Uncoupling Protein-2 Negatively Regulates Polymorphonuclear Leukocytes Chemotaxis via Modulating [Ca^{2+}] Influx

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Objective—Previous studies demonstrated that uncoupling protein 2 (UCP2) plays a negative role in modulating leukocyte inflammatory responses. The mechanism underneath the role of UCP2 in modulating leukocyte inflammatory responses, however, is incompletely understood. Here, we investigated the effect of UCP2 in polymorphonuclear leukocyte (PMN) chemotaxis.

Methods and Results—First, we assessed PMN chemotaxis in zymosan-induced murine peritonitis and found that UCP2^-/- mice had significantly more migrated PMN in peritoneal lavage compared to their wild-type littermates. In vitro transmigration assays using isolated PMN also showed that PMN from UCP2^-/- mice migrated faster than those from wild-type mice in response to N-formyl-methionyl-leucyl-phenylalanine (fMLP). Second, in supporting an inhibitory role of UCP2 in PMN transmigration, migrated PMN had a decreased UCP2 expression compared to nonmigrated PMN. In contrast, in streptozotocin-induced diabetic mice in which UCP2 expression was enhanced, PMN chemotaxis was reduced. Third, comparing to UCP2^+/+ PMN, UCP2^-/- PMN had a stronger upregulation of fMLP-induced surface CD11b/CD18 and CD11a/CD18. Finally, UCP2^-/- PMN showed a quicker and larger fMLP-triggered intracellular calcium mobilization compared to UCP2^+/+ PMN.

Conclusion—Our study demonstrates that UCP2 serves as a brake in controlling PMN chemotaxis and that the effect of UCP2 on PMN chemotaxis may be through modulating calcium influx. (Arterioscler Thromb Vasc Biol. 2010;30:575-581.)

Key Words: chemotaxis • cytosolic calcium influx • neutrophil • transmigration • uncoupling protein 2

Polymorphonuclear leukocytes (PMN) form the first line of host defense against infection by bacterial pathogens and are rapidly recruited to sites of bacterial invasion. Because the majority of pathogens are encountered at mucosal surfaces, PMN must migrate out of the circulation, through the interstitium, and across the epithelium to engage microbes. Despite the importance of PMN migration in the acute inflammatory response, many of the details regarding the regulation of this process remain undefined. Studies on this have revealed that migration of PMN across epithelial barriers involves a concerted series of cell–cell interactions between the PMN and epithelial cells.1–3 Solid evidence indicates that initial PMN–epithelial binding requires leukocyte β2 integrins, especially CD11b/CD18.4–6 In the postadhesion stage, the rate of PMN migration between epithelial cells is dependent on downstream signaling events from binding interactions between epithelial CD47 and PMN-expressed signal regulatory protein α.7 Although the leukocyte β2 integrin CD11b/CD18 and CD11a/CD18 are key adhesive elements that regulate PMN adhesion to epithelia and consequent transepithelial migration, there is evidence that other molecules expressed on either PMN or epithelia participate in PMN transepithelial migration.2 Recently, uncoupling protein 2 (UCP2), a member of mitochondria uncoupling protein family, has been shown to serve as a critical regulator for activation, chemotaxis, and bacteria clearance by leukocytes, including macrophages and monocytes.

UCP2, a member of mitochondrial anion carrier family, is ubiquitously expressed in various tissues and immune cells, including PMN, monocytes, and macrophages.8,9 As an uncoupling protein, mitochondrial UCP2 serves a role in dissipating the proton electrochemical gradient across the mitochondrial membrane when it is specifically activated10 and results in reduced ATP synthesis and less intracellular reactive oxygen species (ROS) production.11,12 Although extensive previous studies have demonstrated that UCP2 may not mediate adaptive thermogen-
esis, it has more general functions such as involvement in the regulation of immune cell inflammatory responses. Supporting evidence came from phenotyping UCP2 knockout mice. These mice were resistant to infection by the intracellular parasite Toxoplasma gondii through a mechanism proposed to involve increased macrophage ROS production. Similarly, low-density lipoprotein receptor knockout mice with UCP2 knockout bone marrow had more oxidative stress and were more susceptible to atherosclerosis than controls, suggesting a protective role of UCP2 against ROS-mediated atherosclerosis in vivo. Previous studies also showed that compared with UCP2 wild-type mice, the UCP2 knockout mouse had accelerated autoimmune diabetes with increased intra-islet lymphocytic infiltration. Macrophages from streptozotocin (STZ)-treated UCP2 knockout mice also had increased IL-1β and more macrophages recruited in islets compared with UCP2 wild-type macrophages. Moreover, directly overexpressing UCP2 in THP-1 monocytes significantly inhibited β2 integrin-mediated monocyte activation, adhesion, and transendothelial migration.

In the present study, we focus on the role of UCP2 in mediating neutrophil chemotaxis and the mechanism underneath this multistep event. Our results indicate that UCP2 serves as a negative regulator in controlling PMN chemotaxis and that the effects of UCP2 on PMN chemotaxis may not be attributable to the alteration of ROS level but may be attributable to the modulation of cytoplasmic calcium mobilization.

Materials and Methods

Reagents and Antibodies
These reagents and antibodies were obtained from: zymosan A, N-formyl-methionyl-leucyl-phenylalanine (fMLP), and mitochondrial fractionation kit (Sigma); Fura-2 AM (Molecular Probes); anti-UCP-2 antibody (C-20; sc5625); horseradish peroxidase–conjugated donkey anti-goat IgG (sc-2020); anti-UCP-2 antibody (C-20; sc6525); and rat anti-mouse CD11b monoclonal antibody (Clone 2D7; BD Biosciences). Peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. All reagents were obtained form Sigma unless indicated others.

Cells
Mouse bone marrow PMN were obtained by flushing the femoral and tibial cavities as previously described. Isolated PMN were resuspended in cold Hank balanced salt solution devoid of Ca²⁺ and Mg²⁺ at a concentration of 5×10⁶ cells/mL and used within 4 hours of isolation.

Animals
Adult C57BL/6 mice (aged 8 to 12 weeks) were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Mice lacking UCP2 were created as described previously. In brief, after targeted clones were identified by Southern blot analysis, cells expanded from targeted embryonic stem clones were injected into C57BL6 blastocysts, and germline transmitting chimeric animals were obtained and then mated with C57BL6 mice. C57BL6 mice lacking UCP1 were obtained from Dr Bradford B. Lowell (Harvard Medical School, Mass) and raised in Model Animal Research Center of Nanjing University. All animal care and handling procedures were performed in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals and approved by the Institutional Review Board of Nanjing University, Nanjing, China.

PMN Transmigration Assay
PMN transmigration was assayed according to previously described methods with a minor modification. PMN migration across collagen-coated transwell filters was initiated with addition of fMLP. In brief, mouse PMN (1×10⁶) in 150 μL Hank balanced salt solution were added to the upper chamber of the monolayer setup. PMN transmigration was initiated by adding 1 mL of 5 μmol/L fMLP (Sigma) into the lower chamber, followed by incubation at 37°C. PMN migrating into the lower chambers were quantified by myeloperoxidase assay.

Immunofluorescence Labeling and Flow Cytometry
For PMN cell surface labeling, cells were incubated with blocking solution (Hank balanced salt solution containing 10% normal goat serum) for 30 minutes at 4°C and then incubated with 1 μg/mL of anti-mouse CD11b or anti-mouse CD11a antibody in blocking solution for 1 hour at 4°C. The epothe-matched mouse IgG was used as control in cell labeling. After 3 washes, cells were briefly fixed with 3.7% paraformaldehyde (3–5 minutes, 20°C) and then incubated with fluorescence-conjugated secondary antibodies in blocking solution (30 minutes, 20°C). After washing off unbound antibody, the samples were analyzed using FACSscan flow cytometry and CellQuest software (BD Biosciences). To measure the intracellular production of ROS, PMN were labeled with 5 μmol/L DCFDA (Molecular Probes) for 30 minutes as described, stimulated with fMLP at 37°C for 5 to 10 minutes, and then cell-associated fluorescence was measured.

STZ Mouse Model
STZ-induced type 1 diabetic model was established as previously described. Briefly, male C57BL6 mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). For STZ-induced type 1 diabetic model, mice received a single injection of 150 mg/kg, intraperitoneally. STZ dissolved in citrate buffer at pH 4.5. Mice were fasted overnight before glucose measurements. Blood samples were taken from the tail vein 3, 7, and 14 days after STZ injection, respectively. Animals were euthanized 14 days after STZ injection. The mice with fasting blood glucose ≥12 mmol/L were considered as having diabetes.

Zymosan Peritonitis
Zymosan-induced mouse intraperitoneal inflammation model (zymosan peritonitis) was used as previously reported. Briefly, control C57BL6 mice, UCP2−/− mice, or STZ-treated C57BL6 mice were injected intraperitoneally with zymosan A (1 mg in 0.5 mL sterile saline, Sigma). Animals were euthanized later by carbon dioxide exposure, and peritoneal cavities were lavaged with 6 mL phosphate-buffered saline containing 2.5 mmol/L EDTA. Aliquots of the lavage fluid were then stained and also assessed for myeloperoxidase activity to determine cell concentration. Normally by this procedure, nearly 90% of the cells recovered were granulocytes, of which the majority is PMN.

Cytosolic Calcium Chemotaxis Influx in PMN
The fMLP-induced cytoplasmic cytosolic calcium [Ca²⁺] immobili-
Briefly, PMN freshly isolated from mouse bone marrow were loaded with the calcium indicator fura-2-AM (Molecular Probes) for 45 minutes at 37°C, followed by 3 washes with Hank balanced salt solution. Labeled PMN were then suspened in 980 mL of Hank balanced salt solution in a cuvette (Starstedt) and placed into the spectrofluorometer that had been thermostated to 37°C. A 4-mm mini-stir bar (Fisher) was placed in the cuvette. After equilibration for 5 minutes, PMN were stimulated with 100 μmol/L fMLP and cytoplasmic [Ca²⁺] changes were immediately recorded with fluorescence emission at 505 nm while the excitation wavelength was switched between 340 and 380 nm 4 times per second. It was then analyzed by Intracellular Cation Software (Hitachi). Levels of intracellular [Ca²⁺] were calculated via the Gryciwietz equation (R−Rmin)/(Rmax−R) × Kd (Kd=2.24×10⁻⁷ M). Rmax and Rmin were measured by adding digitonin (10 mmol/L) and then EGTA (20 mmol/L) to fura-2-AM–loaded PMN, respectively.

**Immunoblot Analysis of UCP2**

For analyzing PMN UCP2, mitochondria fractions were first isolated from PMN. PMN in 2 mL cold buffer (10 mmol/L NaCl; 1.5 mmol/L MgCl₂; 10 mmol/L Tris/HCl, pH 7.5; 1 mmol/L phenylmethylsulfonyl fluoride; 2 μmol/L leupeptin; 1× aprotinin) were homogenized in a Dounce homogenizer with 30 to 50 strokes. The cell homogenate was transferred to a prechilled microcentrifuge tube and spun at 800 g at 4°C. Mitochondria fraction was collected from pellets. The pellet containing mitochondrial protein was lysed using a protein extraction solution and centrifuged at 12 000 g at 4°C. The cell homogenate was homogenized in a Dounce homogenizer with 30 to 50 strokes. The supernatant was transferred to a new prechilled microcentrifuge tube and spun at 10 000 g for 20 minutes at 4°C. Mitochondria fraction was collected from pellets. The pellet containing mitochondrial protein was lysed using a protein extraction solution and centrifuged at 12 000 g for 30 minutes, and the supernatant was saved. For Western blot, 100 μg mitochondrial protein were separated by 12.5% SDS-PAGE and then transferred to the nitrocellulose membrane (Hybond). The membranes were blocked with 5% fat-free milk for 1 hour. Goat polyclonal antibody against UCP-2 (sc-6525) was applied for analysis of UCP-2. The level of UCP2 was detected in both UCP2-deficient (UCP2⁻/⁻) and wild-type (UCP2⁺/⁺) mice. As shown in Figure 1A, Western blot clearly showed the specificity of UCP2 deficiency in PMN chemotaxis.

**Data Analysis**

All data were shown as the means±SD of at least 3 independent experiments. The data were analyzed by paired Student’s t test and P<0.05 were regarded as significant differences.

**Results**

**UCP2 Serves as a Negative Regulator in PMN Chemotaxis**

To determine the role of UCP2 in PMN chemotaxis, we compared the PMN infiltration under zymosan-induced peritonitis in UCP2-deficient (UCP2⁻/⁻) mice and wild-type (UCP2⁺/⁺) mice. As shown in Figure 1A, PMN from UCP2⁻/⁻ mice migrated significantly faster than UCP2⁺/⁺ PMN. At 2 hours after zymosan A injection, the migrated UCP2⁻/⁻ PMN in lavage solution were nearly 2.5-fold as many as had migrated in UCP2⁺/⁺ PMN. Measured by cell count (Figure 1A), migrated UCP2⁻/⁻ PMN were 3.02±0.37-fold of migrated UCP2⁺/⁺ PMN. A similar result was shown by myeloperoxidase assay (3.91±0.42-fold, migrated UCP2⁻/⁻ PMN vs migrated UCP2⁺/⁺ PMN) in Figure 1B. Serving as another control, PMN lacking UCP1 (UCP1⁻/⁻) showed no difference in migration compared to PMN from the littermates (UCP1⁺/⁺). The result implicates the specificity of UCP2 deficiency in PMN chemotaxis.

**Reduction of UCP2 During PMN Migration**

We next assessed the UCP2 level in migrated PMN and nonmigrated PMN. As shown in Figure 2A, Western blot clearly showed that UCP2 is expressed in PMN. PMN UCP2 level was reduced in migrated PMN compared to nonmigrated PMN, suggesting a linking between UCP2 and PMN transmigration. Supporting evidence for UCP2 negatively regulating PMN transmigration was derived from the observation of PMN infiltration in STZ-treated mice. A previous study indicated that STZ treatment caused an increase of UCP2 level in rat brown adipose tissue, white adipose tissue, and skeletal muscle. Here, we showed that UCP2 level in PMN from STZ-treated mice was also increased compared to that in control mice (Figure 3A). Interestingly, these PMN with increased UCP2 level had slower chemotaxis compared to control PMN (Figure 3B).
UCP2 Negatively Regulates fMLP-Induced Upregulation of PMN Surface CD11b/CD18 and CD11a/CD18

CD11b/CD18 and CD11a/CD18 are major β2 integrins expressed in PMN and play a critical role in initial and firm adhesion of PMN, an essential step in PMN transmigration.2,27,28 Under resting condition, CD11b/CD18 and CD11a/CD18 are mainly stored in secondary granules and secretory vesicles of PMN. However, when PMN are stimulated by a chemoattractant, such as fMLP, CD11b/CD18 and CD11a/CD18 will rapidly appear on the cell surface via a degranulation process. In this experiment, UCP2−/− PMN and UCP2+/+ PMN (control) were stimulated with fMLP for 5 to 10 minutes. To estimate surface integrin expression, UCP2−/− PMN and UCP2+/+ PMN were surface-labeled with phycoerythrin-conjugated mouse IgG specific for CD11b and CD11a, and surface expression of β2 integrin was analyzed using flow cytometry. The result showed that fMLP-induced upregulation of CD11b/CD18 and CD11a/CD18 onto PMN surface after 5 minutes of stimulation was significantly larger in UCP2-deficient mice compared to their wild-type littermates (Figure 4A, B).

Figure 3. Increased PMN UCP2 expression in STZ-treated mouse correlates with decreased PMN infiltration. C57BL6 mice were treated with STZ to establish a stable high-glucose level in plasma. PMN infiltration was induced by zymosan A, and PMN influx was assessed after 4 hours after zymosan A injection. A, Enhanced expression level of UCP2 in PMN from STZ-treated mouse. B, Decreased PMN migration in STZ-treated mouse. The data were derived from 6 independent experiments. *P<0.05.
A negative role of UCP2 in regulating ROS production has been widely reported, and enhanced ROS level in UCP2-deficient leukocytes might mediate the signal events that activate leukocytes.\textsuperscript{16,29} We next tested whether short exposure of PMN to fMLP would affect intracellular ROS level in PMN. As shown in Figure 4C, we had not detected significant alteration of ROS level within the same timeframe as fMLP-induced PMN surface upregulation of CD11b/CD18 and CD11a/CD18.

**Effect of UCP2 on fMLP-Induced PMN [Ca$^{2+}$] Influx**

Cytosolic calcium level, as determined by labeling mouse PMN with fura 2-AM, was measured by flow cytometry. Stimulation of the labeled PMN with 10 $\mu$mol/L fMLP triggered a rapid elevation of [Ca$^{2+}$] in PMN. As shown in Figure 5, fMLP-induced [Ca$^{2+}$] influx in UCP2$^{-/-}$ PMN was significantly faster and larger than that in UCP2$^{+/+}$ PMN. The data suggest that modulating the [Ca$^{2+}$] influx process might be the mechanism causing the negative regulatory role of UCP2 protein in PMN transmigration.

**Discussion**

Using in vitro and in vivo PMN transmigration assays, we directly determined the role of UCP2 in regulating mouse PMN chemotaxis. Our results show that the transmigration of PMN was clearly accelerated in UCP2-deficient mice compared with UCP2 wild-type mice. This phenomenon was caused, at least in part, by an enhanced [Ca$^{2+}$] influx in UCP2$^{-/-}$ PMN in response to chemoattractant.

Our results are consistent with previous observations of aggregated inflammatory responses in UCP2-deficient mice.\textsuperscript{9,13,15,29,31,32} Consistent with the negative role of UCP2 in leukocyte inflammatory responses, previous studies also showed that high levels of UCP2 in humans were associated with reduced susceptibility to multiple sclerosis\textsuperscript{33} and reduced risk of diabetic neuropathy in type 1 diabetes.\textsuperscript{34} Here, we further illustrated that in UCP2-deficient mice, leukocyte $\beta_2$ integrins, CD11a/CD18 and CD11b/CD18, 2 major integrins that are responsible for cell initial and firm adhesion, had a more rapid upregulation onto cell surface in UCP2$^{-/-}$ PMN (Figure 3). As a double-edge sword, PMN not only can serve as the first defense line of body innate immunity but also can disrupt tissue/cell structures to cause more inflammation. Therefore, UCP2, serving as a negative regulator in PMN transmigration, provides a novel therapeutic target in speeding the process of bacteria clearance or attenuating the inflammation. The development of strategies to induce or reduce UCP2 levels in immune cells would be promising in modulation of autoimmunity.

Whereas the role of UCP2 in insulin secretion and metabolism process is well investigated,\textsuperscript{19,20,35} the mechanisms by which UCP2 modulates the leukocyte inflammatory responses, particularly PMN chemotaxis, are not completely understood. Because UCP2 seems to link to the mitochondrial ROS production and ROS damage is responsible for several degenerative diseases, such as diabetes, Parkinson disease, and the aging process,\textsuperscript{36,37} ROS production and ROS-mediated signal pathways have been suggested to play a critical role in UCP2 functions.\textsuperscript{16,29,30} However, we did not detect apparent elevation of ROS level in PMN within the timeframe of fMLP-induced upregulation of $\beta_2$ integrins CD11a/CD18 and CD11b/CD18 (Figure 4). In this regard, alteration of ROS level induced by UCP2 knockout might play a minor role in modulating PMN chemotaxis in response to fMLP.

By comparing the intracellular [Ca$^{2+}$] level after fMLP stimulation in UCP2$^{-/-}$ and UCP2$^{+/+}$ PMN, we found that fMLP-induced [Ca$^{2+}$] influx in UCP2$^{-/-}$ PMN was significantly faster and larger than UCP2$^{+/+}$ PMN (Figure 5). The elevation of intracellular free [Ca$^{2+}$] would then activate leukocytes and promote leukocyte inflammatory response, including upregulation of $\beta_2$ integrins onto cell surface and leukocyte chemotaxis. This result is in agreement with the observation that UCP2$^{-/-}$ PMN has an enhanced leukocyte inflammatory responses compared to UCP2$^{+/+}$ PMN. The effect of UCP2 on [Ca$^{2+}$] influx in PMN may be similar to that in pancreatic $\beta$ cells. Previously, we reported\textsuperscript{19} that UCP2 knockout would increase ATP/ADP ratio and shutdown membrane K$_{ATP}$ channel, leading to the opening of membrane potential-sensitive calcium channel and, finally, [Ca$^{2+}$] influx in $\beta$ cells. [Ca$^{2+}$] influx would trigger exocytosis of insulin-containing granules and stimulate insulin release. Our finding that UCP2 negatively modulates intracellular [Ca$^{2+}$] mobilization was also supported by 2 previous reports.\textsuperscript{16,20} Although whether PMN contain the same type of K$_{ATP}$ channel as pancreatic $\beta$ cells is not clear, there is well-established evidence that ATP-sensitive potassium channels play a critical role in modulating PMN inflammatory responses, including the enhanced expression of adhesion molecules and cell migration.\textsuperscript{38,39}

The molecular basis underlying the alteration of UCP2 level in PMN during chemotaxis process remains elusive. However, we speculate that 2 possible mechanisms may be involved in this process. One is similar to the mechanism that governs the reduction of UCP2 in lipopolysaccharide-stimulated macrophages.\textsuperscript{30} In their study, Kizaki et al\textsuperscript{30} reported that activated macrophages had a lower UCP2 level and suggested that the lipopolysaccharide-stimulated signals suppress UCP2 expression in macrophages by...
interrupting the function of the intronic enhancer. The stimulation of PMN by chemoattractants, including fMLP, may also interrupt the intronic enhancer function of UCP2, although so far no evidence is available. Another mechanism may be the selective degradation of UCP2 in migrating PMN because PMN contain a wide range of proteases. In addition, because zymosan is a ligand for toll-like receptor 2,40,41 and because toll-like receptor 2 plays a critical role in PMN activation and chemotaxis, it would be interesting to know whether and how toll-like receptor 2–zymosan interactions modulate UCP2 expression in PMN during chemotaxis.

In conclusion, the present study demonstrated for the first time to our knowledge that mitochondria UCP2 serves as a brake in PMN chemotaxis via negatively regulating [Ca2+] influx induced by chemoattractant.

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Disclosures

None.

References


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