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Prostaglandin E₂ Modulation of p59<sub>fy</sub>n Tyrosine Kinase in T Lymphocytes During Sepsis¹

Mashkoor A. Choudhry,* Shahab Uddin,† and Mohammed M. Sayeed²*

Prostaglandin E₂ (PGE₂) has been implicated in the suppression of T cell IL-2 production and proliferation during burn and sepsis. The present study evaluated the potential intracellular mechanism of suppressed T cell responses by assessing the activation of p59<sub>fy</sub>n kinase in T cells from septic rats as well as the T cells incubated with PGE₂. p59<sub>fy</sub>n is known to regulate T cell functions. Sepsis was induced in rats by implanting fecal pellets containing Escherichia coli (150 CFU) and Bacteroides fragilis (10⁴ CFU) into the abdominal cavity. For the assessment of PGE₂ role in sepsis, a group of septic rats were treated with indomethacin, which inhibits endogenous PGE₂ synthesis. As assessed by immunoblotting or in vitro kinase assay, a more than 40% inhibition of p59<sub>fy</sub>n phosphorylation and kinase activity was observed in septic rat T cells compared with the T cells from sterile or control rats. A similar inhibition in p59<sub>fy</sub>n phosphorylation and kinase activity was observed in PGE₂-treated T cells compared with the T cells incubated in the absence of PGE₂. The septic-related suppression in p59<sub>fy</sub>n phosphorylation and kinase activity in T cells was prevented in rats treated with indomethacin. We observed that the inhibition in p59<sub>fy</sub>n activation in septic or PGE₂-treated T cells was due primarily to a suppression in p59<sub>fy</sub>n phosphorylation and not due to alterations in p59<sub>fy</sub>n protein expression. These findings suggest that PGE₂ released during sepsis could contribute to the sepsis-related suppression in T cell proliferation by attenuating p59<sub>fy</sub>n phosphorylation and its kinase activity. The Journal of Immunology, 1998, 160: 929–935.

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3 Abbreviations used in this paper: PLC-γ, phospholipase C-γ; PGE₂, prostaglandin E₂; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; DAG, 1,2-diaclyglycerol; PTK, protein tyrosine kinase; [Ca<sup>2+</sup>], intracellular [Ca<sup>2+</sup>].

10, 11). Whereas IP<sub>3</sub> stimulates Ca<sup>2+</sup> release from the intracellular stores, DAG activates the protein kinase C activity (12–15). An increase in intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]) sustained for several hours precedes T cell activation and its subsequent proliferation activity (13, 14, 16–19).

A suppression in the T cells’ proliferative response and their production of IL-2 has been observed following sepsis, burn, and trauma (20–22). Recent studies from our and other laboratories have implicated a role of prostaglandin E₂ (PGE₂) in the sepsis-associated suppression of T cell proliferation and IL-2 production (23–26). Additionally, we have shown that PGE₂ suppression of T cell proliferation and IL-2 production could result from a suppression in Ca<sup>2+</sup> signaling (25). The altered Ca<sup>2+</sup> signaling could result from either a direct effect of PGE₂ on [Ca<sup>2+</sup>]<sup>i</sup> homeostasis or via an effect on upstream signaling events such as the phospholipase of PTKs. June et al. (27) have suggested that an inhibition in PTK phosphorylation could prevent TCR-mediated signal transduction including the [Ca<sup>2+</sup>]<sup>i</sup> elevation and production of IL-2. Among the tyrosine kinases that are phosphorylated after TCR stimulation, p59<sup>fy</sup>n, P56<sup>lck</sup>, and ZAP70 have been recognized as playing important roles in T cell signal transduction pathways (3, 28). P56<sup>lck</sup> is associated with CD4 and CD8 molecules (29), and is believed to be important in the generation of signals through these molecules. ZAP70 is associated with the ζ-chain of TCR (30). p59<sup>fy</sup>n has been proposed to be instrumental in TCR-mediated signaling (31). Several studies have shown that the overexpression of fyn protein results in enhanced TCR-mediated Ca<sup>2+</sup> mobilization and T cell IL-2 production (3, 28, 32–34). Rigley et al. (35) suggested that p59<sup>fy</sup>n can regulate Ca<sup>2+</sup> influx via a mechanism that is independent of PIP₂ hydrolysis. That p59<sup>fy</sup>n is an integral component of the TCR-mediated signaling pathways is indicated by the co-immunoprecipitation of p59<sup>fy</sup>n with TCR (31), and the modulation in the signaling cascade with manipulations in the levels of p59<sup>fy</sup>n in transgenic mice (32, 36). The present study evaluated the effects of PGE₂ on the phosphorylation and activation of

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p59fyn. For the assessment of PGE2 effects on the phosphorylation and activation of p59fyn in T cells during sepsis, septic rats were treated with indomethacin to block the endogenous PGE2 synthesis (23–26), and to monitor its effect on activation of p59fyn and its kinase activity toward its target substrate. Further, we ascertained PGE2-related changes in the p59fyn autophosphorylation and its mediation of tyrosine phosphorylation of other proteins in T cells incubated with and without PGE2, in vitro.

Materials and Methods

Bacterial strains and reagents

Escherichia coli (American Type Culture Collection (ATCC), Rockville, MD; 5922) and Bacteroides fragilis (ATCC 25285) were obtained from Dr. Kenneth D. Thompson, University of Chicago, Chicago, IL. Con A, PGE2, enolase, and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO. mAbs to p59fyn (residues between 85–206 of human Fyn protein) were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-phosphotyrosine mAbs were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-re CD3 Abs were purchased from Pharmingen, San Diego, CA. 32P-ATP was obtained from DuPont NEN, Boston, MA. Molecular weight markers were purchased from Sigma and other reagents for the SDS-PAGE were obtained from Bio-Rad, Richmond, CA. Immobilon P membrane (polyvinylidine fluoride) for the transfer of proteins was obtained from Millipore, Bedford, MA. Reagents needed for the preparation of lysis buffer, wash buffer, and kinase buffer were obtained from Sigma. Nylons were purchased from Polysciences, Inc., War-ington, PA. Ficoll-Paque was purchased from Pharmacia, Uppsala, Sweden.

Animal model of sepsis

Male Sprague-Dawley rats (225 to 250 g) obtained from Harlan (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used. Sepsis was induced by implanting 1-cm3 presterilized fecal pellets containing Escherichia coli (150 CFU) and Bacteroides fragilis (105 CFU) into the rat abdomen (22). Rats implanted with sterilized pellets without the bacteria are referred to as sterile. A febrile response (>38°C), decreased sensibilities to touch, diarrhea, eye hemorrhage, and piloerection were characteristic findings in septic rats on days 1 to 3 postimplantation. Whereas no mortality occurred in sterile rats, approximately 45% of all septic rats died between days 1 and 2 after implantation. Rats were killed 48 h after implantation.

Treatment of animals with indomethacin

Indomethacin (2 mg/kg) was administered i.p. initially 2 h prior to implantation, and 24 and 36 h after implantation (25). There was no significant difference in the mortality in the septic rats treated with and without indomethacin.

T cell preparation

Rats were killed to remove their spleens. Splenic T lymphocytes were isolated using the method previously described (25). Briefly, spleens were gently ground to prepare a single cell suspension. The red and dead cells from the suspension were removed by density gradient centrifugation with Ficoll-Paque. Splenocytes appearing at the interface of Ficoll and the medium were collected. To obtain a pure T cell preparation, splenocytes were incubated with nylon wool-packed columns. These columns were prequilled with HBSS supplemented with 10 mM HEPES, 5% FCS, and 50 g/ml of gentamicin per ml. The column containing cells was incubated at 37°C for 50 to 60 min. T cells were obtained by eluting the columns with 30 to 40 ml of HBSS at a flow rate of 1 drop per second. Flow cytometric analysis was performed to assess the purity of the CD3-positive cells using anti-CD3 Abs. In most of the preparation, 90 to 95% of the cells were CD3 positive (data not shown).

Stimulation of T cells and lystate preparation

T cells obtained from control, sterile, and septic rats were stimulated with Con A (10 μg/ml) or anti-CD3 Abs (1 μg/ml) for 180 s at 37°C. The stimulation was stopped by cell solubilization in a phosphorylation lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM MgCl2, 10 mM Na3VO4, 200 μM Na2VO3, 0.5% Triton X-100, and 10% glycerol) on ice for 45 to 50 min. Lysates were centrifuged at 10000 rpm for 5 min at 4°C.

Immunoprecipitation

Lysates were incubated with mAbs to p59fyn protein for 1 h, and then the mixture was incubated with protein G-Sepharose beads for another 2 h (37, 38). These incubations were conducted at 4°C. The precipitates were washed three times in phosphorylation lysis buffer without added glycerol.

Immunoblot

As described elsewhere (37, 38), immunoprecipitated protein after the third wash was analyzed by SDS-PAGE and transferred to Immobilon membranes using a semi-dry Trans-Blot system (Bio-Rad). The membranes were saturated with blocking buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween-20 supplemented with 10% BSA) for 1 h at room temperature or for 16 to 20 h at 4°C followed by an incubation with anti-phosphotyrosine Ab (1:10000 dilution) at 4°C. The membranes were washed three times with wash buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween-20). The membranes were incubated with a secondary Ab conjugated with horseradish peroxidase (1:3000 dilution) followed by washing. After the final wash, membranes were probed using enhanced chemiluminescence dye, and phosphoproteins were autoradiographed.

In vitro kinase assay

This was performed using the method described previously (37, 38). After the final wash, immune complexes were collected and washed two times with in vitro kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MnCl2, 0.1% Triton X-100). After these washes, kinase assays were performed by incubating immune complexes first with 5 μg/ml of acid-treated enolase, and then for 30 min with 10 μCi of 32P-ATP. These incubation were conducted at room temperature (28°C). Samples were analyzed on SDS-PAGE (9%) and the proteins were transferred to the Immobilon membrane. Phosphoproteins were analyzed by autoradiography and the intensity of the bands were assessed using densitometry.

Reprobing the membranes

Membranes were reprobed after stripping the Abs. For stripping, membranes were incubated with stripping buffer (65 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% SDS). Membranes were saturated and immunoblotting was performed essentially following the protocol mentioned in immunoblot section. For the analysis of fyn we have used anti-fyn mAbs instead of anti-phosphotyrosine Abs.

Results

Analyses of p59fyn protein

T cells obtained from control, sterile, and septic rats were lysed in the lysis buffer. The total protein content in each sample was assessed and analyzed for the presence of p59fyn using the immunoblot technique. A representative immunoblot is shown in Figure 1A. No significant change in the p59fyn protein levels was observed in T cells obtained from the control, sterile, and septic groups of rats. The protein contents of p59fyn in T cells from control and experimental animals were also analyzed after immunoprecipitation of p59fyn from the cell lystate with anti-fyn Abs, and were not found to be different between the control and experimental groups of animals (Fig. 1B). Densitometric analyses of the blots from immunoprecipitated fyn proteins are shown in Figure 1C. There seemed to be a decreasing trend in the fyn protein content in T cells from septic and sterile compared with control rats; however, it was not found to be statistically significant.

Tyrosine phosphorylation of p59fyn protein

T cells obtained from control and experimental animals were stimulated with Con A for 3 min at 37°C and lysed. Fyn protein was immunoprecipitated from the cell lysates using 1 μg of anti-fyn Abs and analyzed by SDS-PAGE. Proteins were transferred to the membrane and the phosphorylation of fyn protein was assessed by immunoblotting with anti-phosphotyrosine Abs. A representative immunoblot showing the phosphorylation of p59fyn protein is shown in Figure 2A. No detectable phosphorylation of p59fyn was observed in unstimulated T cells (data not shown). The stimulation of T cells with Con A resulted in the tyrosine phosphorylation of Tyr at residues 240 and 261.
p59fyn protein. There was no change in the tyrosine phosphorylation of p59fyn in sterile rat T cells compared with control rat T cells. In contrast, a significant decrease in the phosphorylation of p59fyn PTK was observed in the T cells obtained from septic rats compared with the sterile and control rats’ T cells. To assure that the observed inhibition in p59fyn phosphorylation was primarily due to the septic injury and not due to different amounts of protein loaded in the lanes, we stripped the blots and reprobed for the content of p59fyn protein in each lane using anti-fyn Abs. The results are shown in Figure 2B. There was no change in the p59fyn protein contents among the lanes. These data suggested that the attenuation in phosphorylation of p59fyn protein was the result of septic injury. Finally, p59fyn phosphorylation data blots were analyzed using densitometric analyses, and the data from four or more similar experimental groups were pooled. The control response was normalized to 1. As shown in Figure 2C, while there was no significant change in the p59fyn phosphorylation in T cells obtained from sterile rats compared with the control rats, a 30 to 40% inhibition in the phosphorylation of p59fyn was observed in T cells obtained from septic rats compared with control and sterile rats.

Assessment of p59fyn tyrosine kinase activity

These experiments evaluated whether or not the above observed inhibition in p59fyn tyrosine phosphorylation could affect its ability to phosphorylate enolase, a known substrate for Src kinases. Phosphoproteins were analyzed using in vitro kinase assay and are shown in Figure 3A. There was no detectable p59fyn autophosphorylation or fyn-mediated phosphorylation of enolase in unstimulated T cells. Con A stimulation of T cells resulted in p59fyn autophosphorylation as well as in enolase phosphorylation by fyn. Both the p59fyn autophosphorylation and enolase phosphorylation by fyn in control T cells were not significantly different from sterile rat T cells. In septic rat T cells, a significant inhibition was observed in both the autophosphorylation of fyn as well as its ability to phosphorylate the exogenous substrate compared with the T cells from control and sterile rats. The data from densitometric analyses pooled from several blots are presented in Figure 3, C and D. The fyn autophosphorylation was inhibited by 40 to 50% in septic rat T cells (Fig. 3C). Similarly, a more than 40% inhibition in enolase phosphorylation by fyn was found in septic rat T cells compared with control and sterile rat T cells (Fig. 3D). For equal loading of fyn in various lanes, blots were stripped and reprobed. No significant change in the amount of fyn was detected in various lanes (Fig. 3B).

Effect of indomethacin on Fyn kinase activity

In these studies, T cells were stimulated with anti-rat CD3 or Con A. The blots showing the fyn autophosphorylation and enolase
phosphorylation by fyn in T cells after their stimulation with Con A or anti-CD3 Abs are given in Figure 4, A and C. There was no apparent change in the pattern of autophosphorylation of fyn as well as fyn-mediated phosphorylation of enolase in T cells after their stimulation with Con A or anti-CD3. Moreover, the kinase activity of p59Fyn in T cells obtained from sterile rats treated with and without indomethacin was not significantly different. As shown above, a significant inhibition in both fyn autophosphorylation as well as the enolase phosphorylation by fyn was observed in septic rat T cells after their stimulation with Con A or anti-CD3. Indomethacin treatment of septic animals significantly prevented the inhibition in fyn autophosphorylation as well as the enolase phosphorylation. The data presented in Figure 4, E and F represent the groups of T cells stimulated with Con A. As shown in the figures, sepsis resulted in a 40 to 50% inhibition of fyn autophosphorylation (Fig. 4E) and fyn-mediated enolase phosphorylation (Fig. 4F) compared with sterile rats. The inhibition in fyn autophosphorylation and its kinase activity was evidently prevented when the rats were treated with indomethacin. Protein-loading control of the blots is shown in Figure 4, B and D. Because we did not find any difference in the stimulatory effects of Con A or anti-CD3 on T cells’ p59Fyn phosphorylation, we presumed the effects of PGE2 blockade on p59Fyn phosphorylation would be similar whether the cells were stimulated with Con A or anti-CD3.

**Effect of PGE2 on control rat T cell Fyn autophosphorylation and its kinase activity**

T cells from control rats were incubated for 2 h with or without PGE2 (1 µM) at 37°C before their stimulation with Con A or anti-CD3. The effects of PGE2 on fyn autophosphorylation and its phosphorylation of enolase are shown in Figure 5, A and C. Stimulation of control rat T cells with Con A or anti-CD3 resulted in increased autophosphorylation of fyn as well as its kinase activity. As shown in Figure 5, A and C, autophosphorylation of p59Fyn induced by Con A or anti-CD3 was significantly suppressed in T cells incubated with PGE2 compared with T cells incubated in its absence. Similarly, phosphorylation of enolase by fyn in PGE2-treated T cells was significantly lower than that observed in untreated T cells. Densitometric analyses of six or more experiments performed with anti-CD3 are shown in Figure 5, E and F. A nearly 40 to 50% inhibition of fyn autophosphorylation was observed in T cells treated with PGE2 compared with the untreated T cells (Fig. 5E). Similarly a 50 to 60% decrease in enolase phosphorylation was observed by fyn from PGE2-treated T cells compared with control T cells (Fig. 5F). Figure 5, B and D shows the equal protein-loading controls in blots shown in Figure 5, A or C, respectively.

**Discussion**

The demonstration by us (25) and others (26, 39, 40) that PGE2 attenuates [Ca2+]i signaling in T cells supports the concept that PGE2-mediated suppression of T cell proliferation could result either from a disturbance in the [Ca2+]i response per se or in a signaling component upstream from it. Recently, our studies have corroborated the aforementioned concept by showing that PGE2-related alterations in Ca2+ signaling and the proliferative response in T cells from septic animals could be overcome by the treatment of cells with ionomycin (41). Similar effects of Ca2+ ionophore on PGE2-exposed T cells have previously been reported (39, 40). The present study has implied that p59Fyn activation could indeed be the signaling component upstream from the [Ca2+]i responses, which is adversely affected in the presence of PGE2. Our study shows that...
PGE2-related suppression in p59fyn activation occurs both in control rat T cells exposed to PGE2 as well as T cells harvested from septic rats. The suppression of the p59fyn response was apparently not due to an alteration in p59fyn protein expression, but rather due to a decrease in the tyrosine phosphorylation of p59fyn as well as its ability to phosphorylate tyrosine residues of other proteins. As anticipated, the sepsis-mediated suppression in T cell fyn auto-phosphorylation and its kinase activity was preventable with the treatment of animals with indomethacin. The indomethacin treatment of sterile animals was without any effect on fyn tyrosine phosphorylation or its kinase activity. These results clearly support the idea that sepsis may primarily cause a suppression in T cell tyrosine phosphorylation of fyn and its ability to phosphorylate its substrates, which in turn attenuates T cells’ [Ca2+]i signaling and the proliferative response.

Although various studies have shown the suppression in T cell proliferation during burn, sepsis, and trauma, the mechanism of the proliferative suppression during these injury states has remained largely unknown (26). Several lines of evidence suggested that the burn-, sepsis-, or trauma-associated inflammatory response is accompanied by increased production of PGE2 from phagocytic cells and monocytes (42, 43). On the basis of our present findings, an increase in the PGE2 levels in the various inflammatory conditions would seem to play a role in the T cell suppression through an attenuation in p59fyn activation followed by disturbances in Ca2+ signaling.

The tyrosine phosphorylation of fyn triggers its enzymatic activity and leads to the phosphorylation of the substrate proteins including PLC-γ1 (28, 34). PLC-γ1 phosphorylation in turn hydrolyzesPIP2 into IP3 and DAG (44). The IP3-mediated release of Ca2+ from intracellular stores and the subsequent Ca2+ influx from the extracellular space are well-established prerequisites for T cell activation, proliferation, and lymphokine production (3, 11–13, 28). An inhibition in the upstream event such as fyn activation could potentially attenuate the [Ca2+]i signal via altered PLC-γ and hence lead to T cell proliferative dysfunction. There is also a possibility that fyn may not interfere with PIP2 hydrolysis but may directly affect Ca2+ signaling using a hitherto unknown pathway suggested by some studies (35, 45).

It remains unknown whether the fyn inhibition is caused by a direct effect of PGE2 or by PGE2 interference with the mitogen-induced signal transduction. PGE2 interaction with its receptor results in an elevation of intracellular cAMP via an up-regulation of adenylate cyclase (40). Previous studies have shown that cholera toxin and forskolin, which up-regulate adenylate cyclase and subsequently increase cAMP levels, inhibit the TCR-mediated signaling via an attenuation in most proximal events of the cascade such as CD3ζ (46) and PLC-γ phosphorylation (10). Whether or not fyn attenuation in septic rat T cells or T cells pretreated with PGE2 is affected by these PGE2-mediated second messengers is not known. However, as observed in cholera toxin-treated T cells, a
FIGURE 5.  p59<sup>yn</sup> autophosphorylation and kinase activity in control (lane 1) or PGE<sub>2</sub>-treated (lane 2) T cells after their stimulation with Con A (panel A); immunoblot with anti-fyn Abs showing equal loading of protein in all lanes (panel B). Panel C shows the fyn autophosphorylation and enolase phosphorylation by fyn in T cells stimulated with anti-CD3, protein loading control for the blot (panel D). Densitometric analyzes of blots showing p59<sup>yn</sup> autophosphorylation (panel E) and the phosphorylation of enolase (panel F) by fyn from control (n = 6) and PGE<sub>2</sub>-treated T cells after their stimulation with anti-CD3 (n = 6); *, p < 0.01, T cells treated with PGE<sub>2</sub> vs control T cells.

PGE<sub>2</sub>-mediated modulation of CD3-ζ could attenuate fyn activation and hence the subsequent signaling events. PGE<sub>2</sub> could also affect the distal events of T cell activation in addition to its effect on [Ca<sup>2+</sup>], and upstream signaling. There are a number of studies suggesting that an inhibition in T cell proliferation and IL-2 production could result from a disturbed transcriptional regulation of IL-2 by PGE<sub>2</sub> and other cAMP-elevating agents (47–49). These studies suggest that PGE<sub>2</sub> can inhibit T cell proliferation and IL-2 production by modulating both the proximal and distal events of T cell activation. The stimulation of TCR serves as the primary signal needed for T cell activation but the cell proliferation and IL-2 production could be influenced by the activation of several other accessory signals generated through other surface molecules (3, 5, 6). Among these, the activation of P56<sup>lck</sup> by CD4, and of phosphotyrosine phosphatases are important (3, 37). Whether or not PGE<sub>2</sub> released during sepsis attenuates the accessory signals is yet to be determined.

In summary, sepsis induced a 40 to 50% inhibition of T cell fyn phosphorylation and its kinase activity. This was similar to the inhibition of fyn kinase activity in PGE<sub>2</sub>-exposed T cells. Indomethacin treatment of rats prevented the septic-related suppression of fyn phosphorylation and kinase activity in T cells. These observations indicate that PGE<sub>2</sub> released during sepsis could contribute to suppressed T cell proliferation and IL-2 production by inhibiting p59<sup>yn</sup> activation. Because p59<sup>yn</sup> activation precedes [Ca<sup>2+</sup>], signaling in T cells, and our earlier studies showed indomethacin prevented the sepsis-related attenuation in T cell [Ca<sup>2+</sup>], responses, our present findings suggest that the septic injury initially induces a p59<sup>yn</sup> down-regulation to lead to the disturbances in the [Ca<sup>2+</sup>], responses, lymphokine production, and proliferation in T cells.

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