Peptidylarginine Deiminase 2 Suppresses Inhibitory \( \kappa B \) Kinase Activity in Lipopolysaccharide-stimulated RAW 264.7 Macrophages*†‡

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Peptidylarginine deiminases (PADs) are enzymes that convert arginine to citrulline in proteins. In this study, we examined PAD-mediated citrullination and its effect on pro-inflammatory activity in the macrophage cell line RAW 264.7. Citrullination of 45–65-kDa proteins was induced when cells were treated with lipopolysaccharide (LPS; 1 \( \mu g/mL \)). Protein citrullination was suppressed by the intracellular calcium chelator BAPTA/AM (30 \( \mu M \)). LPS treatment up-regulated COX-2 levels in cells. Interestingly, overexpressing PAD2 reduced LPS-mediated COX-2 up-regulation by 50%. PAD2 overexpression also reduced NF-\( \kappa B \) activity, determined by NF-\( \kappa B \)-driven luciferase activity. The effect of PAD2 on NF-\( \kappa B \) activity was further examined by using HEK 293 cells transfected with NF-\( \kappa B \)-luciferase, \( \kappa B \)B/\( \gamma \) kinase (IKK\( \gamma /B \)\( /\)\( \gamma \)) subunits, and PAD2. IKK\( \gamma \) increased NF-\( \kappa B \) activity, but this increase was markedly suppressed when PAD2 was present in cells. IKK\( \gamma \)-mediated NF-\( \kappa B \) activation was further enhanced by IKK\( \gamma \) in the absence of calcium ionophore A23187. However, this stimulatory effect of IKK\( \gamma \) was abolished by PAD2. Coinmuno precipitation of cell lysates showed that IKK\( \gamma \) and PAD2 can coimmunoprecipitate in the presence of the Ca\(^{2+}\) ionophore. IKK\( \gamma \) coimmunoprecipitated truncation mutants, PAD2(1–385) and PAD2(355–672). The substitution of Gln-358 (a putative ligand for Ca\(^{2+}\) binding) with an Ala abolished coimmunoprecipitation. Conversely, PAD2 coimmunoprecipitated truncation mutants IKK\( \gamma \)(1–196) and IKK\( \gamma \)(197–419). In other experiments, treating RAW 264.7 cells with LPS induced citrullination in the immunoprecipitates of IKK\( \gamma \). In vitro citrullination assay showed that incubation of purified PAD2 and IKK\( \gamma \) proteins in the presence of Ca\(^{2+}\) citrullinated IKK\( \gamma \). These results demonstrate that PAD2 interacts with IKK\( \gamma \) and suppresses NF-\( \kappa B \) activity.

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‡‡ Peptidylarginine deiminases (PADs)2 (E.C.3.5.3.15) are Ca\(^{2+}\)-dependent enzymes that catalyze the conversion of arginine to citrulline with concomitant production of ammonia (1, 2). Because the conversion results in a change in charge (positive to neutral), protein citrullination or deimination can affect both intramolecular and intermolecular interactions, potentially altering protein structure and function (3–5). Citrullination can engender autoimmune responses such as those seen in rheumatoid arthritis (6, 7), multiple sclerosis (8), and psoriasis (9).

Protein citrullination can be induced by inflammatory stimuli such as LPS and TNF (10). Citrullination of proteins such as histones regulates gene transcription (11, 12). Smoking increases PAD expression and citrullination in human lung (13). Among five different PAD isotypes identified in mammals (2), PAD2 and PAD4 are responsible for citrullination in macrophages (14, 15). PAD2 protein is observed in macrophages, although the mRNA is expressed in monocytes and monocytes-derived macrophages (14). PAD4 is expressed mainly in the granulocytes, lymphocytes, endothelial cells, monocytes, and macrophages (16–18). PAD4 is predominantly found in nuclei in these cells. However, the precise role of the citrullination in diseases and inflammation remains unclear. Moreover, the physiological role of PADs in macrophages has yet to be fully determined.

Macrophages are effector cells that regulate inflammatory responses in a variety of tissues including lung. The lung harbors abundant alveolar macrophages and is constantly challenged by inflammatory stimuli. Macrophages are the major responders to LPS (endotoxin) (19, 20), and LPS-activated macrophages produce pro-inflammatory proteins including cyclooxygenase-2 (COX-2) (21). COX-2 is responsible for formation of prostaglandins (21–23), which promote inflammation through a variety of mechanisms. The synthesis of COX-2 is mediated by nuclear translocation and activation of NF-\( \kappa B \) that normally resides in the cytoplasm and forms a protein complex with inhibitory \( \kappa B \) (I\( \kappa B \)). The dissociation of NF-\( \kappa B \)-I\( \kappa B \) is mediated by I\( \kappa B \) kinase (IKK) signalosome, which consists of two catalytic subunits, IKK\( \alpha \) and IKK\( \beta \), and...
**PAD2-mediated Regulation of IKKγ in Macrophages**

a regulator, IKKγ. IKKγ (also known as NF-κB essential modulator; NEMO) is an important regulatory component of the IKK complex. When cells are stimulated by LPS, IKK signaling is activated to cause NF-κB dissociation from IκBa, and NF-κB is translocated to nucleus, where it activates target genes (24, 25).

In this study, we tested whether PAD2-mediated citrullination affects NF-κB activity in the mouse leukemic monocyte macrophage cell line RAW 264.7. We examined LPS-induced citrullination, NF-κB luciferase activity, and in vitro interaction between PAD2 and IKKγ. Our data demonstrate that PAD2 suppresses NF-κB activity probably by interacting with IKKγ.

**EXPERIMENTAL PROCEDURES**

Reagents—Ultrapure TLR4-specific LPS from Escherichia coli (Alexis Biochemicals, San Diego, CA) was directly added to culture medium. The intracellular calcium chelator BAPTA/AM (Calbiochem) was dissolved in dimethyl sulfoxide culture medium. The intracellular calcium chelator (Alexis Biochemicals, San Diego, CA) was directly added to cells at Vanderbilt University.

**Immunoprecipitation**

Cells at 24 h after transfection were used for experiments. Detection of Citrullinated Proteins—Protein citrullination was analyzed by immunoblot with an antibody specific to modified peptidylcitrulline residues. Cells were lysed with radioimmunoprecipitation assay cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% sodium orthovanadate, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with EDTA-free protease inhibitor cocktails (Roche Diagnostics). Cell debris was removed by microcentrifugation for 10 min, and protein concentration was determined by the Bradford assay reagent (Bio-Rad). Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and then cross-linked by formaldehyde incubation to improve protein retention. Citrullinated proteins were detected using the anti-citrulline (modified) detection kit (Millipore, Billerica, MA) with modified anti-citrulline rabbit polyclonal antibody (1:1000) and a goat anti-Rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (1:5000; Millipore). Citrullinated protein bands were detected using the enhanced chemiluminescence ECL Plus (GE Healthcare). As an internal control, the membrane was stripped and reprobed with β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunoblot for COX-2 in RAW 264.7 Cells**—Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was preincubated in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% nonfat dry milk and then incubated with polyclonal anti-COX-2 (Cayman Chemical) at room temperature for 2 h. The blot was washed with PBS containing 0.05% Tween 20 for 20 min (4 × 5 min) and then incubated with GAR-HRP (1:5000) for 1 h. Tubulin (Santa Cruz Biotechnology) was used as an internal control.

**Immunoprecipitation—HEK 293 cells were co-transfected with (i) HA-tagged IKKγ (WT) and FLAG-tagged PAD2 (WT) or truncation mutants or (ii) FLAG-tagged PAD2 (WT) and HA-tagged IKKγ (WT) or truncation mutants for determining domains responsible for interaction. At 24 h after transfection, cells were treated with either 1 μM A23187 or no A23187 in serum-free medium for 1 h. Cell lysates were pre-

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**RT-PCR and Full-length cDNA for PAD2**—Total RNA was extracted from untreated RAW 264.7 cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA by using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). To confirm the presence of PAD2 and PAD4 in RAW 264.7 cells, we performed PCR. For PCR, two sets of primers were designed based on the published sequence of mouse PAD2 (GenBank™ accession number: NM_008812.1). The forward primer was 5'-CTTCAAGATG-GATGAAAATCACCAGG-3', and the reverse primer was 5'-CACTATGTCACCACCTGAAAGCC-3', which amplify a 246-bp PAD2 product. The forward primer of the other sets was 5'-GTATGTTACGGCCTGGGAGGCATG-3', and the reverse primer was 5'-TACCGATCATGTTCACTAGTGTAGG-3', which amplify a 246-bp PAD2 product. PCR was performed for 30 cycles with Pfui DNA polymerase (Promega, Madison, WI) at 94°C for 40 s, 60°C for 30 s, and 72°C for 40 s. The final extension time at 72°C was 7 min. The products were sequenced in the DNA Sequencing Facility at Vanderbilt University.

The full-length PAD2 cDNA was cloned by PCR with primers corresponding to the 5' and 3' ends of mouse PAD2. The sequence of the full-length PAD cDNA was identical to the published PAD2 of mouse (the amino acid sequence of cloned PAD2 is available in the supplemental material).

**Plasmids**—FLAG-tagged PAD2 was constructed by excising out full-length PAD2 from the plasmid pBlescript/PAD2 with EcoRI and Sall and subcloning into pCMV-Tag 2C Vector, FLAG-tagged truncation mutants PAD(1–385) and PAD(355–672) were made by deletion of corresponding nucleotides. The point mutant PAD(355–672)/Q358A was generated using a site-directed mutagenesis kit (Agilent Technologies). The N-terminal hemagglutinin (HA)-tagged IKKγ was made by ligating IKKγ into pCDNA 3.1(+) plasmid (Invitrogen). HA-tagged truncation mutants IKKγ(1–196) and IKKγ(197–419) were constructed by deletion of correspond-
pared in immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.5 mM EDTA, 3% glycerol, 0.5% Nonidet P-40, 1 mM NaF, and 1 mM Na₂VO₃) supplemented with 1 mM dithiothreitol and EDTA-free protease inhibitor cocktails (Roche Diagnostics). Lysates were incubated with the monoclonal anti-HA antibody (1–2 μg) (Cell Signaling, Danvers, MA) at 4 °C overnight and then with protein A-Sepharose beads (Zymed Laboratories Inc., San Francisco, CA) at 4 °C for 30 min. The beads were washed with immunoprecipitation buffer containing 250 mM NaCl more than five times, and the immunoprecipitates were dissociated from the beads by adding SDS-sample buffer and boiling. The immunoprecipitates were subjected to immunoblotting with monoclonal anti-FLAG M2 antibody (BD Pharmingen). After washes, protein citrullination was determined by the immunoblot described above. Silver staining of the proteins loaded on the gel was performed using the Silver Stain Plus kit (Bio-Rad).

**RESULTS**

**PAD Activity Is Induced by LPS Treatment in RAW 264.7 Cells**—To examine PAD activity in RAW 264.7 cells, we treated cells with LPS (1 μg/ml) in DMEM containing 2 mM Ca²⁺ for 0.25–30 h and lysed for immunoblot analysis with the antibody to modified citrulline. Fig. 1A shows the time course of citrullination after LPS treatment (n = 4). Proteins with the molecular mass of 45–65 kDa were prominently citrullinated, comparable with a previous report (26). Most of time, two or three citrullinated major bands appeared on the Western blot. The reason for this is unclear. Vertical stripes or dots at 0.25, 0.5, 16, and 30 h are immunoblot artifacts. Protein citrullination regardless of LPS treatment was found at 1 h determined by densitometric measurements of citrullinated proteins normalized to β-actin (p < 0.05; one-way analysis of variance with Bonferroni post test) (Fig. 1B). Activation of PAD enzymes requires Ca²⁺ (1, 2, 4), and thus, to further characterize citrullination, we applied LPS to cells for 4 h after pretreating with the Ca²⁺ chelator BAPTA/AM (30 μM) for 1 h. Fig. 1C shows a representative immunoblot result. Control cells without LPS had negligible citrullination regardless of BAPTA/AM in the medium (Fig. 1C, lanes 1 and 2). A slight citrullination in the presence of BAPTA/AM is often observed, the reason for which is unclear. Cells with LPS had typical citrullination in the absence of BAPTA/AM (lane 3). However, the citrullination was suppressed by BAPTA/AM (lane 4). These results demonstrate that LPS induces protein citrullination in RAW 264.7 cells and that citrullination is calcium-dependent.

**PAD2 Reduces COX-2 Expression in LPS-treated Cells**—Among five PAD isotypes characterized in mammals (2), comparison among more than two samples. The p value of less than 0.05 was considered significant. Data were analyzed using Origin 8.1 software (OriginLab Corp., Northampton, MA).
PAD2 and PAD4 are closely associated with rheumatoid arthritis (7). PAD2 is expressed in cytosol, whereas PAD4 is predominantly found in nucleus (27), and thus, we focused on PAD2 in this study to determine the effect of cytosolic citrullination on inflammatory gene expression in macrophage cells. RT-PCR with primers specific to mouse PAD2 detected PAD2 mRNA in RAW 264.7 cells (Fig. 2A). PCR without reverse transcriptase detected none. We also performed RT-PCR with primers for PAD4 but could not detect its mRNA (data not shown). We then cloned the full-length PAD2 cDNA by PCR with primers corresponding to mouse PAD2 (amino acid sequence comparison of the cloned mouse PAD2 with human PAD4 is provided in the supplemental material).

RAW 264.7 cells were transfected with PAD2 and treated with LPS for 4 h, and an immunoblot was performed to determine COX-2 protein expression. Fig. 2B shows that COX-2 expression was induced by LPS, consistent with previous reports (21–23). The induction occurred in both groups of cells transfected with PAD2 or vector only (Mock in the figure), but the COX-2 expression level was substantially lower in cells transfected with PAD2. This decrease corresponded to 50% of the controls ($p < 0.05; n = 3$; Fig. 2C). The internal control tubulin had negligible change.

This finding suggests that PAD2 can suppress COX-2 expression induced by LPS. To further characterize this effect, we compared NF-κB activity between control cells and PAD2-overexpressing cells. Cells were transiently transfected with PAD2 and the NF-κB luciferase construct, and 24 h later, luciferase activity in cells was measured after 4 h of treatment with LPS (Fig. 2D). Controls were cells without PAD2 (Mock in the figure). The application of LPS increased luciferase activity by $3.2 \pm 0.3$-fold ($n = 4$) in control cells. However, in PAD2-expressing cells, LPS increased luciferase activity by $1.7 \pm 0.2$-fold ($n = 4$). The decrease corresponded to 47% of the control ($p < 0.05$).

**PAD2 Inhibits IKK Complex Activity**—We tested whether PAD2 inhibits IKK complex from activating NF-κB. For this analysis, we used HEK 293 cells to ensure robust expression of wild type and truncated mutant DNA plasmids. HEK 293 cells were transiently transfected with IKKβ and IKKγ subunits, PAD2, and NF-κB-luciferase construct. PAD2 activation was achieved by inducing Ca$^{2+}$ influx using 1 μM calcium ionophore A23187 (18). Fig. 3A shows NF-κB activity in cells transfected with or without PAD2 (no subunits of IKK complex). The values were presented relative to the value for cells without PAD2. Overall, NF-κB activities were low in these control cells regardless of PAD2 expression and A23187 treatment ($n = 3$ for each).

Fig. 3B shows NF-κB activity in cells transfected with IKKβ with or without PAD2. IKKβ increased NF-κB activity by 2.1-fold when compared with control cells without IKKβ ($p < 0.05$).
PAD2 mediates Regulation of IKKγ in Macrophages

**FIGURE 4. Interaction of PAD2 and IKKγ.** A, coimmunoprecipitation of PAD2 and IKKγ. HEK 293 cells were transfected with the plasmid containing HA-tagged IKKγ and either FLAG-tagged PAD2 or vector only. Twenty-four hours later, cells were treated with 1 µM A23187 for 1 h, lysed, and immunoprecipitated with the anti-HA antibody (a-HA). The immunoprecipitates (IP) were then immunoblotted (IB) with the anti-FLAG antibody (a-FLAG). B, densitometric measurements of PAD2 bound to IKKγ. The intensity of PAD2 in the immunoprecipitates was normalized to the corresponding intensity in the lysates (n = 4). C, coimmunoprecipitation of truncation mutants of PAD2 with IKKγ. PAD2(1–385) contains the N-terminal 385 amino acids, whereas PAD2(355–672)/Q358A has an Ala instead of Gln-358 (a putative ligand for Ca²⁺ binding). The molecular masses of PAD2(1–385) and PAD2(355–672) are 42 and 35 kDa, respectively. The 50-kDa bands in all lanes are IgG heavy chains, *, PAD2 coimmunoprecipitated with IKKγ. The schematic diagram for truncation mutants is shown in the right panel. Putative ligand sites for Ca²⁺ binding are clustered in two areas (bars), Sub-1, Subdomain-1; Sub-2, Subdomain-2.

In other experiments, we also constructed truncation mutants of IKKγ: IKKγ(1–196), which contains the N-terminal 196 amino acids of the protein, and IKKγ(197–419), which contains the remaining amino acids. Coimmunoprecipitation was performed with cells transfected with PAD2 and these mutants as well as wild type IKKγ. PAD2 was coimmunoprecipitated with these truncation mutants (Fig. 5A). The amount of PAD2 being coimmunoprecipitated with IKKγ(1–196) appeared to be lower, but the amount of IKKγ(1–196) bound to protein A-Sepharose beads was also low. Thus, densitometric measurements of PAD2 relative to IKKγ (Fig. 5B) exhibited similar values among different immunoprecipitates (p > 0.05; n = 4). These results indicate that the interaction of PAD2 and IKKγ involves multiple bindings, at least one in the region of 1–196 residues of IKKγ and another in the region of 197–419 residues.

PAD2 Can Citrullinate IKKγ—The finding of the ability of PAD2 to suppress IKKβ/γ activity and interact with IKKγ led us to postulate that PAD2 might citrullinate IKKγ and/or its associated proteins. To test this hypothesis, we performed two sets of experiments. First, RAW 264.7 cells were treated with LPS after pretreatment with BAPTA/AM (30 µM), and immunoprecipitation was performed with an anti-IKKγ antibody. The immunoprecipitates were then immunoblotted with anti-citrulline antibody. Fig. 6A shows representative
PAD2-mediated Regulation of IKKγ in Macrophages

RESULTS

PAD2 was detected in RAW 264.7 cells by Western blotting (lane 1 of Fig. 5A). Immunoprecipitation of PAD2 from lysates of RAW 264.7 cells showed the presence of IKKγ in the immunoprecipitates (lane 1) but not in the supernatant (lane 2) or the buffer (lane 3). Immunoprecipitation from lysates of RAW 264.7 cells preincubated with LPS and BAPTA/AM showed the presence of IKKγ in the immunoprecipitates (lane 4) but not in the supernatant (lane 5) or the buffer (lane 6). Immunoprecipitation from lysates of RAW 264.7 cells preincubated with LPS and BAPTA/AM showed the presence of IKKγ in the immunoprecipitates (lane 7) but not in the supernatant (lane 8) or the buffer (lane 9).

DISCUSSION

Overview—The major findings from the present study are the following. 1) LPS induces Ca2+-dependent citrullination in the macrophage cells RAW 264.7; 2) PAD2 overexpression in macrophage cells reduces COX-2 production; 3) activated PAD2 suppresses IKKβ/γ activity; 4) PAD2 interacts with IKKγ in the presence of Ca2+; and 5) PAD2 can citrullinate IKKγ. These findings provide novel evidence that PAD2 can reduce inflammatory responses by suppressing IKKβ/γ and interacting with, and probably citrullinating, the regulatory protein IKKγ in macrophage cells.

Based on these findings, we propose a model for the effect of protein citrullination on cellular function in immune effector macrophages. LPS causes intracellular Ca2+ to rise (31), which then induces the conversion of inactive PAD2 to active PAD2 to mediate protein citrullination (supplemental material). Activated PAD2 then suppresses the IKK complex activity by binding to IKKγ. The binding of PAD2/IKKγ may lead to citrullination of IKKγ and/or IKKγ-interacting proteins. The essential part of the model is that PAD2 acts to down-regulate pro-inflammatory gene expression induced by LPS. Activation of PAD2 in inflammation might serve to self-limit inflammatory processes by modifying key LPS-sensing signaling molecules.

LPS Treatment Induces PAD Activity in RAW 264.7 Cells—Many reports show that citrullination occurs in a variety of proteins, including keratin (32), histones (10), vimentin (26), and myelin basic proteins (33). Citrullination also occurs in chemokines (34–36) such as CXCL8–12. Given the fact that the conversion of positively charged arginine into uncharged citrulline causes changes in protein folding, citrullination has been known to alter protein structure and function in cells (1, 2, 4). Citrullination has attained strong pathological and clinical interests because of its close association with autoimmune diseases such as rheumatoid arthritis (7). The goal of our study was to understand whether PAD2-mediated protein citrullination in macrophages is associated with inflammatory responses.

We found that protein citrullination is induced by LPS in RAW 264.7 cells (Fig. 1). Citrullination starts to occur within 1 h after LPS treatment and continues over time. LPS increases intracellular free Ca2+ levels by ~150-fold within 4 min in RAW 264.7 cells (31). This increase is mediated by L-type Ca2+ channels (31). PAD is a Ca2+-dependent enzyme that requires Ca2+ for substrate binding. Thus, Ca2+ influx by LPS is sufficient to bring out PAD activity. Protein citrullination at early time points after LPS treatment is probably due to the conversion of inactive PAD to active PAD by transient increase in intracellular Ca2+. As for citrullination at later time points, PAD activity may be induced from pro-inflammatory proteins produced by LPS. Prostaglandin D2 (4) produced by COX-2 acts to cells in autocrine and paracrine fashions (37). Prostaglandin D2 can induce PAD activity in RAW 264.7 cells (38). Prostaglandin D2 receptor (D2 receptor) activation also results in intracellular Ca2+ increase by Ca2+ influx (39).

PAD2 Reduces NF-κB Activity—The novel finding in our study is that PAD2 can reduce COX-2 production mediated...
by LPS in macrophage cells (Fig. 2, B and C). This reduction is due to the capability of PAD2 to suppress NF-κB activity (Fig. 2D). PAD2-mediated suppression of NF-κB is also observed in HEK 293 cells expressing PAD2 and IKKβ and IKKγ subunits (Fig. 3). IKKβ is a catalytic subunit that can phosphorylate IkBα, which binds to NF-κB and inhibits its function (25). Phosphorylation of IkBα results in ubiquitination. In contrast, IKKγ (also known as NEMO) is the regulatory subunit. IKKγ interacts preferentially with IKKβ and is required for the activation of the IKK complex (40). Our study shows that PAD2 markedly suppresses IKKβ alone as well as IKKβ/γ. The effect of PAD2 on IKKβ occurs regardless of Ca\(^{2+}\) in the medium, whereas the effect on IKKβ/γ appears to be dependent upon Ca\(^{2+}\). Thus, PAD2 appears to be activated to suppress IKKβ/γ activity. Liu et al. (41) reported that PAD4 overexpression in hematopoietic cells induces apoptosis. One might thus think that the reduced expression of COX-2 in our study would be due to apoptotic events caused by PAD2 overexpression in cells. However, in the previous report, the apoptotic event occurs >48 h after PAD4 overexpression, and we found that there is no difference in cell viability 24–32 h after PAD2 transfection (data not shown).

**PAD2 Interacts with IKKγ—**A number of proteins have been identified to interact with IKKγ. The essential function of these IKKγ-binding proteins is to modulate IKK activity. At least 16 IKKγ-binding proteins (30) are reported to enhance IKK complex activity, and at least eight IKKγ-interacting proteins are reported to suppress the complex (29). The mechanisms of their interactions with IKKγ vary depending upon the molecular and cellular nature of individual proteins. Our study shows that PAD2 is another member of the proteins interacting with IKKγ (Fig. 4). The significance of this interaction is its dependence on Ca\(^{2+}\). The interaction does not occur under normal conditions, in which intracellular Ca\(^{2+}\) is low. In response to LPS, however, intracellular Ca\(^{2+}\) levels increase to mediate the conversion of inactive PAD to active PAD2, and activated PAD2 then interacts with IKKγ. Therefore, the interaction is an induced event that occurs in response to inflammatory stimuli.

What would be the consequence of PAD2/IKKγ interaction? IKKγ plays important roles in regulating IKK complex activation. Proteins interacting with IKKγ activate or downregulate NF-κB activity (29, 30). Obviously, PAD2/IKKγ interaction appears to downregulate IKK complex activity. The interaction may serve as an inhibitory pathway to reduce the LPS-mediated signaling cascade. PAD2 decreases COX-2 expression by 50% (Fig. 2C) and NF-κB activity by 47% (Fig. 2D), both of which are substantial changes. These lead us to re-evaluate the pathological role of protein citrullination in inflammation. Citrullination causes changes in protein folding, which lead to abnormal protein structure and function (3–5). Therefore, citrullination induced by LPS is clearly disadvantageous to cells. On the other hand, the activation of PAD2 occurring in inflammation may be a process that limits inflammation. PAD2 may play an anti-inflammatory role by reducing IKKγ activity and/or modifying key molecules responsible for LPS-induced inflammation (Fig. 5). In this sense, we note that Prostaglandin D\(_2\), which can induce citrullination (38), functions as an anti-inflammatory molecule in lung inflammation (42).

**Mechanism of PAD2/IKKγ Interaction—**Given the finding that the mutation of a putative Ca\(^{2+}\) coordination site Gln-358 completely abolishes the interaction (Fig. 4C), we conclude that PAD2/IKKγ interaction requires Ca\(^{2+}\). This result provides valuable information on the molecular mechanism of the interaction. Structural analysis of PAD4 (4) suggests that the binding of Ca\(^{2+}\) to the acidic concave surface in the C-terminal domain is critical for substrate specificity and binding. The binding of Ca\(^{2+}\) to PAD4 leads to conformational changes around the substrate-binding site, which then allows PAD4 to interact with arginine residues in other proteins. PAD4 has many residues for Ca\(^{2+}\) coordination, and one of these residues is Gln-349 (corresponding to Gln-358 in PAD2). Replacing Gln-349 with an Ala abolishes citrullination activity of PAD4. Thus, in our study, Q358A probably inhibits PAD2 from binding Ca\(^{2+}\) and subsequently fails to induce conformational change required for IKKγ binding. Nonetheless, this interpretation makes it difficult to explain the interaction between IKKγ and PAD2(1–385), which does not contain the site for substrate binding. The N-terminal domain of PAD4 is proposed to be responsible for a Ca\(^{2+}\)-dependent regulation or protein-protein interaction (4). Thus, the interaction between IKKγ and PAD2 not only requires the C-terminal substrate-enzyme interaction, but it may also involve N-terminal protein-protein interaction.

**Summary—**Our study shows that protein citrullination is induced by LPS in macrophages and that citrullination can suppress up-regulation of pro-inflammatory gene expression. Activated PAD2 can interact with IKKγ and may citrullinate IKK subunits. PAD2-mediated suppression of IKK complex may be a novel mechanism that regulates inflammatory response. The exact mechanism underlying PAD2-mediated suppression needs further investigation.

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PAD2-mediated Regulation of IKKγ in Macrophages

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