Protein Kinase Cδ Oxidation Contributes to ERK Inactivation in Lupus T Cells

Gabriela J. Gorelik,[1] Sushma Yarlagadda,[1] and Bruce C. Richardson[2]

Objective. CD4+ T cells from patients with active lupus have impaired ERK pathway signaling that decreases DNA methyltransferase expression, resulting in DNA demethylation, overexpression of immune genes, and autoimmunity. The ERK pathway defect is due to impaired phosphorylation of T^{505} in the protein kinase Cδ (PKCδ) activation loop. However, the mechanisms that prevent PKCδ T^{505} phosphorylation in lupus T cells are unknown. Others have reported that oxidative modifications, and nitration in particular, of T cells as well as serum proteins correlate with lupus disease activity. We undertook this study to test our hypothesis that nitration inactivates PKCδ, contributing to impaired ERK pathway signaling in lupus T cells.

Methods. CD4+ T cells were purified from lupus patients and controls and then stimulated with phorbol myristate acetate (PMA). Signaling protein levels, nitration, and phosphorylation were quantitated by immunoprecipitation and immunoblotting of T cell lysates. Transfections were performed by electroporation.

Results. Treating CD4+ T cells with peroxynitrite nitrated PKCδ, preventing PKCδ T^{505} phosphorylation and inhibiting ERK pathway signaling similar to that observed in lupus T cells. Patients with active lupus had higher nitrated T cell PKCδ levels than did controls, which correlated directly with disease activity, and antinitrotyrosine immunoprecipitations demonstrated that nitrated PKCδ, but not unmodified PKCδ, was refractory to PMA-stimulated T^{505} phosphorylation, similar to PKCδ in peroxynitrite-treated cells.

Conclusion. Oxidative stress causes PKCδ nitration, which prevents its phosphorylation and contributes to the decreased ERK signaling in lupus T cells. These results identify PKCδ as a link between oxidative stress and the T cell epigenetic modifications in lupus.

Systemic lupus erythematosus (SLE) is an incompletely understood chronic, relapsing autoimmune disease. Persuasive evidence indicates that genetic factors contribute to disease development. However, a lack of complete disease concordance in identical twins and the relapsing nature of the disease indicate that environmental factors are important as well (1). The full repertoire of the environmental factors, and the mechanism(s) by which the environmental agents contribute to the onset of lupus and lupus flares, remain unknown. Our group has reported that T cell DNA demethylation contributes to the pathogenesis of human lupus by causing overexpression of immune genes, leading to T cell autoreactivity and the development of lupus in genetically predisposed hosts (2). This suggests that the environment may trigger lupus flares by inhibiting T cell DNA methylation. The mechanisms by which environmental agents cause T cell DNA demethylation in lupus are also unknown.

DNA methylation patterns are replicated each time a cell divides by DNA methyltransferase 1 (DNMT-1) (3), whose levels are regulated in part by the ERK signaling pathway (4–6). We reported that treating proliferating T cells with ERK pathway inhibitors, such as hydralazine and U0126, or with DNMT inhibitors, such as procainamide, is sufficient to demethylate the DNA and that the demethylated T cells are sufficient to cause a lupus-like disease in murine models (7,8). Further, transgenic mice with an inducible T cell ERK pathway signaling defect develop a lupus-like disease (9). We also reported that T cells from patients with

active lupus have impaired ERK pathway signaling, low DNMT-1 levels, and overexpress methylation-sensitive genes (10), suggesting that impaired ERK pathway signaling contributes to DNA demethylation in human lupus. Interestingly, CD4+ lupus T cells demethylate the same genetic regulatory elements demethylated by ERK pathway inhibitors and DNMT inhibitors (11), and the level of lupus disease activity is directly related to the degree of signaling impairment and DNA demethylation (10). These studies thus suggest an important role of impaired ERK pathway signaling in triggering lupus flares.

More recent studies traced the lupus ERK pathway signaling defect to impaired protein kinase Cδ (PKCδ) phosphorylation (12). PKCδ is a key molecule that is phosphorylated in response to a variety of signals, and thus activates other signaling pathways including the ERK pathway (13–15). PKCδ in T cells from patients with active lupus or in hydralazine-treated T cells is not properly phosphorylated in response to phorbol myristate acetate (PMA) or other stimuli, and the defect correlates with the lupus ERK pathway defect. Transfecting human T cells with a kinase-inactive PKCδ causes decreased ERK phosphorylation, indicating that PKCδ is critically required for ERK pathway activation. Additionally, the transfected cells exhibited demethylation and overexpression of the TNFSF7 (CD70) gene, similar to findings in lupus patients (12). However, the mechanisms inhibiting PKCδ phosphorylation in lupus T cells are unknown.

Inflammation is associated with the generation of reactive oxygen species (ROS), and patients with active lupus have increased levels of ROS and reactive nitrogen intermediates (RNIs), as well as decreased levels of oxidant scavengers (16–18). This imbalance causes increased levels of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), all highly reactive metabolites that cause direct toxicity by inducing chemical modifications in lipids, proteins, and DNA (19). $ONOO^-$ nitrates Tyr residues to prevent phosphorylation (20) and may thus affect signaling pathways. However, the consequences of oxidative stress on T cell signaling are also poorly understood.

Since T cell PKCδ kinase activity is decreased in patients with active lupus and active lupus is characterized by the generation of ROS/RNIs and oxidative protein damage, we hypothesized that PKCδ may be covalently modified by ROS/RNIs in lupus T cells, preventing its activation. The goal of this study was to determine if oxidative modifications produced by $ONOO^-$ contribute to impaired T cell PKCδ phosphorylation, causing the decreased ERK pathway signaling observed in patients with active lupus.

MATERIALS AND METHODS

Reagents. Hydralazine was purchased from VWR and peroxynitrite from Calbiochem. All other chemicals were from Sigma.

Antibodies. The following primary antibodies were used: polyclonal rabbit anti–phospho-PKCα (at $T^{638/641}$), anti–phospho-PKCθ (at T^{538}), anti–phospho-PKCδ (at T^{505}), anti–phospho-PKCδ (at Y^{311}), and anti–phospho–3′-phosphoinositide–dependent protein kinase 1 (anti–phospho–PDK-1) (at Ser^{241}), at 1:1,000 dilution (Cell Signaling Technology). For immunoprecipitation, antinitrotyrosine, clone 1A6 agarose conjugate, was used (Millipore). Rabbit polyclonal anti–active MAPK (1:5,000) was from Promega, and anti–total PKCδ was from Santa Cruz Biotechnology. Secondary antibodies included horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (1:2,000 dilution; Cell Signaling Technology) and HRP-conjugated anti-mouse IgG (1:4,000 dilution; Amersham).

Subjects. Sixteen lupus patients (mean age 42 years, range 27–64 years) with active or inactive disease were recruited from the outpatient rheumatology clinics and inpatient services at the University of Michigan, and 21 healthy controls were recruited by advertising. All lupus patients met the American College of Rheumatology revised criteria for SLE (21). Lupus disease activity was quantified using the SLE Disease Activity Index (SLEDAI) (22), and the range was 4–10 (mean 6.2) for the patients with active lupus and 0–2 (mean 0.5) for the patients with inactive disease. Controls were matched to the lupus patients for age, race, and sex. These protocols were reviewed and approved by the University of Michigan Institutional Review Board for Human Subject Research. The demographics of the patients and the medications they received are summarized in Table 1.

T cell isolation. Peripheral blood mononuclear cells were isolated from healthy donors or SLE patients by Ficoll-Hypaque density-gradient centrifugation. CD4+ T cells were then purified by negative selection using magnetic beads (CD4+ T cell isolation kit; Miltenyi Biotec) as previously reported (12).

Table 1. Demographics, disease activity, and treatment in the SLE patients*

SLEDAI score	
Patients with active disease	6.2 ± 2.3
Patients with inactive disease	0.5 ± 1.0
Age, years	41.8 ± 11.1
Female:male ratio	14:2
Medications, %	
Prednisone	62
Antimalarials	62
Azathioprine	25
Mycophenolate mofetil	44
Methotrexate	0

* Except where indicated otherwise, values are the mean ± SD. SLEDAI = Systemic Lupus Erythematosus Disease Activity Index.

T cell stimulation and protein isolation. CD4+ T cells were suspended in RPMI 1640 supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, and penicillin/streptomycin, and then were left unstimulated or were stimulated with 50 ng/ml PMA for 15 minutes at 37°C. Treatment with peroxynitrite was performed at the concentrations and times specified in each experiment and before PMA stimulation. Following stimulation, whole cell lysates were obtained according to previous protocols (12), and protein content was quantified using the BCA Protein Assay (Pierce).

Transient transfection. T cells from normal donors were immediately transfected with small interfering RNA (siRNA) or the indicated mutants using Amaxa nucleofection technology according to previous protocols (12). After 24 hours, the cells were treated as indicated, harvested, and cell lysates were obtained as above. Small interfering RNA protein phosphatase 2Ac (PP-2Ac) and PP-2Ac mutants H118N and L199P were kindly provided by Dr. G. Tsokos and used as described by Katsiari et al (23). The mean ± SD transfection efficiency was 63 ± 6% of the total cell number and was verified by fluorescence microscopy of cells transfected with the positive control vector pmaxGFP, which encodes a green fluorescent protein (GFP) and was provided with the kit.

Immunoblot analysis. Immunoblots were performed as previously described (12). Briefly, 20 μg of whole cell protein was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (Schleicher & Schuell), and then stained with ponceau S (Sigma) to verify equal protein loading between lanes. After incubation with the kinase-specific antibody and then with the secondary antibody, protein bands were visualized by chemiluminescence (Amersham). The bands on x-ray films were scanned and analyzed with ImageQuant 5.2 software (Amersham) for quantification. Where indicated, blots were stripped and reprobed with the corresponding antibody. Values were normalized to β-actin or the corresponding kinase, as indicated.

Immunoprecipitation. Immunoprecipitation was performed according to the manufacturer's instructions. Briefly, lysates from normal or lupus T cells were incubated with the agarose-conjugated antinitrotyrosine antibody overnight at 4°C. Following centrifugation, an aliquot of the supernatant (containing non-nitrated proteins) and the beads were resuspended in Laemmli sample buffer and boiled for 5 minutes. Proteins from each fraction were then analyzed in parallel by SDS-PAGE and immunoblot analysis.

Statistical analysis. The significance of the difference between means was determined using Student's *t*-test, regression analysis, or analysis of variance as appropriate. *P* values less than or equal to 0.05 were considered significant.

RESULTS

PDK-1 does not contribute to the PKCδ signaling defect in lupus T cells. We examined the mechanisms regulating PKCδ phosphorylation to determine the causes of the lower levels of phosphorylated T^{505} in PKCδ in PMA-stimulated lupus T cells. PDK-1 controls the phosphorylation of T^{505} in the PKCδ catalytic loop
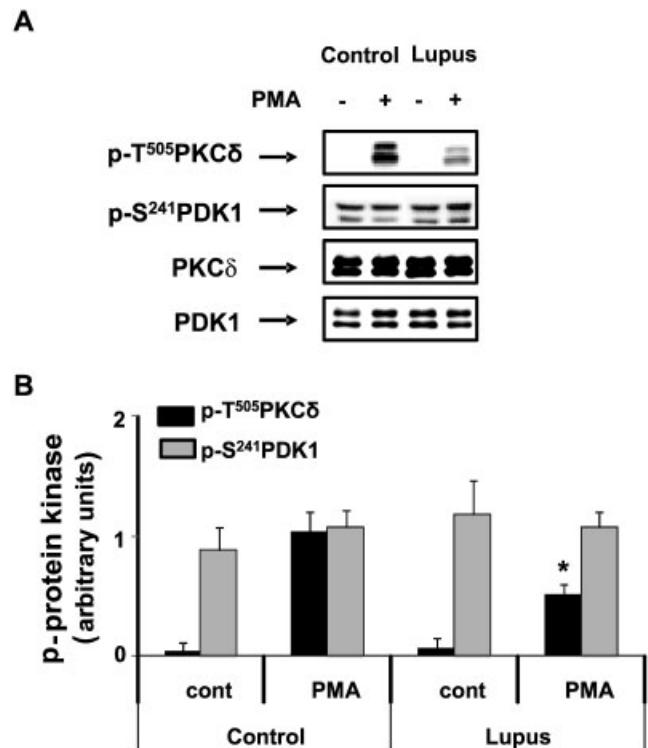
Figure 1. The molecule 3'-phosphoinositide–dependent protein kinase 1 (PDK-1) is not affected in lupus T cells. CD4+ T cells from healthy donors (control) or from lupus patients were left unstimulated (cont) or stimulated with phorbol myristate acetate (PMA) for 15 minutes. Twenty micrograms of whole cell lysates was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with a polyclonal antibody against phosphorylated T^{505} in protein kinase Cδ (PKCδ), as described in Materials and Methods. Membranes were also stripped and reprobed with a polyclonal antibody against phosphorylated S^{241} in PDK-1. PKCδ and PDK-1 were used as controls for the corresponding total protein expression. **A,** Representative blot comparing phosphorylation of PDK-1 and PKCδ both in a lupus patient and in a healthy donor. **B,** Results of 4 similar experiments performed in different cell preparations from healthy donors and lupus patients. Values are the mean ± SEM. * = $P \leq 0.02$ versus PMA-stimulated T cells from healthy donors.

(24). We therefore examined PDK-1 activation. PDK-1 has 5 sites that are phosphorylated (Ser^{25}, Ser^{393}, Ser^{396}, Ser^{410}, and Ser^{241}), but only Ser^{241} is located in the activation loop and is required for PDK-1 activity (25). PDK-1 activation was therefore studied using antibodies to PDK-1 phospho-Ser^{241} and immunoblotting. There was no significant difference in PDK-1 phosphorylation between control and lupus T cells, although phosphorylation of T^{505} in PKCδ was decreased in PMA-stimulated CD4+ T cells from lupus patients with active disease (Figure 1A). The densitometric analysis of 4
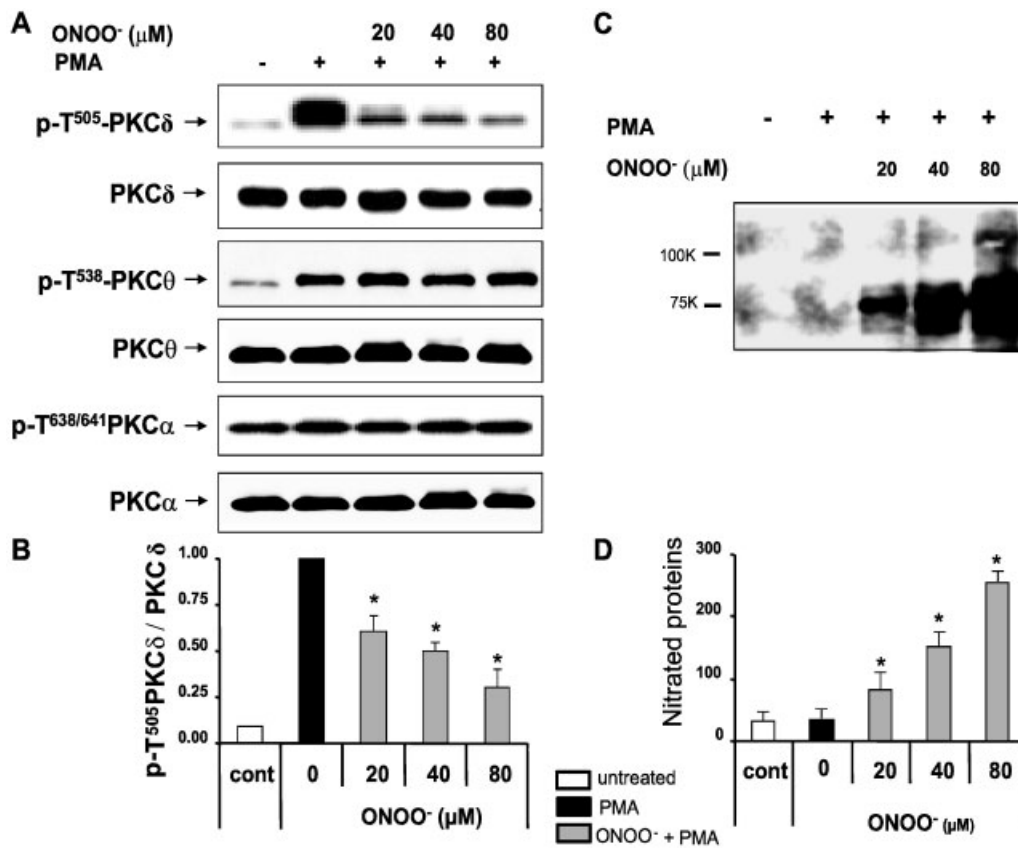
Figure 2. Peroxynitrite (ONOO⁻) decreases PKCδ T[505] phosphorylation while increasing protein nitration in T cells. CD4+ T cells from healthy donors were left untreated (cont) or treated with 20–80 μM ONOO⁻ for 15 minutes and then stimulated with PMA as indicated. Whole cell proteins were obtained and subjected to Western blot analysis. **A,** Representative immunoblots showing phosphorylation at the activation loops of PKCδ, PKCθ, and PKCα using antibodies specific to the phosphorylated PKC isoforms as indicated. **B,** Quantitative analysis of PKCδ phospho-T[505] in CD4+ T cells from 4 healthy donors in 4 different experiments. Values were normalized to total PKCδ. PMA-stimulated CD4+ T cells without ONOO⁻ treatment were assigned a value of 100%. **C,** Representative experiment using anti–3-nitrotyrosine antibody to probe PMA-stimulated T cell lysates. **D,** Relative quantitation of 3-nitrotyrosine proteins from 4 similar experiments. Untreated cells were used as controls. Values in **B** and **D** are the mean ± SEM. ∗ = $P < 0.01$ versus PMA-stimulated cells not treated with ONOO⁻. See Figure 1 for other definitions.

similar experiments confirmed no significant difference in PDK-1 activation between lupus patients and controls ($P > 0.05$) (Figure 1B). Our observation that phospho–PDK-1 is expressed at similar levels in resting and PMA-stimulated T cells is concordant with the observation that PDK-1 is constitutively active due to autophosphorylation of its activation loop (26). These results indicate that a PDK-1 activity defect is not likely to be causing the decreased phosphorylation of T[505] in PKCδ in CD4+ lupus T cells.

ONOO⁻ inhibits phosphorylation of T[505] in PKCδ. Oxidative stress plays an important role in triggering lupus flares, and others have reported that serum protein oxidation correlates with disease activity in lupus patients and that serum proteins are abnormally nitrated in patients with active lupus (16,27). Since ONOO⁻ is a

potent oxidizing agent that nitrates proteins, causing functional loss (20), we sought to determine whether ONOO⁻ causes the PKCδ T[505] phosphorylation defect observed in lupus T cells. CD4+ T cells from healthy subjects were treated with increasing concentrations of ONOO⁻ and then stimulated with PMA. Treatment with ONOO⁻ caused a concentration-dependent decrease in PKCδ T[505] phosphorylation, while PKCα and PKCθ activation loop phosphorylation was not appreciably affected (Figure 2A). Figure 2B shows the quantitative densitometric analysis of 4 serial repeat experiments similarly measuring the effects of ONOO⁻ on the phosphorylation of T[505] in PKCδ. These results indicate that the inhibitory effects of ONOO⁻ on PKCδ are isoform specific, since phosphorylation of other isoforms was unaffected.

Since ONOO$^-$ nitrates Tyr residues to form 3-nitrotyrosine (20), we confirmed the effect of ONOO$^-$ by measuring 3-nitrotyrosine protein derivative formation. CD4+ T cells from a healthy donor were treated with increasing concentrations of ONOO$^-$ and then stimulated with PMA. Proteins modified by 3-nitrotyrosine were studied by immunoblotting. We observed a concentration-dependent increase in 3-nitrotyrosine derivative formation (Figure 2C). Figure 2D shows the mean ± SEM values from densitometric analyses from 4 similar experiments, confirming that T cell proteins are nitrated by ONOO$^-$ and that their nitration levels correlate with the ONOO$^-$-induced inhibition of phosphorylation of T^{505} in PKCδ shown in Figure 2B.

We also studied the phosphorylation of Y^{311} in the PKCδ molecule, because this amino acid is phosphorylated in cells undergoing oxidative stress (28,29). Figure 3A shows a representative immunoblot comparing the effects of ONOO$^-$ on PMA-stimulated PKCδ T^{505} and Y^{311} phosphorylation. In untreated (0 μM ONOO$^-$) CD4+ T cells, PMA caused a substantial increase in T^{505} phosphorylation, but had no appreciable effect on Y^{311} phosphorylation. It is important to note that phospho-Y^{311} was almost undetectable in resting CD4+ T cells (results not shown), similar to phospho-T^{505}. However, ONOO$^-$ induced a concentration-dependent increase in Y^{311} phosphorylation in PMA-stimulated cells that was inversely related to T^{505} phosphorylation ($P \leq 0.01$ for ONOO$^-$ versus no ONOO$^-$; n = 4 experiments). Figure 3B clearly demonstrates a different pattern of PKCδ phosphorylation that is stimulus dependent. While PMA promoted phosphorylation on T^{505}, Y^{311} was almost insensitive. In contrast, ONOO$^-$ increased Y^{311} phosphorylation with a concomitant decrease in phospho-T^{505}. The lack of additional effects caused by NaOH excluded nonspecific effects due to pH changes caused by the ONOO$^-$ solution (Figure 3A).

PP-2Ac does not participate in the ONOO$^-$-induced PKCδ defect. ONOO$^-$ increases PP-2Ac activity in some cell types (30), and PP-2Ac has the highest specific phosphatase activity toward PKCδ (31). Further, PP-2Ac expression and activity are increased in T cells from lupus patients (23). We tested whether increased PP-2Ac activity could be responsible for the PKCδ dephosphorylation caused by ONOO$^-$. T cells from healthy donors were transfected either with 5 μg of plasmids encoding the dominant-negative PP-2Ac mutants H118N or L199P (32) or with 100 nM PP-2Ac–specific siRNA according to Katsiari et al (23). Cells transfected with an irrelevant siRNA were used as
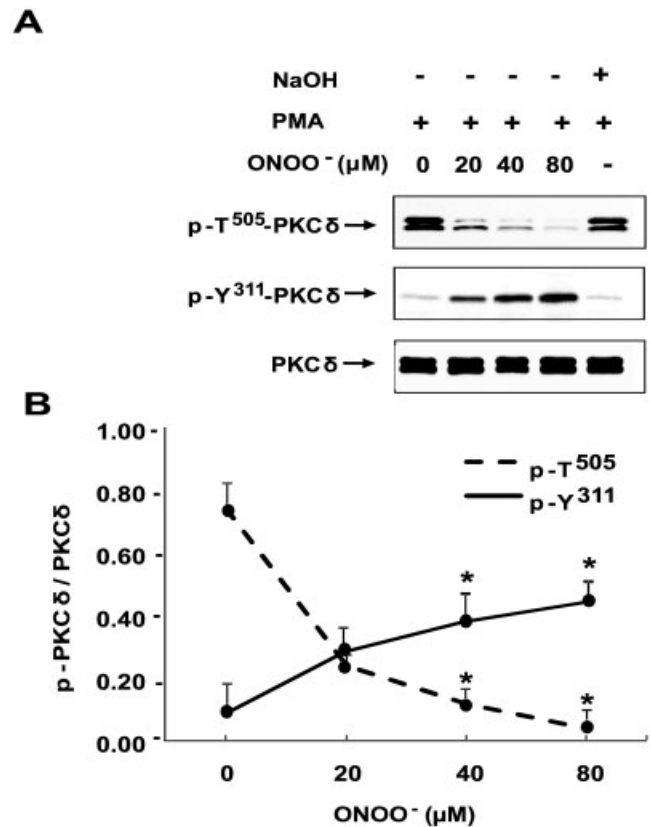
Figure 3. Differential PKCδ phosphorylation induced by peroxynitrite (ONOO$^-$). **A,** Representative experiment showing the effect of different ONOO$^-$ concentrations on PKCδ phosphorylation. CD4+ T cells were isolated from healthy controls and treated with ONOO$^-$ at the concentrations specified. Following treatment the cells were stimulated with 50 ng/ml PMA for 15 minutes, and PMA-stimulated cells not treated with ONOO$^-$ were used as control. Protein lysates were then subjected to electrophoresis and transferred to nitrocellulose membranes, and the membranes were probed with an antibody against phosphorylated T^{505} in PKCδ. The blot was then stripped and reprobed with an antibody against phosphorylated Y^{311} in PKCδ. NaOH was used as vehicle control and added to the culture for 15 minutes before PMA stimulation at the same final concentration as in the ONOO$^-$ solution. **B,** Differential dose-response curves of PKCδ phospho-T^{505} and PKCδ phospho-Y^{311} following ONOO$^-$ treatment. Values are the mean ± SEM of 4 independent experiments. $* = P \leq 0.04$ versus cells not treated with ONOO$^-$. See Figure 1 for other definitions.

control, and 2 μg of the empty GFP vector was transfected as an internal control for transfection efficiency in each experiment. Twenty-four hours later, the cells were treated with ONOO$^-$ for 15 minutes followed by PMA stimulation. In 3 serial repeat experiments, relative to PMA-stimulated T cells (set at 100%), phosphorylation at T^{505} in PKCδ was not increased in ONOO$^-$-treated cells overexpressing the catalytically inactive PP-2Ac

mutants (for H118N, mean $\pm$ SEM 23.39 $\pm$ 4.2%; for L199P, mean $\pm$ SEM 31.58 $\pm$ 4.5%) or the siRNA (mean $\pm$ SEM 28.36 $\pm$ 5.2%) relative to nontransfected cells (mean $\pm$ SD 21.01 $\pm$ 4.8%) ($P = 0.62$ for nontransfected cells versus transfected cells; n = 3 experiments), confirming that PP-2Ac is not causing the PKCδ T^{505} phosphorylation defect in this system.

PKCδ nitration correlates with decreased ERK phosphorylation. We previously reported that PKCδ T^{505} phosphorylation is decreased in T cells from patients with active lupus (12). The decreased PKCδ T^{505} phosphorylation correlates with decreased ERK phosphorylation in CD4+ lupus T cells and in CD4+ T cells treated with pharmacologic PKCδ inhibitors or transfected with PKCδ siRNA or with a dominant-negative PKCδ. To determine whether PKCδ nitration also inhibits ERK phosphorylation, CD4+ T cells from healthy controls were treated with different concentrations of ONOO$^-$ for varying times, then stimulated with PMA. Figure 4A shows that, as expected, PMA-stimulated ERK phosphorylation declined with increasing ONOO$^-$ concentrations and paralleled the decrease in PKCδ T^{505} phosphorylation. This is consistent with our previous results showing that PKCδ T^{505} phosphorylation is upstream of ERK (12) and its impairment decreases ERK pathway signaling. Figure 4B shows the quantification of 4 serial repeat experiments confirming this observation.

PKCδ nitration in lupus. Lupus is characterized by increased oxidative stress (16,33,34). T cells from patients with active lupus generate large amounts of ROS (35), and serum proteins are nitrated in these patients (36). We therefore hypothesized that PKCδ is inactivated in T cells from patients with active lupus through nitration, similar to ONOO$^-$-treated T cells.

To address this hypothesis, CD4+ T cells from 6 lupus patients with active disease (SLEDAI score $\geq$4) were stimulated with PMA, and nitrated proteins were immunoprecipitated from the lysates with anti–3-nitrotyrosine antibodies. Results were compared to similarly treated CD4+ T cells from 4 lupus patients with inactive disease. Controls included CD4+ T cells from 3 healthy subjects that were left untreated or were treated with ONOO$^-$ followed by PMA stimulation. Total PKCδ and PKCδ phospho-T^{505} content were then compared by immunoblotting.

Nitrated and non-nitrated PKCδ were analyzed as the total PKCδ content in the precipitate and supernatant, respectively. The levels of total PKCδ (supernatant plus immunoprecipitate) were similar in untreated
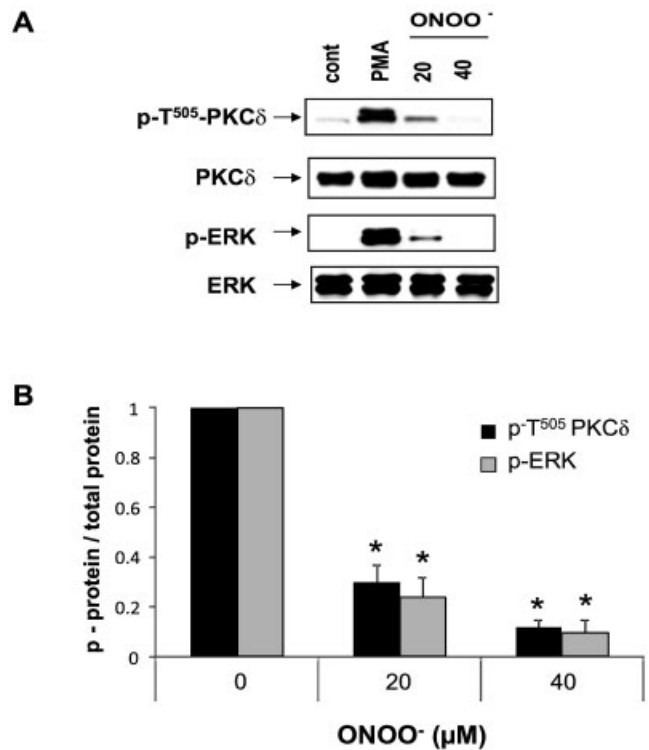
Figure 4. Peroxynitrite (ONOO$^-$) decreases ERK phosphorylation. **A,** CD4+ T cells from normal donors were left untreated (control [cont]) or were treated for 15 minutes with ONOO$^-$ at the indicated concentrations and then stimulated with PMA. Whole cell extracts were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting using an antibody against phospho-T^{505} in PKCδ. The blot was then stripped and reprobed with an antibody against phospho-T^{202}/Y^{204} in ERK. No variation in total PKCδ or ERK protein expression was observed. The blot is representative of 4 independent experiments. **B,** Shown is quantitative immunoblot analysis of 4 different experiments. PMA-stimulated cells not treated with ONOO$^-$ were assigned a value of 1. Values are the mean $\pm$ SEM. $*$ = $P \leq 0.001$ versus cells not treated with ONOO$^-$. See Figure 1 for other definitions.

or ONOO$^-$-treated T cells from control donors and lupus patients (Figures 5A and B). These results confirm previous data showing no differences in PKCδ protein expression in control donors and lupus patients (12). As anticipated, the amount of nitrated PKCδ (precipitated) was higher in T cells from patients with active lupus relative to controls ($P \leq 0.03$) and with a pattern similar to that in ONOO$^-$-treated control T cells (Figure 5A). In contrast, Figure 5B shows that the patterns of nitration and phosphorylation of PKCδ did not differ significantly in CD4+ T cells from patients with inactive lupus when compared to CD4+ T cells from healthy controls. Importantly, the level of T cell PKCδ nitration in lupus
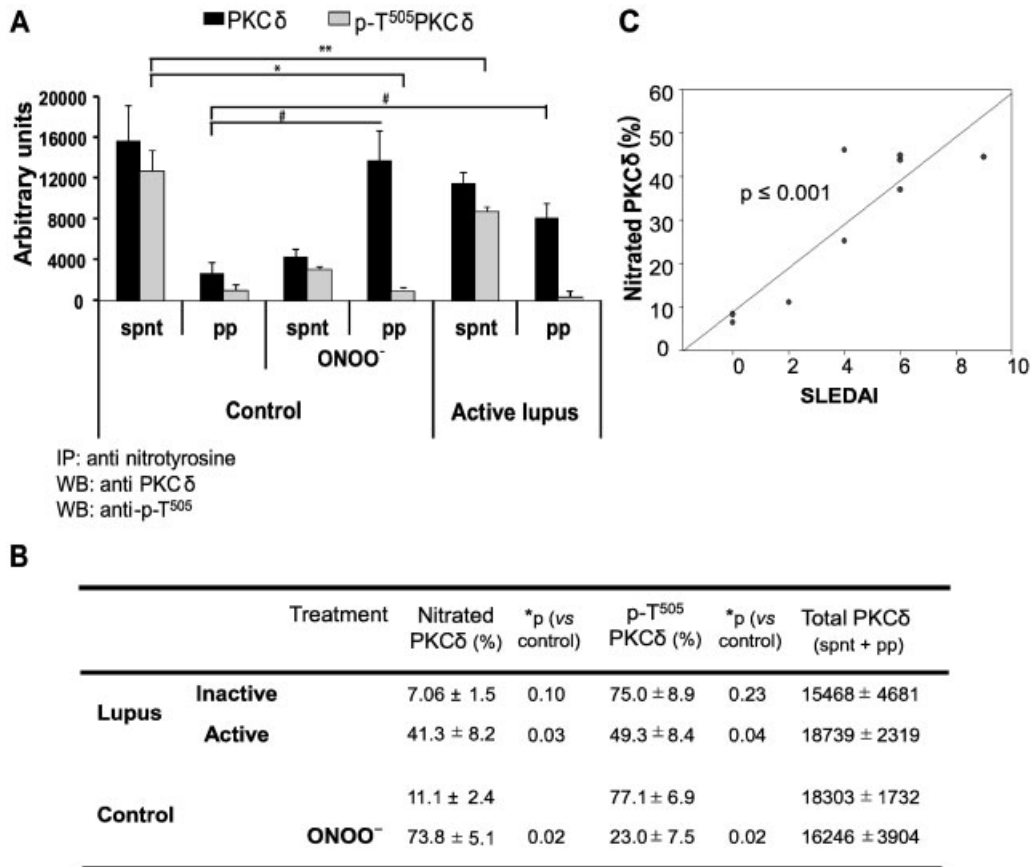
Figure 5. PKCδ nitration in lupus T cells. CD4+ T cells from 3 healthy donors (control) were left untreated or were treated with peroxynitrite (ONOO⁻) followed by stimulation with PMA. In parallel, CD4+ T cells from 6 patients with active lupus and 4 patients with inactive lupus were stimulated with PMA. Total lysates were immunoprecipitated, and then supernatants (spnt) and precipitates (pp) were immunoblotted with an antibody against PKCδ phospho-T^{505}. Membranes were reprobed with an antibody against total PKCδ. **A,** Quantitative densitometric analysis of total PKCδ and PKCδ phospho-T^{505} in the supernatant and the precipitate using CD4+ T cells from healthy donors and patients with active disease. Values are the mean ± SEM of 6 independent experiments. ∗ = $P \leq 0.01$; ∗∗ = $P \leq 0.04$; # = $P \leq 0.03$. **B,** Nitrated PKCδ (total PKCδ content in precipitate) and PKCδ phospho-T^{505} (phosphorylated PKCδ in supernatant plus precipitate) as a percentage of the total PKCδ (expressed in arbitrary units) in supernatant and precipitate under each experimental condition. Values are the mean ± SEM of 6 experiments. P values are versus control cells not treated with ONOO⁻. **C,** Correlation between nitrated PKCδ levels in lupus patients and scores on the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). The P value was determined by analysis of variance. IP = immunoprecipitation; WB = Western blotting (see Figure 1 for other definitions).

patients correlated directly with the SLEDAI scores (Figure 5C).

However, overall T^{505} phosphorylation in PKCδ was decreased in stimulated T cells from patients with active lupus relative to those from healthy controls ($P = 0.04$) (Figure 5B), and the nitrated PKCδ fraction from the patients with active lupus was almost completely refractory to PMA-stimulated phosphorylation (mean ± SEM 4 ± 2% of nitrated PKCδ) ($P \leq 0.04$ for patients with active lupus versus healthy controls) (Figure 5A). As predicted, 74% of the PKCδ in T cells treated with ONOO⁻ was nitrated, but only 6% of this fraction

was phosphorylated at T^{505} after PMA stimulation (Figure 5A). In contrast, non-nitrated PKCδ was phosphorylated in T cells from healthy controls and from patients with active or inactive lupus (supernatant) (Figure 5A). These experiments thus demonstrate more extensive PKCδ nitration, and less T^{505} phosphorylation, in T cells from patients with active lupus relative to those from patients with inactive lupus and healthy controls, suggesting that T^{505} phosphorylation decreases in proportion to the extent of nitration and that the degree of nitration is directly related to the disease activity.

DISCUSSION

The goal of this study was to investigate mechanisms causing the PKCδ phosphorylation defect responsible for decreased ERK pathway signaling in lupus T cells. PKCδ belongs to the PKC family of related serine/threonine kinases with active roles in growth regulation and apoptosis. PKC isoforms contain a highly conserved C-terminal catalytic domain. However, they are subdivided into 3 subfamilies according to their N-terminal regulatory domains. Conventional isoforms comprise PKCα, PKCβ, and PKCγ, bind diacylglycerol (DAG)/PMA in their C1 domain, and bind anionic phospholipids in a calcium-dependent manner in their C2 domain. Novel isoforms include PKCδ, PKCε, PKCη, PKCμ, and PKCθ and are activated by DAG/PMA without a calcium requirement. Atypical isoforms, PKCζ and PKCλ/ι, are DAG/PMA and calcium independent (37).

PKCδ is ubiquitously expressed among cells and tissues and is the only isoform that can be activated by 3 different mechanisms: through Ser/Thr phosphorylation, through tyrosine phosphorylation, and by caspase 3–dependent proteolytic cleavage. These are independent mechanisms that regulate PKCδ activity, substrates, and cellular localization and play critical roles during cell growth, differentiation, and programmed cell death as well as the cellular response to oxidative stress (38).

Our previous work identified decreased PKCδ phospho-T^{505} levels in stimulated lupus T cells, which correlated with the decreased ERK pathway signaling previously reported in lupus T cells. We also demonstrated that PKCδ is upstream of ERK and that impaired PKCδ/ERK pathway signaling in T cells causes demethylation and overexpression of methylation-sensitive genes (12). Importantly, transgenic mice lacking T cell PKCδ activity develop a lupus-like disease with decreased ERK signaling, overexpression of methylation-sensitive genes, and production of anti–double-stranded DNA antibodies similar to those observed in lupus patients (ref. 39 and Gorelik GJ, et al: unpublished observations), strongly supporting the hypothesis that defective PKCδ signaling is sufficient to cause lupus.

PDK-1 phosphorylates PKCδ at T^{505} in the activation loop, promoting alignment of these residues with the catalytic pocket and controlling catalytic activity of the enzyme (24). We demonstrated that phosphorylation of Ser^{241}, which is required for PDK-1 kinase activity, is not appreciably affected in lupus T cells relative to T cells from healthy donors and under the same assay conditions in which PKCδ phospho-T^{505} levels were decreased. This implies that another mechanism inhibits phosphorylation at T^{505} in PKCδ in lupus T cells.

Chronic inflammation is associated with oxidative stress, and a prooxidative state has been described in patients with lupus (27,34). In this prooxidative state, higher levels of ROS/RNIs are present and can directly cause toxicity through posttranslational modifications of proteins and alterations of lipids and DNA. Nitric oxide (NO) is overproduced in lupus (40) and can combine with O_2^- to form $ONOO^-$, a highly reactive and potentially pathogenic molecule. Multiple markers of protein oxidation have been found in SLE patient sera, including protein carbonyls, protein-bound methionine sulfoxide, decreased levels of protein thiols (27), and increases in protein 3-nitrotyrosines that correlate with worsening disease status (16,33,41), indicating that protein oxidation and, in particular, nitrating pathways may play an important role in the pathogenesis of SLE (33,34,36). However, while it is recognized that overproduction of ROS/RNIs alters and modifies T cell signaling, their target molecules and the intracellular mechanisms that they affect are not well understood.

Based on these observations, we investigated whether the defective PKCδ activation in lupus T cells was caused by oxidative damage. $ONOO^-$ was used as the oxidizing agent and caused PKCδ nitration that resulted in decreased phosphorylation of T^{505}. The fact that PKCδ was the only PKC isoform catalytically affected indicates that the $ONOO^-$ inhibitory effect is selective and specific to PMA-stimulated PKCδ phospho-T^{505}.

PKCδ is not phosphorylated at the activation loop (T^{505}) in resting T cells, but phosphorylation increases following PMA stimulation and translocation to the cytoplasmic membrane. In our studies, oxidation modified the phosphorylation pattern, increasing tyrosine phosphorylation while decreasing threonine phosphorylation, indicating specific effects on PKCδ phosphorylation regulation. This differential pattern of PKCδ phosphorylation also modifies its intracellular translocation, resulting in changes to the interaction of PKCδ with downstream targets (42). Recent studies indicate that ROS can also modulate PKCδ activity in other cell types. In keratinocytes, oxidation increases PKCδ tyrosine phosphorylation, resulting in reduced enzymatic activity (43), whereas in other cells, oxidation enhances the enzymatic activity (44) or even modifies the enzymatic specificity (45), indicating that oxidative modifications of PKCδ activity may be cell type dependent and/or phosphorylated residue dependent (28). The precise effects of oxidation on T cell PKCδ have not

been explored, but it is possible that oxidation of certain residues in the molecule causes distortions in the secondary and tertiary structure, resulting in decreased accessibility of T^{505} to phosphorylation. It is also possible that the rate of dephosphorylation increases with oxidation.

The phospho-serine/threonine phosphatases PP-1c, PP-2Ac, and PP-2Cα dephosphorylate PKCδ, with PP-2Ac demonstrating the highest specific activity toward PKCδ (31). However, our results show that T cells transfected with catalytically inactive PP-2Ac mutants or with the corresponding siRNA did not restore PKCδ phosphorylation following $ONOO^-$ treatment, suggesting that modifications of PP-2Ac activity are unlikely to be responsible for the decreased T^{505} phosphorylation observed in oxidized PKCδ. This observation is consistent with a study demonstrating that even though the messenger RNA, protein, and enzymatic activity of the PP-2A catalytic subunit (PP-2Ac) is increased in patients with lupus, the defect is independent of disease activity (23), while the PKCδ phosphorylation impairment is directly related to disease activity (12). The present work shows that oxidation of PKCδ results in a selective decrease in PKCδ phospho-T^{505} levels and directly correlates with decreased phospho-ERK levels in T cells, consistent with our previous results.

The consequences of an oxidative environment on ERK activity are not clearly defined and vary depending on the cell system and stimulus. Decreased phosphorylation of ERK induced by $ONOO^-$ in kidneys of β^s sickle cell mice has been reported (46), and similarly, an alteration of the cellular redox state leads to increased MAPK phosphatase 1 expression in fibroblasts, resulting in ERK inactivation (47). This suggests that additional mechanisms may contribute to the decreased ERK phosphorylation caused by oxidative stress in lupus T cells.

$ONOO^-$ may alter protein structure and function by interacting with different amino acids. By oxidizing cysteine residues (S-nitrosylation), $ONOO^-$ inactivates many enzymes involved in energetic processes in addition to protein tyrosine phosphatases that may enhance tyrosine phosphorylation–dependent signaling (20). $ONOO^-$ also oxidizes tyrosine residues (O-nitration) to form 3-nitrotyrosine derivatives (20). Tyrosine nitration is considered to be a major cause of $ONOO^-$-mediated cytotoxicity because it affects protein structure and function that may result in the generation of antigenic epitopes (48), changes in the catalytic activity of enzymes, and impaired cell signal transduction (49). Nitra-

tion is a highly selective process limited to specific tyrosine residues on a limited number of proteins. In general, protein nitration is associated with loss of function (46). Others have reported an increased amount of NO-mediated oxidation products, such as 3-nitrotyrosine proteins, due to elevated levels of $ONOO^-$ in the serum of lupus patients (41).

We found higher levels of nitrotyrosine-containing proteins in T cells from patients with active lupus than in T cells from patients with inactive lupus or in T cells from healthy controls. The levels of nitrated PKCδ were also greater in T cells from patients with active lupus, and PMA-stimulated T^{505} phosphorylation of the nitrated proteins was impaired. Similar results were observed in control T cells pretreated with $ONOO^-$. Therefore, the presence of significant levels of nitrated PKCδ in T cells from patients with active lupus may explain the decrease in PKCδ phospho-T^{505} levels observed in T cells from lupus patients, the consequent decrease in ERK signaling pathway, and their correlation with disease severity. Protein nitration is often associated with pathologic states related to inflammation, but is also recognized as a normal physiologic process (20), suggesting an explanation for the presence of nitrated proteins in T cells from healthy donors and patients with inactive lupus. The relatively lower level of PKCδ phosphorylation that we found in normal T cells in the nitrated fraction could be due to a lesser degree of oxidation that normally takes place during physiologic metabolism. However, our studies also demonstrate that human T cell PKCδ is directly modified by oxidation. The oxidation selectively decreases PMA-stimulated PKCδ T^{505} phosphorylation and is directly related to a decrease in ERK signaling, suggesting that higher levels of nitration cause structural changes in PKCδ that make it catalytically inactive.

Our study reveals that the impaired T cell PKCδ kinase activation observed in patients with active lupus is likely due to oxidative damage and causes the impaired ERK pathway signaling in T cells from lupus patients. These studies point to PKCδ as a link between oxidative stress, caused by environmental agents, and the epigenetic changes observed in lupus T cells. Other poorly understood autoimmune diseases also result from gene–environment interactions and involve epigenetic mechanisms that include not only DNA methylation, but also histone modifications and microRNA (50). Oxidative stress may also contribute to these disorders through mechanisms such as altered signaling.

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