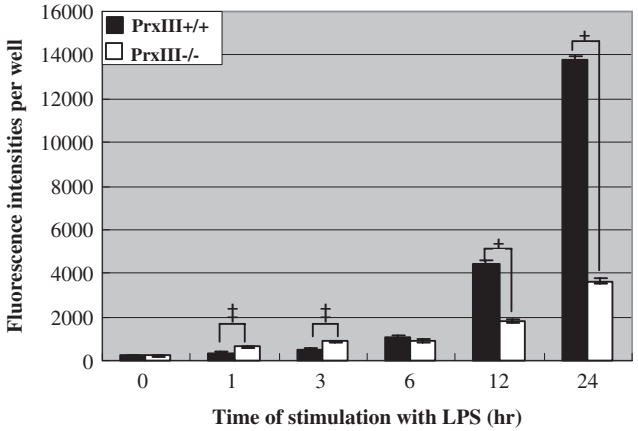

Fig. 1. **ROS production in macrophages after LPS stimulation.** Data were presented as mean ± SD of three separate experiments and compared by analysis of variance. The ROS level at 1- and 3-h points was higher in PrxIII$^{-/-}$ macrophages than in wild-type controls (‡PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.05$), while a significantly low level of ROS was observed in PrxIII$^{-/-}$ macrophages at 12- and 24-h points (†PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$).

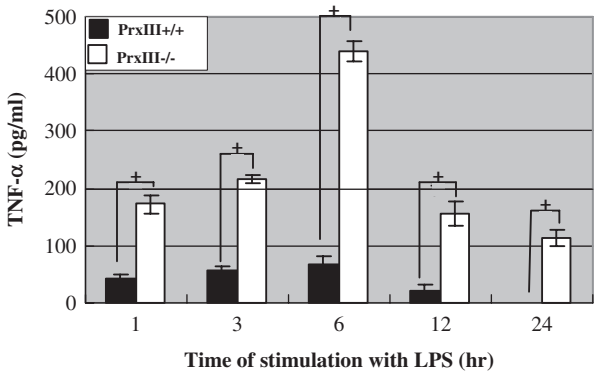

Fig. 2. **Detection of TNF-α level in macrophages after LPS stimulation.** Data were presented as mean ± SD of three separate experiments and compared by analysis of variance. The TNF-α level was significantly higher in PrxIII$^{-/-}$ macrophages than in wild-type controls (†PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$).

Biosciences Pharmingen). Colour was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate system (KPL) and absorbance was measured at 450 nm in a spectrophotometer (Amarsham Pharmacia). As shown in Fig. 2, TNF-α level in the medium of PrxIII$^{-/-}$ macrophages was significantly higher than in that of wild-type controls (PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$) and reached its peak at 6 h after LPS stimulation. TNF-α was undetectable in macrophages under basal condition (data not shown). In addition, we performed western blotting to detect TNF-α expression in macrophages treated with LPS for 6 h. Cellular proteins (20 μg) were separated on 15% SDS–PAGE and TNF-α was recognized with anti-murine TNF-α polyclonal antibody (Biovision). β-Actin was used as internal control and ECL western blotting detection system (Amersham) was used for visualization. As shown

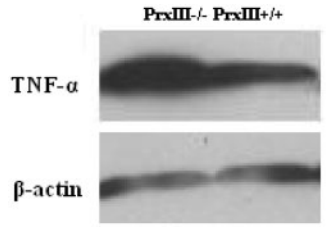

Fig. 3. **Western blot analysis for expression of TNF-α in macrophages treated by LPS for 6 h.** The signal for TNF-α protein was significantly enhanced in PrxIII$^{-/-}$ macrophages as compared with that in wild-type controls.

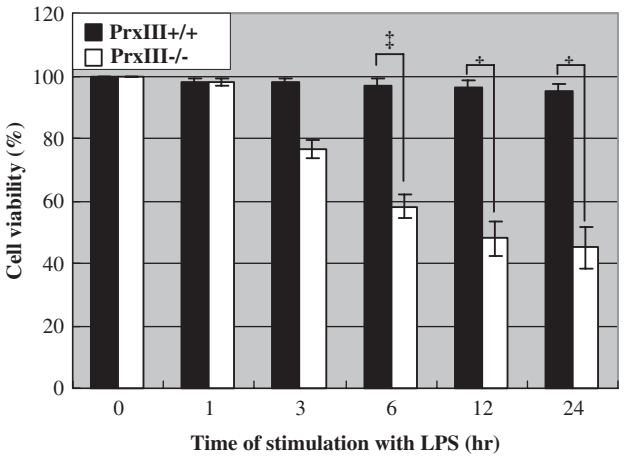

Fig. 4. **Cell viability by trypan blue exclusion assay**. The number of viable PrxIII$^{-/-}$ macrophages was significantly decreased 6 h after LPS stimulation (‡PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.05$; †PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$).

in Fig. 3, the TNF-α level was significantly increased in PrxIII$^{-/-}$ macrophages as compared with that in wild-type controls.

To understand the possible mechanism underlying the pattern of ROS production in PrxIII$^{-/-}$ macrophages exposed to LPS, we investigated cell viability by trypan blue exclusion assay and found significant reduction of surviving PrxIII-deficient macrophages (Fig. 4). After LPS treatment for 24 h, apoptotic cell death was measured with a quantitative sandwich-ELISA assay (Cell Death Detection ELISA Plus, Roche) detecting mono- and oligonucleosomes according to the kit instruction. The relative value of optical density at 405 nm was 4.06 ± 0.62 for PrxIII$^{-/-}$ macrophages and 1.59 ± 0.24 for wild-type controls. There was a significant increase of apoptosis in PrxIII$^{-/-}$ macrophages compared with wild-type controls (PrxIII$^{-/-}$ *vs.* PrxIII$^{+/+}$: $P < 0.01$).

In the present study, we observed close pattern of ROS production between PrxIII-deficient and wild-type macrophages under basal conditions. Nevertheless, LPS stimulation caused transient excess of ROS release, augmentation of TNF-α accumulation and increase of apoptosis in PrxIII$^{-/-}$macrophages. Our results indicate that PrxIII$^{-/-}$ macrophages are more sensitive to LPS stimulation than wild-type controls and suggest that PrxIII is an important regulator of intracellular ROS level in macrophages under oxidative stress.

As an inflammatory agent from outer membrane of Gram-negative bacteria, LPS induces phagocytes to produce ROS (*5*, *6*). In addition to destroying injurious agents, ROS is produced for signal transduction that activates transcriptional factors and induces subsequent release of pro-inflammatory cytokines (*7–9*). Nevertheless, macrophages require antioxidants to harness ROS as a beneficial mediator. Excessive amount of ROS would result in overwhelming immune response and direct damage to protein, DNA, and lipid (*10*, *11*). In addition, the cross talk and biphasic activation between inflammatory mediators further contributes to the deterioration of oxidative damage (*12*). It might be the excessive ROS production in PrxIII-deficient macrophages that caused subsequent TNF-α augmentation. Thereafter, ROS level was decreased due to reduced number of surviving macrophages as demonstrated by detection of cell viability and apoptosis. There might be multiple mechanisms that contribute to LPS-induced apoptosis of PrxIII-deficient macrophages. First, excessive ROS induces direct injury of protein, DNA and lipid and subsequent apoptosis. Second, oxidative damage on mitochondria causes mitochondrial dysfunction and subsequent apoptosis. As a mitochondrial scavenger of ROS, PrxIII plays an important role in maintaining mitochondrial homeostasis (*13*). It has been demonstrated that PrxIII depletion or inactivation causes elevation of ROS level, decrease of mitochondrial membrane potential, release of cytochrome c and activation of caspases, which ultimately results in apoptosis (*14*, *15*). Last, ROS-induced TNF-α secretion triggers apoptotic process directly (*16*).

Our results has provided explanation for the previous finding that intratracheal inoculation of LPS causes serious inflammation and oxidation in lungs of PrxIII$^{-/-}$ mice (*4*), which suggests that PrxIII is an active scavenger of ROS under oxidative stress. In fact, PrxIII is among the antioxidants of early response to oxidative stress according to the report by Brown *et al*. (*17*). Together with our recent report on the role of PrxIII in placentas (*18*) where ROS is increasingly produced with gestation progression, we conclude that PrxIII executes its antioxidant function predominantly under oxidative conditions.

We previously showed that ROS level in PrxIII-deficient peritoneal macrophages were higher than that of wild-type controls at basal conditions. One possibility for the discrepancy might be individual features of macrophages due to different origins but the precise mechanism remains to be investigated.

CONFLICT OF INTEREST

None declared.

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