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Protein disulfide isomerase suppresses the transcriptional activity of NF-κB

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Abstract

We report here that the transcriptional activity of NF-κB is negatively regulated by protein disulfide isomerase (PDI). Overexpression of PDI in RAW 264.7 cells strongly suppressed the LPS-induced production of inflammatory cytokines as well as NF-κB-dependent luciferase activity. This negative regulation of NF-κB was reversed by bacitracin, a PDI inhibitor. Interestingly, NF-κB/DNA complex formation and phosphorylation of NF-κB subunits was intact in PDI-expressing cells following stimulation with LPS. In addition, PDI and another redox regulator, thioredoxin (TRX), had opposite effects on NF-κB-dependent gene expression: activation of the NF-κB pathway by TRX was suppressed by expression of PDI in a dose-dependent manner. Finally, PDI expression was induced by the anti-inflammatory cytokine IL-10, and IL-10-mediated inhibition of LPS-induced IL-6 expression was reduced by bacitracin. These findings clearly demonstrate that PDI is a negative regulator of NF-κB, and may act downstream of IL-10 in this signaling pathway.
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Keywords: Protein disulfide isomerase; NF-κB; Thioredoxin; LPS; RAW 264.7; IL-10; Bacitracin

Inflammatory cytokines such as IL-6, IL-1β, or TNF-α contribute to the development of symptoms associated with the acute and chronic phases of various immune and regeneration disorders. The production of these cytokines is highly dependent upon the activity of NF-κB transcription factors [1,2]. NF-κB is a homo- or hetero-dimer complex composed of Rel (c-Rel), p65 (Rel A), Rel B, p50 (NF-κB), and p52 (NF-κB-2). All of these proteins contain a conserved region of approximately 300 amino acids at the N-terminal region, which is called the Rel homology domain. The Rel domain acts as a DNA binding domain and a homo/hetero-dimerization motif in a variety of species. NF-κB dimers associate with I-κB to form an inactive complex which localizes in the cytoplasm of unstimulated cells. When cells are stimulated by agonists, I-κB is inactivated through enzymatically modifications such as phosphorylation and ubiquitination, followed by proteolysis. NF-κB becomes activated after dissociation from I-κB and translocates to the nucleus [1,2]. The transcriptional activities of NF-κB are under the control of several specific key molecules including protein kinase A [3], p38 MAP kinase [4], and co-activators such as HMG I(Y), Bcl-3, and p300 [5–7]. Other transcription factors, such as glucocorticoid receptors, Sp1, and some members of the bZip family including C/EBP and AP-1, are also involved in regulating NF-κB transcriptional activity [8–11]. Those findings highlight the importance of accurate cellular regulation of the NF-κB signaling pathway.

Previous studies have proposed that reactive oxygen species (ROS) may contribute to NF-κB activation. This hypothesis is based on the observations that the NF-κB signaling pathway is activated by oxidant molecules such as hydrogen peroxide or butyl peroxide, and inhibited by anti-oxidant molecules such as pyrrolidinedithiocarbamate (PDTC) or *N*-acetyl-L-cysteine (NAC) [12,13]. However, a recent study demonstrated that the inhibition of NF-κB by both PDTC

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and NAC is independent of their antioxidative function and endogenously produced ROS do not mediate NF-κB activation [14]. On the other hand, the redox status of disulfides on NF-κB itself is correlated with the DNA binding activity of NF-κB. For instance, thioredoxin (TRX) has been shown to enable DNA binding activity by reduction of a disulfide bond on NF-κB [15,16].

Protein disulfide isomerase (PDI; E.C. 5.3.4.1) contains two redox catalytic motifs (-WCGHCK-) with homology to TRX [17,18]. PDI is a multifunctional protein catalyzing the oxidation, reduction, and isomerization of disulfide bridges within a molecule during the post-translational modification steps [19], as well as being a component of the enzymes prolyl 4-hydroxylase [20] and microsomal triglyceride transfer protein [21]. Although PDI is mainly localized in the endoplasmic reticulum, it also is detected in nuclear envelopes, plasma membranes, mitochondria, and other organelles of mammalian cells [22,23]. For example, PDI on the surface of platelets mediates platelet aggregation and secretion by activation of glycoprotein IIbIIIa receptor [24]. Moreover, in plant chloroplasts, the redox regulation by PDI is essential for the translational gene expression in photosynthesis [25]. In contrast, in mammalian cells, the role of PDI in the regulation of gene expression is poorly understood.

Recently, we have shown that the expression of PDI was up-regulated in renal tissues of patients with immunoglobulin A nephropathy, which is a renal disease causing glomerular inflammation and sclerosis [26]. This observation suggests that PDI may play a critical role in immune and inflammatory responses. Here, we demonstrate that PDI has an inhibitory effect on cytokine production as well as a negative regulatory effect on NF-κB-dependent gene expression in RAW 264.7 cells. Moreover, the anti-inflammatory cytokine IL-10 induced PDI production, and its effect was reduced by bacitracin, an inhibitor of PDI. Thus, PDI may be one of the intracellular effector molecules involved in anti-inflammatory reactions.

Materials and methods

Reagents and plasmids. LPS, pyrrolidinedithiocarbamate (PDTC), and bacitracin were purchased from Sigma (St. Louis, MO). Mouse IL-10 was purchased from R&D Systems (Minneapolis, MN). The plasmid NF-κB-Luc [27], which contains three repeating tandem copies of the NF-κB binding oligonucleotide derived from the immunoglobulin κ gene sequence upstream of the luciferase structural gene, was a kind gift of Dr. T. Fujita (The Tokyo Metropolitan Institute of Medical Science, Japan). The expression plasmids pME-PDI and pME-TRX were constructed by cloning the mouse PDI cDNA (Accession No. J05185) and the human TRX cDNA (Accession No. J04026), respectively, into the mammalian expression vector pME18S, which carries a strong chimerical promoter of SRα, including SV40, P_E, and HTLV-1 LTR R-5U sequences [28]. The pME18S vector was a gift from Dr. K. Maruyama (University of Tokyo, Japan). The expression plasmid pcDNA3.1-PDI was constructed by cloning the mouse PDI cDNA into mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) downstream of the cytomegalovirus promoter.

Cell culture and cytokine assay. Mouse macrophage RAW 264.7 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were seeded into a 96-well tissue culture plate at 2×10^4 cells per well. After 18 h of culture, cells were treated with 1 μg/ml LPS and/or 100 ng/ml IL-10 for another 20 h incubation period. The amounts of murine IL-6 and GM-CSF in culture supernatants were measured by ELISA according to manufacturer's protocol (Genzyme, Cambridge, MA). For the RT-PCR analysis of cytokines, RAW 264.7 cells were treated for 3 h with 1 μg/ml LPS. Total RNAs were isolated with Isogen RNA Extraction Kit (Nippon Gene, Tokyo). Genomic DNA was removed with a Message Clean Kit (GenHunter, Nashville, TN). Reverse transcription of total RNA was carried out using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA) according to manufacturer's protocol. PCR conditions were as follows: denaturation, 94 °C for 45 s; annealing, 60 °C for 45 s; and extension, 72 °C for 2 min; 20–35 cycles. A model 9600 thermal cycler (Perkin–Elmer, Foster City, CA) was used. The primer sets were as follows; for mouse IL-6 mRNA: 5′-ACTGATGCTGGTGACAAC-3′ and 5′-TCCACAAACTGATATG CT-3′, for mouse G3PDH mRNA: 5′-TGAAGGTCGGTGTGAACG GATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Following PCR, PCR products were analyzed by agarose gel electrophoresis.

Transfection and luciferase assay. The transfection of expression vectors was performed by the electroporation method. RAW 264.7 cells (5×10^7) were washed with DMEM supplemented with 10% FCS without antibiotics and resuspended in 200 μl DMEM supplemented with 10% FCS with a total of 10 μg plasmid DNA (1–8 μg pME-PDI and/or pME-TRX, 1 μg NF-κB-Luc, and 1 μg pRL-TK (Promega, Madison, WI) as a transfection efficiency control). The total concentration of DNA was adjusted with the empty plasmid (pME18S). Electroporations conditions were as follows: 750 V cm^{-1} and 960 μF, using a Gene Pulser (Bio-Rad, Hercules, CA). Transfected cells were resuspended in 12.5 ml DMEM supplemented with 10% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Five hundred microliters of cell suspension medium was transferred to each well of a 24-well tissue culture plate and incubated for 18 h. After the incubation, 1 μg/ml of LPS was added to the experimental wells. After 20 h, luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The relative luciferase activity was normalized for the activity of the internal control. For the preparation of stable PDI transfectants, RAW 264.7 cells were electroporated with 10 μg pcDNA3.1-PDI as described above. Transfectants were cultured for 4 days, followed by selection in G418 (Nacalai Tesque, Kyoto, final concentration of 700 μg/ml). G418 containing medium was replaced 2–3 times over a 2-week period. Some drug-resistant clonal lines were prepared from this trial by limiting dilution into a 96-well tissue culture plate.

Immunoblotting. RAW 264.7 cells were washed with PBS and lysed in 100 μl of cold lysis buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF) for 10 min on ice with occasional mixing. The lysates were centrifuged at 10,000*g* for 10 min. The resulting supernatant was used as a cytosol fraction sample. Cytosolic extracts (5 μg of protein) were fractionated by SDS–PAGE (4–20% gradient gel) and electrotransferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were probed with rabbit anti-PDI polyclonal antiserum (SPA-890; Stressgen, Victoria, BC) at a 1:1000 dilution and visualized with the enhanced chemiluminescence system (Amersham–Pharmacia Biotech, Buckinghamshire).

Electrophoretic mobility shift assays. RAW 264.7 cells were treated with 300 μM PDTC for 2 h before stimulation with 1 μg/ml LPS for 20 min. After two washing steps with PBS, nuclear extracts were

prepared as previously described [29]. Ten micrograms of nuclear extracts was added to 25 μl of incubation buffer (10 mM Tris–HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 4% glycerol, and 80 μg/ml salmon sperm DNA), and 100,000 cpm of ^{32}P-labeled NF-κB double strand DNA probe (AAA TCG TGG AAT TTC CTC TGA C). The incubation period was 20 min at room temperature. These samples were separated by electrophoresis in a 6% polyacrylamide gel that was run in 0.25× TBE buffer for 2 h. The gels were dried and analyzed using BAS 2000 image analyzer (Fuji Film, Tokyo).

Phosphorylation of NF-κB subunit proteins. RAW 264.7 cells were metabolically labeled with $^{32}P_i$ for 2 h. Labeled cells were pretreated with 300 μM PDTC for 2 h before stimulation with 1 μg/ml LPS for 20 min. The cells were lysed, NF-κB subunit proteins were immunoprecipitated twice with either anti-p50 (NLS) or anti-p65 (C-20) antibodies (Santa Cruz, Palo Alto, CA) and separated by SDS–PAGE, and detected by autoradiography, using previously published protocols [30].

Results

PDI suppresses LPS-induced cytokine production

In order to investigate the role of PDI on immune and inflammatory responses, we examined the effect of PDI on the production of inflammatory cytokines in LPS-stimulated RAW 264.7, a murine macrophage cell line. We established several clones that stably over-express PDI in RAW 264.7 cells (Fig. 1A). These PDI-transfected cell lines, Nos. 3–6, exhibited a significant decrease in IL-6 production compared to the control vector-transfected cell lines, Nos. 1 and 2 (Fig. 1B). Levels of GM-CSF cytokine in the supernatant were

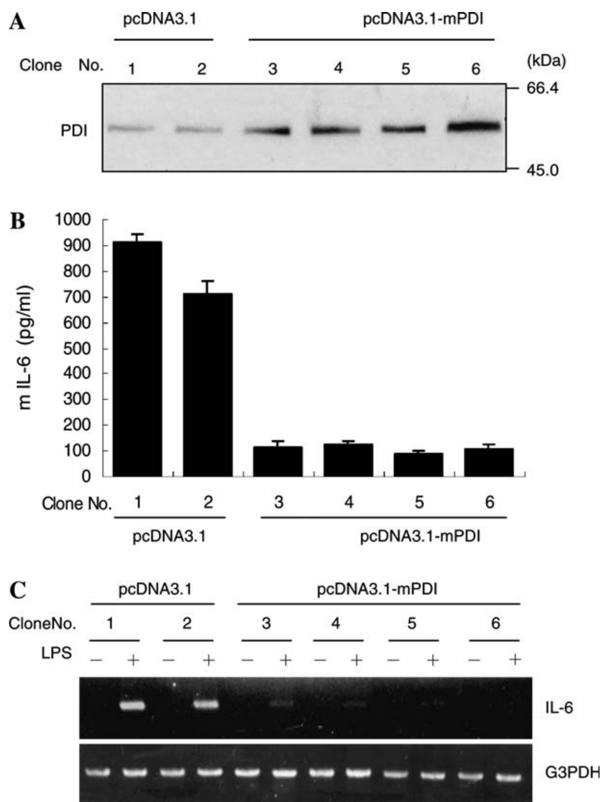

Fig. 1. PDI inhibits LPS-induced cytokine expression. (A) RAW 264.7 cells were transfected with pcDNA3.1 (Nos. 1 and 2) or pcDNA3.1-PDI (Nos. 3–6). The transfectants were lysed and subjected to immunoblot analysis using a rabbit anti-PDI polyclonal antiserum. (B) Quantitation of IL-6 in the culture supernatants of PDI-transfected cell lines. Transfectants were stimulated with 1 μg/ml LPS for 20 h, and IL-6 in the supernatants was measured by ELISA. Data are expressed as means and standard deviations of triplicate samples. (C) RT-PCR analysis of LPS-induced IL-6 mRNA expression in PDI-transfected cell lines. The transfectants were treated for 3 h with 1 μg/ml LPS. Total RNA was extracted and RT-PCR was carried out. The amplified products were analyzed by agarose gel electrophoresis. G3PDH was used as an internal control.

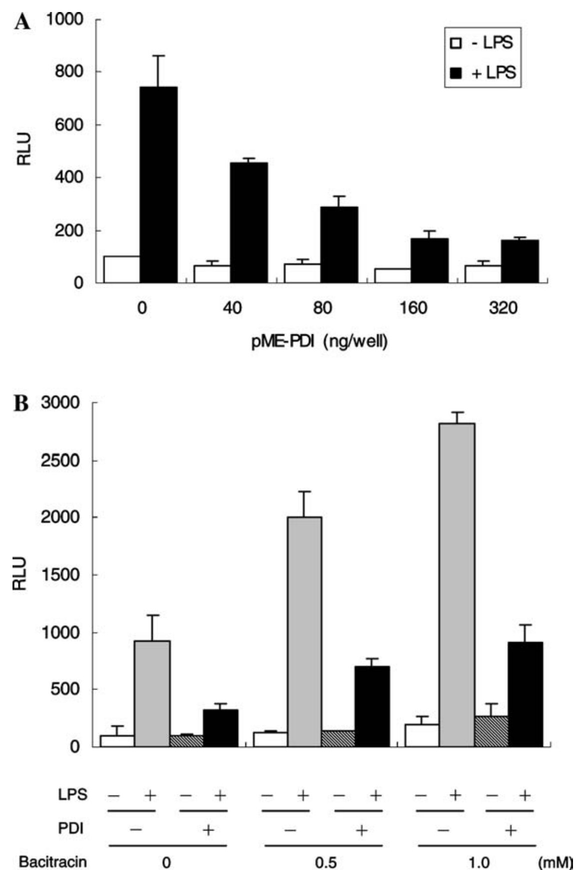

Fig. 2. PDI inhibits LPS-induced NF-κB-dependent transactivation. (A) RAW 264.7 cells were co-transfected with varying concentrations of pME-PDI together with NF-κB-Luc. Eighteen hours after transfection, the cells were treated for 20 h with 1 μg/ml LPS. The cells were then lysed, and the cell extracts were assayed for luciferase activity. (B) Effect of bacitracin on the transcriptional activities from NF-κB promoters. RAW 264.7 cells were transfected in the presence or absence of 8 μg (320 ng/well) pME-PDI together with NF-κB-Luc. Eighteen hours after transfection, the cells were treated for 20 h with 1 μg/ml LPS in the presence of the indicated amounts of bacitracin. Cell extracts were prepared and assayed for luciferase activity. Data are expressed as means and standard deviations of triplicate samples.

also dramatically reduced in these PDI-transfected cell lines (data not shown). To examine whether the inhibitory effect of PDI was caused at the transcriptional level or following post-translational modification of these cytokines, RT-PCR analysis was performed. Based on the RT-PCR results, the amounts of IL-6 mRNA in the PDI-transfected cell lines were significantly lower than those in the control vector-transfected cell lines (Fig. 1C). These results show that PDI acts at the transcriptional level to inhibit cytokine production in murine macrophages.

PDI has an inhibitory effect on NF-κB-dependent gene expression

As described previously, the transcription factor NF-κB is an important molecule for the regulation of inflammatory cytokine genes [1,2]. Therefore, we examined the effect of PDI on NF-κB-dependent gene expression. RAW 264.7 cells were transiently co-transfected with the PDI expression plasmid (pME-PDI) together with a reporter gene construct (NF-κB-Luc). We found that co-expression of the PDI gene resulted in suppression of LPS-induced NF-κB-dependent luciferase activity in a dose-dependent manner (Fig. 2A). Moreover, we found that the inhibitory effects by exogenous and endogenous expression of PDI on the NF-κB activity were blocked by the treatment of bacitracin, an inhibitor of PDI [31] (Fig. 2B).

PDI does not affect DNA-binding activity or phosphorylation of NF-κB

To investigate the mechanism by which PDI inhibits the NF-κB transcriptional pathway (Fig. 1A), the formation of the NF-κB/DNA complex in the PDI-transfected cell lines was examined using the EMSA method with an NF-κB-specific ^{32}P-labeled oligonucleotide. When cells were stimulated with LPS, an inducible protein/DNA complex was present in nuclear extracts. Competition experiments with increasing amounts of unlabeled oligonucleotides showed the inhibition of NF-κB/DNA complex formation, confirming its specificity. PDTC, which has been shown to inhibit the I-κB-ubiquitin ligase activity [14], completely blocked the formation of the LPS-induced NF-κB/DNA complex in RAW 264.7 cells (Fig. 3A). No significant decrease in NF-κB/DNA complex formation was observed in any of the PDI-transfected cell lines (Fig. 3A). These results suggest that PDI does not interfere with either the releasing step of NF-κB from I-κB, the nuclear translocation steps of the NF-κB, or

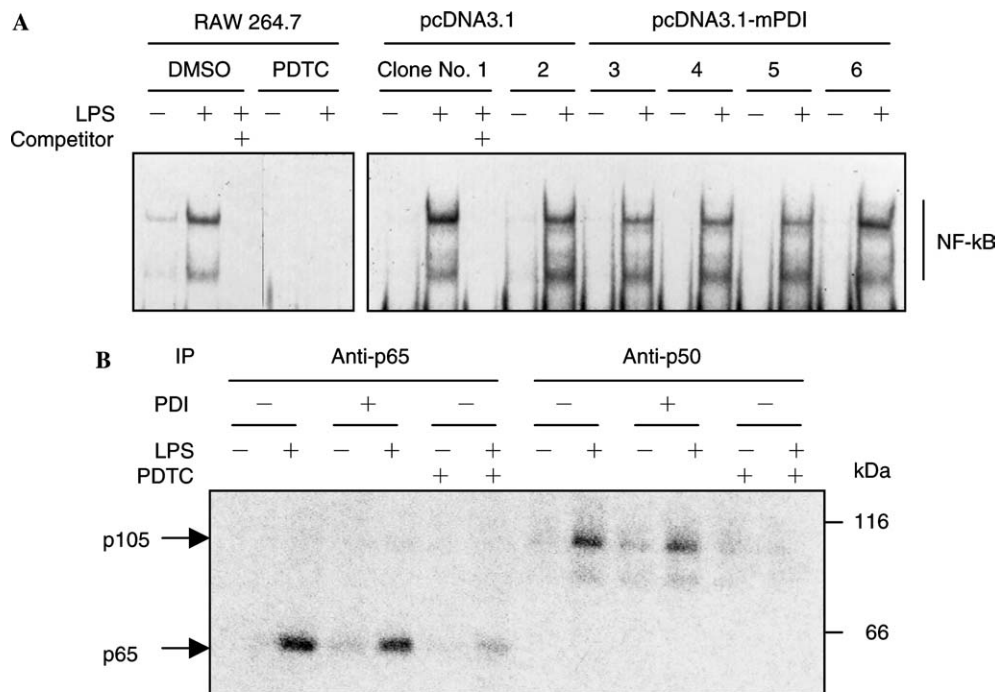

Fig. 3. PDI does not inhibit the LPS-induced DNA-binding activity or phosphorylation of NF-κB. (A) Ten micrograms of nuclear extracts was prepared and analyzed in an EMSA with a ^{32}P-labeled NF-κB probe in RAW 264.7 cells or the transfected cell lines. The cells were stimulated with 1 μg/ml LPS for 20 min. PDTC (300 μM) was given 2 h before LPS stimulation. Twenty-five times molar excess of unlabeled NF-κB competitor DNA was included in the reaction mixture. (B) LPS-induced phosphorylation of NF-κB p65 and p105, p50 precursor, subunits. $^{32}P_i$-labeled transfectants (PDI−, No. 1 and PDI+, No. 3) were stimulated for 20 min with 1 μg/ml LPS in the absence or presence of 300 μM PDTC which was given 2 h before LPS. The cell lysates were immunoprecipitated two times with an anti-p65 or anti-p50 antibodies, separated by SDS–PAGE, and detected by autoradiography. The positions of p65 and p105 are indicated by arrows.

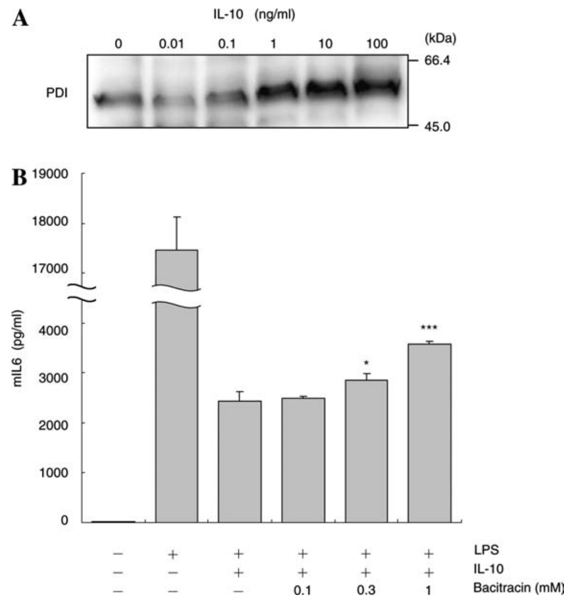

Fig. 4. Effect of bacitracin on the biological activity of IL-10. (A) The production of PDI is induced by IL10. RAW 264.7 cells were treated with the indicated amounts of IL-10 for 20 h. Cells were lysed and subjected to immunoblot analysis using a rabbit anti-PDI polyclonal antiserum. (B) The cells were treated with 1 μg/ml LPS for 20 h in the presence of 100 ng/ml IL-10 and with the indicated amounts of bacitracin. The amount of IL-6 in the supernatants was measured by ELISA following incubation. Data are expressed as means and standard deviations of triplicate samples. Statistically significant stimulations of IL-10-inhibited condition by bacitracin are indicated (*$p < 0.05$; ***$p < 0.001$ vs bacitracin free, Student's t test).

the binding steps with the genomic DNA of NF-κB target sites.

Second, because NF-κB has been reported to be activated by phosphorylation [3,32], we examined whether PDI inhibits the phosphorylation of NF-κB subunits following LPS stimulation. The PDI-transfected cell lines were metabolically labeled with $^{32}P_i$ and then immunoprecipitated using anti-p50 or anti-p65 antibodies. In agreement with previous reports, PDTC inhibited the LPS-induced phosphorylation of both p65 and p105 [3], while PDI had little effect on phosphorylation (Fig. 3B). These results suggest that the inhibitory effect of PDI is not the result of inhibition of phosphorylation of NF-κB subunits. Taken together, these observations suggest that PDI inhibits NF-κB activity via a novel mechanism.

Bacitracin disrupts the biological activity of IL-10

Because IL-10, IL-4, and TGF-β are known to be negative regulators of NF-κB-dependent gene expression [33,34], we examined how PDI contributes to the negative regulatory activity of these cytokines. First, we examined the protein level of PDI in response to these cytokines by immunoblotting. PDI levels were elevated by IL-10 in a dose-dependent manner in RAW 264.7 cells (Fig. 4A). Treatment with IL-10 reduces IL-6 production following activation with LPS (Fig. 4B). When bacitracin was added to this cytokine induction assay system, 0.3–1 mM of bacitracin could slightly reduce the IL-10-induced gene suppression (Fig. 4B). These phenomena suggest the PDI may be a downstream intermediate of IL-10 signaling pathway.

PDI is a dominant regulator of the NF-κB-dependent gene expression

TRX has been reported to activate gene expression via NF-κB by changing its redox state and by increasing the amount of NF-κB/DNA binding in the nucleus [15,16]. It has also been reported that TRX has a redox regulatory motif, similar to PDI [17,18]. Therefore, we compared the effects of PDI and TRX on NF-κB-dependent gene expression. In Fig. 5A, NF-κB-dependent

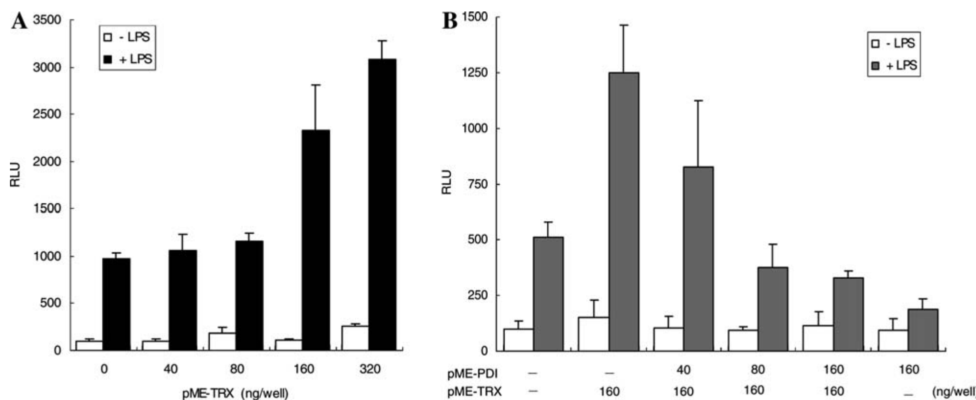

Fig. 5. PDI suppresses TRX-induced gene expression. (A) Stimulation of NF-κB-dependent transactivation by TRX. RAW 264.7 cells were co-transfected with the indicated amounts of pME-TRX together with NF-κB-Luc. Eighteen hours after transfection, the cells were stimulated with 1 μg/ml LPS for 20 h, and the expression of the reporter luciferase was quantified. (B) RAW 264.7 cells were co-transfected with the indicated quantities of pME-PDI and pME-TRX together with NF-κB-Luc. Eighteen hours after transfection, LPS stimulation of cells was performed for 20 h. Cell extracts were prepared and luciferase activities were measured. Data are expressed as means and standard deviations of triplicate samples.

transactivation was observed following transfection with TRX. However, TRX-induced gene expression was suppressed by co-transfection with PDI in a dose-dependent manner (Fig. 5B). These results indicate that PDI plays a dominant regulatory role in the NF-κB-mediated gene expression pathway. These results suggest that PDI acts as a qualitative transcriptional regulator of NF-κB, and will have an effect on the expression of many genes important in the inflammatory response.

Discussion

Aberrant activation of the NF-κB signaling pathway is likely to contribute to the development and progression of various immune and inflammatory disorders; therefore, tight regulation could be necessary for these pathways. In the present study, we demonstrate that over-expression of PDI suppresses NF-κB-dependent transcriptional activity. PDI has previously been reported to be a regulator of gene expression through the modulation of signal transduction pathways. In green algae *Chlamydomonas reinhardtii* [25] chloroplasts, PDI helps to change the redox state of signaling molecules and controls translational gene expression following the photosynthetic reaction. PDI directly modulates the binding activity of chloroplast polyadenylate-binding protein (cPABP) (by reversibly changing its redox status) to the 5′ untranslated region of the *psbA* mRNA, which encodes the photosynthetic reaction center protein D1. The same kind of gene switching mechanism involving the redox system may also be present in mammalian cells. PDI has been reported to activate the DNA binding ability of NF-κB in vitro [35]. However, the over-expression of PDI in RAW 264.7 cells did not affect the LPS-induced DNA binding activity of NF-κB in our hands (Fig. 3A). In our working hypothesis, PDI may control NF-κB activity by changing the redox status of NF-κB proteins itself and/or by modulating NF-κB-associated proteins in the NF-κB-activating signaling pathway. Recently, we found that PDI is always present in the cytoplasm fraction, even after treatment of cells with LPS, bacitracin, or IL-10 (data not shown). This observation suggests two possibilities: PDI could directly interact with the NF-κB in the reducing environment of the cytoplasm and induce conformational changes. Alternatively, PDI modifies the activity of indirectly through co-factors or kinases which are structurally modified by PDI. In addition, a recent study showed that PDI associated with ubiquilin, which has a ubiquitin-like domain and a ubiquitin-associated domain [36]. PDI may have a role in controlling the degradation of key molecules via the ubiquitin–proteasome pathway. Further analysis should include a molecular physiologic approach, to clarify the interaction between PDI and the NF-κB signaling pathway.

IL-10 is a potent, endogenous anti-inflammatory molecule that has been shown to control inflammatory reactions, partly by inhibition of TNF-α, IL-6, and IL-1β production [34]. In Fig. 4, we show that the PDI expression is induced by IL-10 and bacitracin could slightly, but significantly, reduce the inhibitory effect of IL-10. Bacitracin is not a strong inhibitor for PDI enzymatic S–S conversion activity but the only known candidate inhibitor to reduce the PDI activity in cell culture system. According to our results, PDI appears to be a negative regulator of NF-κB-dependent gene expression and may act downstream of IL-10. Recent studies have shown that the suppressor of cytokine signaling 3 (SOCS-3) functions as an intracellular mediator of the inhibitory effect of IL-10 on LPS-induced macrophage activation [37]. Further investigations will focus on the relation between PDI and SOCS-3 functions, and the IL-10 signaling pathway. A better understanding of the NF-κB-dependent gene switching mechanisms in mammalian cells may result in novel approaches to control human immune and inflammatory diseases.

Acknowledgments

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References

[1] M.J. May, S. Ghosh, Signaltransduction through NF-kappa B, Immunol. Today 19 (1998) 80–88.
[2] S. Ghosh, M. Karin, Missing pieces in the NF-kappa B puzzle, Cell 109 (2002) S81–S96.
[3] H. Zhong, H. SuYang, H. Erdjument-Bromage, P. Tempst, S. Ghosh, The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism, Cell 89 (1997) 413–424.
[4] R. Beyaert, A. Cuenda, W. Vanden Berghe, S. Plaisance, J.C. Lee, G. Haegeman, P. Cohen, W. Fiers, The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor, EMBO J. 15 (1996) 1914–1923.
[5] D. Thanos, T. Maniatis, The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene, Cell 71 (1992) 777–789.
[6] F.G. Wulczyn, M. Naumann, C. Scheidereit, Candidate proto-oncogene bcl-3 encodes a subunit-specific inhibitor of transcription factor NF-kappa B, Nature 358 (1992) 597–599.
[7] N.D. Perkins, L.K. Felzien, J.C. Betts, K. Leung, D.H. Beach, G.J. Nabel, Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator, Science 275 (1997) 523–527.
[8] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 270 (1995) 286–290.

[9] N.D. Perkins, A.B. Agranoff, E. Pascal, G.J. Nabel, An interaction between the DNA-binding domains of RelA(p65) and Sp1 mediates human immunodeficiency virus gene activation, Mol. Cell. Biol. 14 (1994) 6570–6583.
[10] B. Stein, A.S. Baldwin Jr., Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-kappa B, Mol. Cell. Biol. 13 (1993) 7191–7198.
[11] B. Stein, A.S. Baldwin Jr., D.W. Ballard, W.C. Greene, P. Angel, P. Herrlich, Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function, EMBO J. 12 (1993) 3879–3891.
[12] N. Li, M. Karin, Is NF-kappa B the sensor of oxidative stress?, FASEB J. 13 (1999) 1137–1143.
[13] J.C. Epinat, T.D. Gilmore, Diverse agents act at multiple levels to inhibit the Rel/NF-kappaB signal transduction pathway, Oncogene 18 (1999) 6896–6909.
[14] M. Hayakawa, H. Miyashita, I. Sakamoto, M. Kitagawa, H. Tanaka, H. Yasuda, M. Karin, K. Kikugawa, Evidence that reactive oxygen species do not mediate NF-kappaB activation, EMBO J. 22 (2003) 3356–3366.
[15] J.R. Matthews, N. Wakasugi, J.L. Virelizier, J. Yodoi, R.T. Hay, Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62, Nucleic Acids Res. 20 (1992) 3821–3830.
[16] T. Hayashi, Y. Ueno, T. Okamoto, Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin, J. Biol. Chem. 268 (1993) 11380–11388.
[17] A. Holmgren, Thioredoxin, Annu. Rev. Biochem. 54 (1985) 237–271.
[18] J.C. Edman, L. Ellis, R.W. Blacher, R.A. Roth, W.J. Rutter, Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin, Nature 317 (1985) 267–270.
[19] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulphide isomerase: building bridges in protein folding, Trends Biochem. Sci. 19 (1994) 331–336.
[20] T. Pihlajaniemi, T. Helaakoski, K. Tasanen, R. Myllyla, M.L. Huhtala, J. Koivu, K.I. Kivirikko, Molecular cloning of the beta-subunit of human prolyl 4-hydroxylase. This subunit and protein disulphide isomerase are products of the same gene, EMBO J. 6 (1987) 643–649.
[21] J.R. Wetterau, K.A. Combs, S.N. Spinner, B.J. Joiner, Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex, J. Biol. Chem. 265 (1990) 9801–9807.
[22] S. Akagi, A. Yamamoto, T. Yoshimori, R. Masaki, R. Ogawa, Y. Tashiro, Distribution of protein disulfide isomerase in rat hepatocytes, J. Histochem. Cytochem. 36 (1988) 1533–1542.
[23] M.P. Rigobello, A. Donella-Deana, L. Cesaro, A. Bindoli, Distribution of protein disulphide isomerase in rat liver mitochondria, Biochem. J. 356 (2001) 567–570.
[24] D.W. Essex, M. Li, Protein disulphide isomerase mediates platelet aggregation and secretion, Br. J. Haematol. 104 (1999) 448–454.
[25] J. Kim, S.P. Mayfield, Protein disulfide isomerase as a regulator of chloroplast translational activation, Science 278 (1997) 1954–1957.
[26] I. Waga, J. Yamamoto, H. Sasai, W.E. Munger, S.L. Hogan, G.A. Preston, H.W. Sun, J.C. Jennette, R.J. Falk, D.A. Alcorta, Altered mRNA expression in renal biopsy tissue from patients with IgA nephropathy, Kidney Int. 64 (2003) 1253–1264.
[27] T. Fujita, G.P. Nolan, H.C. Liou, M.L. Scott, D. Baltimore, The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers, Genes Dev. 7 (1993) 1354–1363.
[28] Y.C. Liu, M. Kawagishi, T. Mikayama, Y. Inagaki, T. Takeuchi, H. Ohashi, Processing of a fusion protein by endoprotease in COS-1 cells for secretion of mature peptide by using a chimeric expression vector, Proc. Natl. Acad. Sci. USA 90 (1993) 8957–8961.
[29] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, Nucleic Acids Res. 11 (1983) 1475–1489.
[30] M. Naumann, C. Scheidereit, Activation of NF-kappa B in vivo is regulated by multiple phosphorylations, EMBO J. 13 (1994) 4597–4607.
[31] R.A. Roth, Bacitracin: an inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase, Biochem. Biophys. Res. Commun. 98 (1981) 431–438.
[32] D. Wang, A.S. Baldwin Jr., Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529, J. Biol. Chem. 273 (1998) 29411–29416.
[33] P. Wang, P. Wu, M.I. Siegel, R.W. Egan, M.M. Billah, Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms, J. Biol. Chem. 270 (1995) 9558–9563.
[34] Y.Q. Xiao, K. Malcolm, G.S. Worthen, S. Gardai, W.P. Schiemann, V.A. Fadok, D.L. Bratton, P.M. Henson, Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta, J. Biol. Chem. 277 (2002) 14884–14893.
[35] D.R. Clive, J.J. Greene, Cooperation of protein disulfide isomerase and redox environment in the regulation of NF-kappaB and AP1 binding to DNA, Cell Biochem. Funct. 14 (1996) 49–55.
[36] H.S. Ko, T. Uehara, Y. Nomura, Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death, J. Biol. Chem. 277 (2002) 35386–35392.
[37] C. Berlato, M.A. Cassatella, I. Kinjyo, L. Gatto, A. Yoshimura, F. Bazzoni, Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation, J. Immunol. 168 (2002) 6404–6411.