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Phosphorylation of Pleckstrin Increases Proinflammatory Cytokine Secretion by Mononuclear Phagocytes in Diabetes Mellitus

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The protein kinase C (PKC) family of intracellular enzymes plays a crucial role in signal transduction for a variety of cellular responses of mononuclear phagocytes including phagocytosis, oxidative burst, and secretion. Alterations in the activation pathways of PKC in a variety of cell types have been implicated in the pathogenesis of the complications of diabetes. In this study, we investigated the consequences of PKC activation by evaluating endogenous phosphorylation of PKC substrates with a phosphospecific PKC substrate Ab (pPKC(s)). Phosphorylation of a 40-kDa protein was significantly increased in mononuclear phagocytes from diabetics. Phosphorylation of this protein is downstream of PKC activation and its phosphorylated form was found to be associated with the membrane. Mass spectrometry analysis, immunoprecipitation, and immunoblotting experiments revealed that this 40-kDa protein is pleckstrin. We then investigated the phosphorylation and translocation of pleckstrin in response to the activation of receptor for advanced glycation end products (RAGE). The results suggest that pleckstrin is involved in RAGE signaling and advanced glycation end product (AGE)-elicited mononuclear phagocyte dysfunction. Suppression of pleckstrin expression with RNA interference silencing revealed that phosphorylation of pleckstrin is an important intermediate in the secretion and activation pathways of proinflammatory cytokines (TNF-α and IL-1β) induced by RAGE activation. In summary, this study demonstrates that phosphorylation of pleckstrin is up-regulated in diabetic mononuclear phagocytes. The phosphorylation is in part due to the activation of PKC through RAGE binding, and pleckstrin is a critical molecule for proinflammatory cytokine secretion in response to elevated AGE in diabetes. The Journal of Immunology, 2007, 179: 647–654.

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To investigate the PKC activation under hyperglycemic conditions, we have used a relatively newly developed Ab, phospho-(Ser) PKC substrate Ab (p-PKC(s)) to detect the phosphorylation of PKC substrates. This Ab detects phosphoserine or threonine in a typical sequence context specific for the substrate of the conventional PKC isoforms (α, β, βII, γ). The substrate sequence contains serine or threonine, with arginine or lysine at the −3, −2, and +2 positions, and hydrophobic amino acids at position +1. Its ability to identify potential substrates of PKC such as MARCKS and the 47-kDa subunit of the phagocyte oxidase (p47phox) was confirmed in polymorphonuclear leukocytes (37). Using this Ab, we were able to identify the unique phosphorylation of a 40-kDa protein, identified as pleckstrin. Characterization of the RAGE signaling pathway revealed pleckstrin as a critical intermediate in elevated cytokine secretion by mononuclear phagocytes of diabetics.

Materials and Methods

**Materials**

PBS without Ca/Mg (PBS) was purchased from Invitrogen Life Technologies. The THP-1 cell line, RPMI 1640 culture medium, and FCS were purchased from American Type Culture Collection. The mAbs for human pleckstrin and bovine S100B protein were purchased from BD Transduction Laboratories; 10× cell lysis buffer, 3× sample buffer, and PKC(s) Ab were purchased from Cell Signaling Technology. Protein A/G agarose beads, β-actin Ab, and secondary Abs were purchased from Santa Cruz Biotechnology. Bicinchoninic acid (BCA) protein assay reagent and SuperSignal West Pico Chemiluminescent substrate were obtained from Pierce. All other reagents including Histopaque 1119, Histopaque 1077, PMA, 100 μM PMSF, 100× protease inhibitor mixture, and 100× phosphate inhibitor mixture were purchased from Sigma-Aldrich.

**Study population**

For this study, 13 diabetic patients and 12 systemically healthy individuals were recruited at the Clinical Research Center of Boston University Goldman School of Dental Medicine. Control subjects were age-, gender-, and race-matched normoglycemic individuals with no systemic or local infections (e.g., periodontitis). All subjects signed an informed consent and the study was approved by the Boston University Institutional Review Board. Patients with diabetes mellitus (DM) were selected according to the criteria of the National Diabetes Data Group (38). Demographic data for the diabetic patients including type of diabetes, age, gender, and race were recorded (Table I). The glycemic control of diabetes was assessed by the glycosylated hemoglobin (HbA1c). Based on their glycemic control values, diabetic patients were further grouped into three groups in subsequent experiments: well controlled (HbA1c < 7%; n = 3), moderately controlled (HbA1c 7–8%; n = 3), and poorly controlled (HbA1c > 8%; n = 7) (39).

**Isolation and purification of peripheral blood monocytes**

To evaluate PKC activation in control and diabetic monocytes, samples from 11 pairs of subjects were used. Heparinized (10 IU/ml) peripheral blood was layered on a Ficoll-Hypaque discontinuous gradient system and centrifuged at 1200 × g for 30 min. Mononuclear cells (mostly monocytes and lymphocytes) at the interface of plasma and Ficoll-Hypaque were collected and washed twice in PBS. Mononuclear phagocytes were further separated from lymphocytes using an indirect magnetic cell sorting system (MCS; Miltenyi Biotech) which uses negative selection to isolate unstimulated monocytes from human PBMC. Briefly, T cells, NK cells, B cells, dendritic cells, and basophils were labeled using a biotin-conjugated Ab mixture with Abs specifically against CD3, CD7, CD16, CD19, CD56, CD123, and glycoporphin A and bound to anti-biotin microbeads. A MACS column with a coated, cell-friendly matrix was placed in a permanent magnet. The magnet retained the target cells labeled with microbeads and the unstimulated monocytes passed through the column. Monocytes were then collected and washed twice with PBS.

**THP-1 cell culture**

THP-1 cells, a commonly used human macrophage-like monocytes cell line, were cultured in RPMI 1640 medium, supplemented with 10% FBS, 10 mM HEPES, and 0.05 mM 2-ME. Cells were fed every 2–3 days, and passed every 1–2 wk. Passages 2–20 were used in this study. THP-1 cells were differentiated into monocytes/macrophage-like cells with 10 ng/ml 1,25(OH)2D3 (the active form of vitamin D3) for 48–72 h (40, 41). Differentiation was confirmed by attachment, morphology, and CD14 expression (data not shown). Because PMA was used as an activator of PKC, it was not used to differentiate the THP-1 cells into monocyte/macrophage-like cells.

**Cell stimulation, lysis, and fractionation**

THP-1 cells were stimulated with PMA or S100B. Before stimulation, differentiated THP-1 cells (5 × 10⁶/ml) were resuspended in warm (37°C) PBS medium (135 mM NaCl, 2.7 mM KCl, 16.2 mM Na₂PO₄, 0.90 mM CaCl₂, 0.50 mM MgCl₂, and 7.5 mM d-glucose (pH 7.35)). Cells were stimulated with 5 μg/ml S100B or 200 nM PMA at a final concentration at 37°C. Cells treated with 0.25% (v/v) DMSO in PBS served as negative control. Stimulation was stopped by addition of ice-cold PBS into the tubes in an ice bath. Cells were washed twice in ice-cold PBS and centrifuged for 100 × g for 10 min at 4°C.

When whole cell lysates were needed, cells were directly lysed in 1× cell lysis buffer (20 ml Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μM sodium fluoride, 1× PMSF, 1× phosphoserine phosphatase inhibitor, and 1× protease inhibitor mixture) after the washing. The cells were sonicated four times for 10 s each, and centrifuged at 10,000 × g for 15 min to spin down cellular debris. The supernatant was collected as the “whole cell lysates.”

Subfractionation of mononuclear phagocytes was conducted by the method described by Wolfsen et al. (42) with minor modifications. Cells were pelleted and resuspended in ice-cold hypotonic extraction buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, 1× protease inhibitor mixture, and 1× protease inhibitor mixture. The cells were sonicated four times for 10 s each and centrifuged at 100,000 × g for 60 min. The supernatant was collected as the “cytosolic fraction.” The pellet was resuspended in the cell lysis buffer by sonication four times for 10 s each, incubated for 30 min on ice, and centrifuged at 13,000 rpm for 15 min. The resulting supernatant was used as the “membrane fraction.” The plasma membrane marker Na/K-ATPase was confirmed by Western blotting to be abundantly present in the membrane fraction, not in the cytosolic fraction (43).

**PKC activity assay**

A nonradioactive PKC activity assay kit (Calbiochem) was used to evaluate PKC activity in the samples based on the capacity of endogenous PKC to phosphorylate the synthetic substrate on the provided pseudosubstrate peptides. After treatment, the cells were lysed as described above. A total of 108 μl of reaction buffer (25 mM Tris-HCl (pH 7.0), 3 mM MgCl₂, 0.1 mM ATP, 2 mM CaCl₂, 50 μg/ml phosphatidylserine, 0.5 mM EDTA, 1 mM EGTA, and 5 mM 2-ME) was placed in each well of a polyvinyl plate and preincubated at 25°C for 5 min. Samples (12 μl), including PKC standards, and membrane or cytosol preparations were added to each well in duplicate. Each sample was incubated at 25°C for 15 min. The reaction mixture was transferred to pseudosubstrate-coated wells with a multichannel pipette. After incubation at 25°C for 15 min, the reaction mixture was removed from the plate, and the plate was washed five times with PBS. Biotinylated Ab ZB9 (100 μl) directed to the phosphorylated pseudosubstrate was added to each well and incubated at 4°C for 60 min. The wash was repeated. Peroxidase-conjugated streptavidin (100 μl) was then added to each well and incubated for another 60 min. The wash was repeated after incubation and 100 μl of substrate solution (o-phenylenediamine) was added to each well. Stop solution (100
tein A/G agarose beads (20 μl) was added after 3–5 min and the 96-well plate was read at 492 nm in a microplate reader. PKC activity is presented as nanograms of active PKC per microgram of total protein (BCA Protein Assay Kit; Pierce).

Western blotting

Cell lysates (whole cell, membrane or cytosol) containing 60 μg of total protein were mixed with SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromphenol blue), and boiled in a water bath for 5 min to denature the protein. After cooling on ice, the samples and the molecular mass standards (Bio-Rad) were loaded and separated by SDS-PAGE on 9.0% (w/v) polyacrylamide slab gels. The separated proteins were immediately transferred electro-photographically to a polyvinylidene difluoride membrane using TB buffer (25 mM Tris, 192 mM glycine, and 20% (w/v) methanol) at 0.25 V/cm for 2 h, then destained thoroughly (0.2% methanol, 0.5% TCA). The gel region corresponding to 40 kDa was excised using a sterile razor blade. As a control, a similar size background slice was excised. The slices were washed with 50% HPLC grade acetonitrile/water two times for 10 min with occasional vortex mixing. The proteins contained in the 40-kDa gel slice and the nonspecific protein background were analyzed by microcapillary reverse-phase HPLC nanoelectrospray tandem MS (Waters). Proteins in gel pieces were digested using trypsin and the tryptic peptides were separated by reverse-phase liquid chromatography using a capillary column. The mass of the eluting peptides was determined with a nanoelectrospray ion trap mass spectrometer (Thermo Finnegan). Mass spectrometric data were analyzed by peptide mapping using TurboSequest (Matrix Science). Proteins in the membrane fraction were fractionated into membrane and cytosol fractions. PKC activity in both fractions was measured by a nonradioactive PKC activity assay as described in Materials and Methods and expressed as nanograms of active PKC per microgram of total protein (BCA protein assay). All values represent the average ± SD (p < 0.01, one-way ANOVA). Because PKC in the membrane fraction represents the activated pool of PKC, the increase suggests that PKC is activated in unstimulated circulating mononuclear phagocytes of diabetics.

To identify the 40-kDa protein with MS, proteins in the membrane fraction were first separated by SDS-PAGE on a 10.0% (w/v) gel and stained with Coomassie blue (0.2% methanol, 0.5% TCA, 0.1% (w/v) Coomassie blue) for 2 h, then destained thoroughly (0.2% methanol, 0.5% TCA). The gel region corresponding to 40 kDa was excised using a sterile razor blade. As a control, a similar size background slice was excised. The slices were washed with 50% HPLC grade acetonitrile/water two times for 10 min with occasional vortex mixing. The proteins contained in the 40-kDa gel slice and the nonspecific protein background were analyzed by microcapillary reverse-phase HPLC nanoelectrospray tandem MS (Waters). Briefly, proteins in gel pieces were digested using trypsin and the tryptic peptides were separated by reverse-phase liquid chromatography using a capillary column. The mass of the eluting peptides was determined with a nanoelectrospray ion trap mass spectrometer (Thermo Finnegan). Mass spectrometric data were analyzed by peptide mapping using TurboSequest (Matrix Science). Proteins in the membrane fraction were fractionated into membrane and cytosol fractions. PKC activity in both fractions was measured by a nonradioactive PKC activity assay as described in Materials and Methods and expressed as nanograms of active PKC per microgram of total protein (BCA protein assay). All values represent the average ± SD (p < 0.01, one-way ANOVA). Because PKC in the membrane fraction represents the activated pool of PKC, the increase suggests that PKC is activated in unstimulated circulating mononuclear phagocytes of diabetics.

HPLC/mass spectrometry (MS)

To identify the 40-kDa protein with MS, proteins in the membrane fraction were first separated by SDS-PAGE on a 10.0% (w/v) gel and stained with Coomassie blue (0.2% methanol, 0.5% TCA, 0.1% (w/v) Coomassie blue) for 2 h, then destained thoroughly (0.2% methanol, 0.5% TCA). The gel region corresponding to 40 kDa was excised using a sterile razor blade. As a control, a similar size background slice was excised. The slices were washed with 50% HPLC grade acetonitrile/water two times for 10 min with occasional vortex mixing. The proteins contained in the 40-kDa gel slice and the nonspecific protein background were analyzed by microcapillary reverse-phase HPLC nanoelectrospray tandem MS (Waters). Briefly, proteins in gel pieces were digested using trypsin and the tryptic peptides were separated by reverse-phase liquid chromatography using a capillary column. The mass of the eluting peptides was determined with a nanoelectrospray ion trap mass spectrometer (Thermo Finnegan). Mass spectrometric data were analyzed by peptide mapping using TurboSequest (Matrix Science). Proteins in the membrane fraction were fractionated into membrane and cytosol fractions. PKC activity in both fractions was measured by a nonradioactive PKC activity assay as described in Materials and Methods and expressed as nanograms of active PKC per microgram of total protein (BCA protein assay). All values represent the average ± SD (p < 0.01, one-way ANOVA). Because PKC in the membrane fraction represents the activated pool of PKC, the increase suggests that PKC is activated in unstimulated circulating mononuclear phagocytes of diabetics.

Immunoprecipitation (IP)

To further confirm the identity of pleckstrin as the PKC substrate, we treated cells with or without PMA to activate PKC. Then, cells (10^6 cells/well) were either added with 1 μM of 50% bead slurry) were added and incubated with whole cell lysates or lysed with hypertonic buffer and subjected to fractionation to collect, spun down at 100,000 g at 4°C. The pellet was washed five times with 500 μl of 1× cell lysis buffer, and resuspended with 20 μl of 3× SDS sample buffer. The pellets were vortexed and microcentrifuged for 30 s. The supernatant was heated to 100°C for 5 min before loading on SDS-PAGE gels for the detection of pleckstrin by Western blotting.

RNA interference gene silencing

To further investigate the role of pleckstrin in cellular events, we used small-interfering RNA (siRNA) duplex targeting pleckstrin mRNA or siCONTROL (nontargeting siRNA) (Ambion). Pleckstrin siRNA targets exon 3 of the mRNA. THP-1 cells, which were within the 2–10th passages, were differentiated with VitD3, 2 days before the transfection. siRNA was introduced by electroporation using a Nucleofector instrument and specific Nucleofector kit for THP-1 Cells (Amaxa Biosystems). Briefly, 1 × 10^6 THP-1 cells were carefully resuspended in 100 μl of Nucleofector solution provided in the kit. siRNA (0.5 μg) was added to the solution and mixed gently. The cell solution was then transferred to a cuvette and electroporated with the predesigned program V-01. Transfected cells were cultured in RPMI 1640 medium for an additional 24 h. Western blotting was performed to evaluate the efficiency of the gene-specific silencing on protein expression, which was 30%.

ELISA

To investigate the impact of suppression of pleckstrin on the inflammatory cytokine release by monocytes/macrophages, THP-1 cells (10^6 cells/well) were transfected with siRNA, cultured for 24 h, and treated with or without LPS (1 μg/ml) SI100B. An ELISA kit (R&D Systems) was used to measure the amount of TNF-α and IL-1β present in the cell culture medium. The kit used a quantitative "sandwich" technique, where a mAb specific for the TNF-α or IL-1β to be measured was precoated on the 96-well microtiter plate. Brieﬂy, the supernatant of THP-1 cells was harvested after treatment under different conditions in a 24-well plate. The standards or unknown sample (100 μl) were then added to the wells in duplicate. After 2-h incubation at room temperature, the wells were washed three times followed by addition of a second-specific cytokine-conjugated Ab. The plate was incubated at room temperature for 2 h and the wash repeated. A substrate solution was then added to all the wells and incubated for 20 min followed by 50 μl of stop solution. Plates were read using an ELISA plate reader (Molecular Devices) at a wavelength of 490 nm and compared with a standard curve. To normalize the concentration of proinflammatory cytokines, we used the total protein content in each well. This method was chosen as an accurate indicator of cellular content (44). Briefly, after treatment at different conditions, cells in the supernatant of each well were collected, spun down at 100 × g for 10 min and lysed with 80 μl of cell lysis buffer. Attached cells were directly lysed in the well with 20 μl of cell lysis buffer. Protein concentration was measured with BCA protein assay. Total protein was calculated based on the protein concentration and the volume. The average total protein levels for cells transfected with siRNA was 160 μg/well and there was no significant difference between cells transfected with control siRNA and PLEK siRNA.

Results

PKC is activated in diabetic mononuclear phagocytes

A nonradioactive PKC activity assay was performed to detect the endogenous activity of PKC in diabetic mononuclear phagocytes
FIGURE 1. Seven pairs of subjects were recruited for this experiment and monocytes were immediately lysed and fractionated after isolation. The levels of cytosolic PKC activity were similar in control and diabetic mononuclear phagocytes (control/H11005 8.54/H11006 2.98, DM/H11005 8.7/H11006 3.07 ng of active PKC per microgram of total protein). However, there was significant elevation of PKC activity in the membrane fraction of diabetic monocytes (control/H11005 2.03/H11006 0.96, DM/H11005 6.32/H11006 2.82 ng of active PKC per microgram of total protein, p < 0.01). Because PKC in the membrane portion represents the activated pool of PKC, the increase suggests that PKC is activated in unstimulated circulating mononuclear phagocytes of diabetics.

Hyperphosphorylation of 40-kDa protein in diabetic mononuclear phagocytes

To investigate the substrates of the activated PKC in diabetic mononuclear phagocytes, mononuclear cell homogenates were assessed by immunoblotting to monitor the phosphorylation of conventional PKC (cPKC) substrates with specific PKC substrate (pPKC(s)) Ab. Unstimulated mononuclear phagocytes from diabetic patients and nondiabetic age-, race-, and gender-matched controls were isolated and immediately lysed for Western blotting. Fig. 2 reveals the phosphorylation pattern of cPKC substrates in monocytes of healthy controls, well-controlled, moderate-controlled, and poorly controlled diabetic patients. Phosphorylation of proteins with apparent molecular weights of 28, 30, 40, 47, and 60 kDa was detected in unstimulated monocytes. Among these, the 40-kDa protein exhibited enhanced phosphorylation in moderate-controlled diabetic cells compared with nondiabetic controls and well-controlled diabetics. The phosphorylation of the 40-kDa protein was dramatically increased in poorly controlled diabetic subjects compared with the nondiabetic matched healthy controls, respectively.

Identification of the 40-kDa protein as pleckstrin

To identify the 40-kDa protein, MS was performed. To reduce contamination of cytosolic proteins of similar molecular mass, a membrane fraction of differentiated THP-1 cells stimulated with PMA for 20 min was harvested. Proteins in the membrane fraction were then separated on a 10% SDS-PAGE gel. The band corresponding to 40 kDa (arrow in A) was excised from the SDS-PAGE gel stained with Coomassie blue for analysis. B, The results of MS analysis. Five tryptic peptides (A–E) mapping to pleckstrin protein sequence were found in MS. Three (A–C) are statistically significant as demonstrated by the Xcorr value >2.0. The A–E peptide sequences are shown along with the total protein coverage of pleckstrin (bold type).
on a 10% SDS-PAGE gel and the band corresponding to the 40-kDa protein was cut from the Coomassie blue-stained SDS-PAGE gel (Fig. 3A) and sent for HPLC/MS analysis. Analysis from HPLC/MS revealed five matched tryptic peptides (designated A–E) identified by peptide mapping (Fig. 3B). Three of these peptides (A–C) are statistically significant matches with Xcorr >2.0. Pleckstrin is the predominant protein in the 40-kDa band. Besides pleckstrin, several other proteins ~40 kDa were detected in the gel band by MS analysis. These proteins are PWPI-interacting protein 4 (40.5 kDa, AAK69110.1), mutant β-actin (41.8 kDa, CAA45026.1), FcRn α-chain (39.3 kDa, AAG31421.1), and MHC class I Ag (40.1 kDa, AAG27626.1). Pleckstrin is the only known PKC substrate among these candidates.

IP was performed to further confirm that the identity of the 40-kDa protein was pleckstrin (Fig. 4). Immunoprecipitates were obtained from membrane protein isolated from differentiated THP-1 cells treated with DMSO or PMA for 5 min with pPKC(s) as the precipitating Ab. Nonimmune rabbit IgG was added in the same manner as the Ab described above as a negative control. Proteins harvested from immunoprecipitation were those phosphorylated PKC substrates recognized by pPKC(s). The presence of pleckstrin in the immunoprecipitates was detected by Western blotting with a mAb against human pleckstrin. Pleckstrin (40 kDa) was detected in precipitates from both unstimulated and PMA-stimulated THP-1 cells and the level of phosphorylated pleckstrin was up-regulated by PMA stimulation (Fig. 4). Taken together, the results suggested that the 40-kDa protein recognized by pPKC(s) is pleckstrin.

**PMA induces the phosphorylation and translocation of pleckstrin in human mononuclear phagocytes**

It has been previously shown that pleckstrin is rapidly phosphorylated and translocates to the membrane in response to inflammatory stimuli such as fMLP, PMA, and OPZ in neutrophils (45). IP demonstrated that the phosphorylated pleckstrin accumulated on the membrane after PMA stimulation. To further confirm these findings and define the translocation of pleckstrin from cytosol to membrane, a mAb against human pleckstrin was used for direct detection of subcellular distribution of pleckstrin after PMA treatment by Western blotting. Fractionated cell lysates were obtained from THP-1 cells with or without PMA stimulation (Fig. 5). In unstimulated cells, pleckstrin was localized predominantly in the cytosol. Within 30 s and up to 20 min after PMA stimulation, pleckstrin rapidly redistributed from the cytosol to the membrane fraction.

To confirm that the phosphorylation of pleckstrin is associated with PKC activation, PMA, a direct activator of PKC, was used to stimulate mononuclear phagocytes taken from healthy controls. Upon 200 nM PMA stimulation, phosphorylation of the 40-kDa protein was elevated in whole cell lysates from both human monocytes and THP-1 cells in a similar pattern (Fig. 6 A). The subcellular distribution of these phosphorylated PKC substrates was also monitored, with the cell lysates fractionated into cytosolic and membrane fractions. It was further observed that phosphorylation of pleckstrin is increased in a time-dependent manner after PKC activation and phosphorylated pleckstrin is primarily located on the membrane fraction (Fig. 6B).

**S100B induces phosphorylation and translocation of pleckstrin**

We have recently reported that RAGE activation activates PKC (46). To study the role of pleckstrin in this process, we used a RAGE ligand, S100B, to treat monocytes/macrophages. Cells were treated with 5 µg/ml S100B for 5–20 min. Membrane proteins were extracted and pleckstrin phosphorylation was analyzed by

![FIGURE 5. PMA induces the translocation of pleckstrin to the membrane. THP-1 cells were treated with PBS containing 0.25% DMSO or with 200 nM PMA for 5 min, lysed with hypertonic buffer, and fractionated into membrane and cytosol fractions. The amount of pleckstrin in these two fractions was detected by Western blotting with an Ab against pleckstrin. Results are representative of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 6. PMA induces the phosphorylation of pleckstrin. A. Normal human mononuclear phagocytes and THP-1 cells were treated with PBS containing 0.25% (v/v) DMSO (resting) or with 200 nM PMA for 5 min. Western blot analysis was performed to detect phosphorylation of PKC substrates in the whole cell lysates. The arrow indicates the increased phosphorylation of pleckstrin. B. THP-1 cells were stimulated with 200 nM PMA for 5 and 20 min. Cells were lysed with hypertonic buffer for cell fractionation. Both membrane and cytosolic fractions were analyzed by Western blotting to detect phosphorylation of cPKC substrates and their cellular distribution. The arrow indicates that the phosphorylated 40-kDa pleckstrin is located in the membrane fraction after PMA stimulation. The amount of total protein loaded in each lane was confirmed by Coomassie blue staining. Results are representative of three independent experiments.](http://www.jimmunol.org/)
Western blotting using pPKC(s) Ab. Fig. 7 demonstrates that, similar to PMA, S100B induced phosphorylation and translocation of pleckstrin to the membrane-related fraction.

The role of pleckstrin in proinflammatory cytokine secretion induced by S100B

Based on the fact that RAGE signal transduction includes phosphorylation of pleckstrin, possibly through PKC, the purpose of the next experiment was to determine the role of pleckstrin in proinflammatory cytokine secretion. siRNA was used to temporarily decrease pleckstrin expression to evaluate cytokine secretion after down-regulation of pleckstrin. Pleckstrin siRNA targeting exon 3 was transfected into the THP-1 cells; down-regulation (30%) of total pleckstrin was confirmed by Western blotting (Fig. 8A). In addition to the suppression of pleckstrin at the total protein level, we also investigated inhibition of activation of pleckstrin in siRNA-transfected cells. Fig. 8B demonstrates that phosphorylation of pleckstrin was also decreased by 30% in siRNA-transfected THP-1 cells. Cell death evaluated with trypan blue staining in pleckstrin siRNA-transfected and control siRNA-transfected cells showed no significant difference.

Fig. 9 demonstrates that the decreased expression of pleckstrin resulted in marked decrease in secretion of TNF-α (A) elicited by S100B (1 μg/ml). Similar to TNF-α, another major proinflammatory cytokine, IL-1β (B), was significantly reduced in response to inhibition of pleckstrin (control siRNA = 9.56 ± 1.2, pleckstrin siRNA = 3.09 ± 0.09 pg/ml per microgram of total protein, p < 0.001; one-way ANOVA; Fig. 9B) elicited by S100B (1 μg/ml). Collectively, these
data demonstrate that decreased pleckstrin levels and activity lead to significant reduction in RAGE-mediated proinflammatory cytokine release by monocytes/macrophages.

**Discussion**

It has been previously reported that activity of PKC is elevated in various tissues of diabetic animals (47). In this study, we have analyzed the mechanism of PKC activation in mononuclear phagocytes of diabetics. Our investigations revealed that poor glycemric control results in increased PKC activity. In addition, as a consequence of PKC activation, the downstream substrates of cPKC are also activated. Pleckstrin is the major PKC substrate phosphorylated in diabetic monocytes/macrophages. To our knowledge, this is the first report of altered phosphorylation of pleckstrin as a consequence of a human disease. The phosphorylation of pleckstrin was specific in diabetics; few other proteins revealed an up-regulation of phosphorylation in mononuclear phagocytes of diabetic individuals. The degree of phosphorylation increased with declining diabetic control (increased HbA1c). Because it is difficult to standardize the individual variations among human subjects with respect to monocyte/macrophage functions, we used THP-1 cells to study the mechanism of hyperglycemia-induced changes in PKC activation and involvement of pleckstrin in vitro. Preliminary experiments by our group and others confirm the expression of RAGE on THP-1 cells as well as functions such as oxidative burst and cytokine production (41, 46). Furthermore, a similar phosphorylation pattern of PKC substrates responding to PMA stimulation between THP-1 cells and human monocytes (Fig. 6) also confirms that THP-1 cells are a suitable model for the study of signaling pathways of PKC, RAGE, and cytokine production.

Pleckstrin was first identified as the major PKC substrate in platelets, while it is found to be expressed in all cells of the hematopoietic system. The protein consists of two pleckstrin homology (PH) domains at the N and C termini, bridged by three PKC-phosphorylation sites (48, 49). The PH domain is a prototypic structural motif presenting in a large number of signaling molecules that mediate protein-protein and protein-phospholipid interactions. Between the two PH domains, there is a so-called disheveled, egl-10, pleckstrin domain; its molecular function in pleckstrin remains unclear (50). Because pleckstrin has not been found to display enzymatic activity, it was hypothesized that pleckstrin may function as an intracellular adaptor, perhaps by bridging proteins and/or lipids via its dual PH domains in response to particulate stimuli.

There are several observations in leukocytes suggesting that pleckstrin might be important in the innate immune response. In neutrophils, the chemotactic agent fMLP induces phosphorylation and subcellular redistribution of pleckstrin (51). In mononuclear phagocytes, expression of pleckstrin can be induced by LPS/IFN-γ. During phagocytosis of IgG-opsonized zymosan particles, pleckstrin translocates to the phagosome membrane (45), which suggests that it might be important in orchestrating phagocytosis of pathogens. Our data show that pleckstrin activation is increased under diabetic conditions leading to an increased proinflammatory cytokine release by monocytes/macrophages and suggests that pleckstrin, at least in part, is involved in inflammatory response in diabetics.

In this study, we used S100B, which is a known agonist for RAGE, to stimulate RAGE-mediated events in monocytes/macrophages (8). AGE are nonenzymatically glyated molecules that are elevated in the serum of diabetics (46, 52). Many cell types express RAGE (53) and it has been demonstrated that binding of AGE to innate immune cells leads to increased inflammation (54–56). RAGE binds a variety of ligands including the various forms of AGE as well as the S100 family of calgranulins (57). In neutrophils, RAGE signals through PKC (20). Using S100B as the RAGE agonist, we demonstrate that phosphorylation and translocation of pleckstrin can be induced by RAGE-mediated PKC activation (Fig. 7). These data suggest that pleckstrin is an important signaling molecule downstream of PKC in the RAGE-signaling pathway, and that activation of RAGE is responsible, at least in part, for the hyperphosphorylation of pleckstrin observed in diabetic mononuclear phagocytes leading to activation and a hyper-inflammatory phenotype. The PKC substrate Ab (p-PKC(s)) used in this study is designed for the substrates of conventional PKCs including PKC α, β, βII, and γ (57–59). Therefore, within the limits of this study, we speculate that conventional PKCs are the most likely candidates to phosphorylate pleckstrin. Further investigations with specific PKC isoform inhibitors are required to clarify which isoform of PKC is responsible for pleckstrin phosphorylation in response to hyperglycemia.

To further implicate RAGE and hyperphosphorylation of pleckstrin in the inflammation associated with poorly controlled diabetics, we investigated induction of the proinflammatory cytokine release by mononuclear phagocytes. siRNA gene silencing was used to temporarily knockdown pleckstrin message and protein expression. Fig. 8B shows that suppression of total amount of pleckstrin expression using siRNA is accompanied by reduced phosphorylation of pleckstrin responding to S100B. Reduction of pleckstrin by 30% at both the protein and phosphorylation levels markedly blocked secretion of TNF-α by 80% and IL-1β by 75% in response to AGE demonstrating that pleckstrin is of fundamental importance in proinflammatory cytokine production induced by RAGE ligand (Fig. 9). Because it has been previously reported that pleckstrin was involved in phagosome maturation, it is also possible that there might be abnormalities of phagosomal formation in diabetic mononuclear phagocytes. Considering the importance of pleckstrin in inflammatory cytokine secretion, the hyperphosphorylation of pleckstrin observed in diabetic mononuclear phagocytes likely contributes to the development of excessive inflammation that leads to diabetic complications. The lack of enzymatic activity of the molecule favors the hypothesis that pleckstrin serves as an adapter protein to organize and facilitate protein-protein and protein-lipid interaction on the plasma membrane or phagosomal membrane. In particular, new therapies targeting PKC and excess inflammation to prevent diabetic complications would benefit from a readily obtained surrogate end point, rather than waiting years for the development of elevations of creatinine or microalbuminuria. In summary, the data presented suggest that phosphorylation of pleckstrin is up-regulated in diabetic mononuclear phagocytes in part due to activation of PKC by RAGE agonists and pleckstrin is a critical molecule in modulating proinflammatory cytokine secretion in the presence of RAGE ligands.

**Disclosures**

The authors have no financial conflict of interest.

**References**


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