Regulation of Neutrophil Adhesion by Pituitary Growth Hormone Accompanies Tyrosine Phosphorylation of Jak2, p125FAK, and Paxillin

Hoon Ryu, Jung-Hee Lee, Kwon Seop Kim, Seong-Min Jeong, Pyeung-Hyeun Kim and Hun-Taeg Chung

J Immunol 2000; 165:2116-2123; doi: 10.4049/jimmunol.165.4.2116
http://www.jimmunol.org/content/165/4/2116

References
This article cites 61 articles, 41 of which you can access for free at:
http://www.jimmunol.org/content/165/4/2116.full#ref-list-1

Subscription
Information about subscribing to J Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of Neutrophil Adhesion by Pituitary Growth Hormone Accompanies Tyrosine Phosphorylation of Jak2, p125FAK, and Paxillin

Hoon Ryu,2,3,*† Jung-Hee Lee,2*† Kwon Seop Kim,¶ Seong-Min Jeong,‡ Pyeung-Hyeun Kim,† and Hun-Taeg Chung4‡

Neutrophil adhesion is fundamentally important during the onset of inflammatory responses. The adhesion signaling pathways control neutrophil arrest and extravasation and influence neutrophil shape and function at sites of inflammation. In the present study the intracellular signaling pathways for the adhesion of human neutrophils by pituitary growth hormone (GH) were examined. Pituitary GH triggered the tyrosine phosphorylation of Janus kinase 2 (Jak2) and STAT3 in neutrophils. In addition, pituitary GH treatment resulted in the morphological changes and the tyrosine phosphorylation of focal adhesion kinase (p125FAK) and paxillin. Preincubation with genistein, a tyrosine kinase inhibitor, blocked the GH-stimulated adhesion and Jak2, STAT3, p125FAK, and paxillin phosphorylation. Confocal microscopy revealed that pituitary GH stimulates the focal localization of p125FAK, paxillin, phosphotyrosine, and filamentous actin filament into the membrane rufflings and uropods of human neutrophils. Immunoprecipitation experiments revealed a physical association of Jak2 with p125 FAK via STAT3 in vivo. Also an in vitro kinase assay showed an augmentation of p125FAK autophosphorylation as a result of pituitary GH treatment. These results suggest that pituitary GH modulates neutrophil adhesion through tyrosine phosphorylation of Jak2, p125FAK, and paxillin and actin polymerization. The Journal of Immunology, 2000, 165: 2116–2123.

1 This work was supported by a postdoctoral fellowship (to H.R.) from the Korean Science and Engineering Foundation and a grant (to H.T.C.) from the Korean Ministry of Education.
3 H.R. and J.H.L. contributed equally to this work.
4 Address correspondence and reprint requests to Dr. Hun-Taeg Chung, Medicinal Resources Research Center and Department of Microbiology and Immunology, Wonkwang University School of Medicine, Iksan, Chonbuk, Republic of Korea
5 Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00
vascular endothelial growth factor, and platelet-derived growth factor (PDGF) have been shown to augment the tyrosine phosphorylation of p125<sub>FaK</sub> (24-30, 40).

The aim of the present study was to determine how neutrophil adhesive functions are modulated by pituitary GH. Herein we provide data that pituitary GH treatment potentiates neutrophil adhesion and triggers the tyrosine phosphorylation of Jak2, p125<sub>FaK</sub>, and paxillin and F-actin formation that may involve in the adhesion signaling of human neutrophils.

Materials and Methods

Reagents

Human pituitary GH, genistein, and phalloidin-FITC were purchased from Sigma (St. Louis, MO). Protein A-Sepharose, dextran T-500, and Ficoll-Paque were purchased from Pharmacia Biotech (Piscataway, NJ). Anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) was used for Western blotting, and anti-phosphotyrosine Ab PB20-agarose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation studies. Rabbit polyclonal anti-Jak2, anti-p125<sub>FaK</sub>, and anti-paxillin mAb (H-250) were purchased from Santa Cruz Biotechnol- ogy. Anti-p125<sub>FaK</sub> mAb (clone 77) and anti-paxillin mAb was obtained from Upstate Biotechnology and Transduction Laboratories (Lexington, KY), respectively. The enhanced chemiluminescence (ECL) Western blotting system was obtained from Amersham (Arlington Heights, IL). Du- Pont-New England Nuclear (Boston, MA) was the source of [γ-<sup>32</sup>P]ATP. Four-well multidish culture plates and 100- and 35-mm dishes were purchased from Nunc (North Aurora Road, IL). RPMI containing 1-arginine (200 mg/ml), HBSS, and FBS and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD).

PMN isolation

Human venous blood (10 ml) was collected from healthy adult volunteers into heparinized tubes (12). To obtain neutrophils from peripheral blood, the heparinized blood was centrifuged for 10 min at 1000 rpm to remove platelet-rich plasma. After 2% dextran sedimentation of erythrocytes for 30 min, neutrophils were isolated under sterile conditions by density gradient centrifugation on Ficoll-Paque cushions in cornical tubes. The tubes were centrifuged at 2500 rpm for 30 min in swing-out buckets at room temperature. Contaminating erythrocytes were lysed with hypotonic solution containing NH<sub>4</sub>Cl-EDTA and then washed twice. The cells were gently resuspended in magnesium-free HBSS containing 1.6 mM CaCl<sub>2</sub> and then the cell number and viability were determined. The entire procedure was conducted in sterile conditions at room temperature. The final cell preparation comprised at least 97% neutrophils and 0.2% monocytes, as assessed by trypan blue exclusion (12).

Immunoprecipitation and Western blot analysis

Lysates (1 ml) obtained as described above were precleared by the addition of 30 μl of protein A-Sepharose (50% (v/v) slurry) for 1 h at 4°C. The precleared lysates were incubated with 5 μg of free Abs for 4–6 h at 4°C on an oscillating platform. Twenty-five microliters of protein A-Sepharose was then added into lysates and left for 1 h at 4°C. In the case of immunoprecipitates of phosphotyrosine (pY), this was followed by the addition of 10 μl of protein A-agarose-conjugated-anti-pY Ab (Santa Cruz Biotechnology) for 4–6 h at 4°C. All beads were collected by centrifugation and washed twice with modified extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin, 1 mM N-ethylmaleimide, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM sodium pyrophosphate, and 50 mM NaF and once with 0.2% dextran supernatant which was removed carefully. 45 μl of 30% cold PBS and then resuspended (100 μl<sup>10</sup>/cells) in an ice-cold cell extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin, 1 mM N-ethylmaleimide, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM sodium pyrophosphate, and 50 mM NaF. Lysates were centrifuged at 15,000 rpm at 4°C for 30 min. The clear cytosol was separated from the insoluble pellet fractions and immediately used for immunoprecipitation or Western blot.

Immunoﬂuorescence staining and confocal microscopy

Indirect labeling methods were undertaken to detect the localization of p125<sub>FaK</sub>, paxillin, and phosphotyrosine in neutrophils. The cellular distri-

bution of F-actin was directly stained using FITC-labeled phalloidin. Ad-

herent cells (4 × 10<sup>5</sup> cells/ml) were seeded onto two-well chamber slides and incubated for 30 min with serum-free RPMI 1640 medium. Cells were then stimulated with GH (100 ng/ml) for the indicated period of time, and the cell layers were washed twice with PBS and fixed for 15 min with 4% paraformaldehyde (PFA) at room temperature. After washing with PBS, fixed cells were blocked for 1 h with blocking solution containing 0.3% paraformaldehyde solution (Vector), and images were analyzed using a confocal microscope (model LSM 410, Carl Zeiss, Thornwood, NY). The following control experiments showed that staining was specific; no signal was visualized when primary Abs were not added.

In vitro kinase assay

Anti-p125<sub>FaK</sub> immunoprecipitates were isolated from neutrophil lysates treated with GH for 0–60 min. Immunoprecipitates were washed three times with a lysis buffer and then divided into two equal parts. Each part was resuspended in an equal volume (30 μl) of kinase assay buffer (50 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM HEPES (pH 7.4) containing [γ-<sup>32</sup>P]ATP (5 μCi/ml) for 30 min at 37°C. The kinase reaction was stopped by the addition of 5× sample buffer for SDS-PAGE, separated on 8% gel, and visualized by autoradiography. The other part of immunoprecipitates was separated on 8% gel and probed with an anti-p125<sub>FaK</sub> Ab.

Flow cytometry

For the effect of GH on F-actin formation, we used the method described by Lee et al. (42). Briefly, neutrophils were incubated for 1 h at 37°C with GH (100 ng/ml). Reactions were terminated, and cells were fixed by the

Downloaded from http://www.jimmunol.org/ by guest on Apr 29, 2017
addition of 4% PFA for 15 min. Cells were washed twice with PBS, and phalloidin-FITC (100 nM) was added for 5 min. The changes in fluorescence activity were measured using FACStar (Becton Dickinson, Mountain View, CA).

Data analysis

Data are expressed as the mean and SEM. Differences were compared using ANOVA (repeated measures) and the Mann-Whitney test. A significant level was designated at the 95% level (p < 0.05). Statistical calculations were completed using the StatView 512 software package (Abacus Concepts, Berkeley, CA).

Results

Pituitary GH increases neutrophil adhesion

In the first series of experiments the time- and dose-dependent effects of pituitary GH on neutrophil adhesion were determined. The time course for the direct adherence of neutrophils to the plastic substratum of cell cultureware was assessed with or without GH. Maximal adherence of neutrophils was observed at 30 min for both control and GH-treated samples (Table I). GH augmented the neutrophil adhesion in a bell-shaped dose-response fashion, with the peak effect observed at 100 ng/ml (Fig. 1A). The effect of GH on neutrophil adhesion was modest, and maximally a 32% increase was observed 30 min after GH stimulation. Then we examined the effect of genistein, a typical tyrosine kinase inhibitor, on GH-stimulated neutrophil adhesion. Fig. 1B shows that 10 μM genistein decreased the adherence of neutrophils by itself and blocked the effect of pituitary GH. Concentrations ranging from 1 to 10 μM genistein significantly blocked the effect of GH on neutrophil adhesion (data not shown). Photomicrographs of Fig. 1C showed that neutrophils in response to GH (100 ng/ml) for 30 min adopted more elongated shapes (Fig. 1C, b and d) than control cells, which show a round shape (Fig. 1C, a and c). Interestingly, GH-treated neutrophils showed membrane ruffles and exhibited more spreading compared with control neutrophils. The bipolar shape and uropod formation were also observed (Fig. 4, C, F, and I). The sustained effect on the shape change by GH was most evident in the distribution of cell adherence by 30 min. These data indicated that the signaling by GH is an effective stimulus for shape change of neutrophils.

Pituitary GH stimulates tyrosine phosphorylation of p125FAK and paxillin

Next, the effect of GH stimulation on tyrosine phosphorylation of Jak2, STAT3, p125FAK, and paxillin was examined. Neutrophils were incubated at 37°C with different concentrations of GH (10–500 ng/ml) for 30 min and lysed with detergent. GH induced the tyrosine phosphorylation of Jak2 (Fig. 2A), STAT3 (Fig. 2B), p125FAK (Fig. 2C), and paxillin (Fig. 2D) in a bell-shaped, concentration-dependent manner. The half-maximal and maximal increases in the level of tyrosine phosphorylation of Jak2 and

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Pituitary GH increases the adhesion of human neutrophils. A, Dose-dependent effect of pituitary GH on the adhesion of human neutrophils (n = 8). The neutrophils (4 × 10^6) were incubated with medium alone or with the indicated concentration of GH for 30 min in a 96-well plate. B, Inhibitory effect of genistein on the GH (100 ng/ml)-stimulated adhesion of neutrophils (n = 6). The cells were preincubated with genistein (10 μM) for 30 min. Values show the change in absorbency at 570 nm. *, Significantly different from the control (no treatment of GH), p < 0.01. †, Significantly different from the value of GH-stimulated cells, p < 0.05. C, Morphological changes in neutrophils induced by pituitary GH. Cells were incubated in chamber slides with GH (100 ng/ml) for 30 min and examined by phase contrast light microscopy (a and b). Cells were fixed with 4% paraformaldehyde and stained with Wright-Giemsa solution (c and d). a and c, Medium control; b and d, pituitary GH treatment.
STAT3 occurred at 50 and 100 ng/ml, respectively. Furthermore, GH caused a striking increase in the tyrosine phosphorylation of p125FAK and paxillin. The maximal tyrosine phosphorylation of p125 FAK and paxillin was observed at 100 ng/ml of GH. Subsequent blotting with respective Abs demonstrated that equal amounts of the protein were loaded in each pair of lanes.

To examine the kinetics of phosphorylation of Jak2, STAT3, p125 FAK, and paxillin, cells were stimulated with GH (100 ng/ml) for 5, 15, 30, and 60 min before lysis. As illustrated in Fig. 3A, increased tyrosine phosphorylation of Jak2 was detected 5 min after stimulation, reached its maximum within 15 min, and declined thereafter. The tyrosine phosphorylation of STAT3 (Fig. 3B) was also evident after 5 min of stimulation and reached its maximum at 15 min of treatment with GH. After 30 min, the tyrosine phosphorylation of STAT3 was almost reduced. It was noted that GH-stimulated tyrosine phosphorylation of p125FAK and paxillin reached a maximum at 30 min and declined at 60 min. Pretreatment with genistein (10 mM) inhibited the tyrosine phosphorylation of p125FAK (Fig. 3C) and paxillin (Fig. 3D).

Localization of p125FAK, paxillin, and phosphotyrosine in pituitary GH-stimulated neutrophils

Previous studies have shown that the initial effect of growth factors such as IGF-I and PDGF is to promote the formation of ruffles and the extension of filopodia or lamellipodia (29, 43, 44). We also tested whether GH could modulate the morphological changes in neutrophils. Our data obtained from confocal microscopy showed that pituitary GH stimulates typical membrane rufflings and uropod formation in neutrophils (Fig. 4, C, F, and I). Interestingly, p125FAK and paxillin were localized and concentrated at these focal adhesion sites, such as peripheral rufflings (Fig. 4, B and E). These peripheral complexes were clearly distinguished from control cells both morphologically and in the intensity of staining (Fig. 4, A and D). Otherwise, pY staining showed the punctate structures in control and GH-stimulated neutrophils (Fig. 4, G and H). Although the pY was densely stained in membrane rufflings in the presence of GH (Fig. 4H), the distribution of pY showed different patterns from p125FAK and paxillin in neutrophils (Fig. 4, B and E). In part, these data showed that several proteins other than p125 FAK and paxillin are tyrosine phosphorylated upon GH stimulation in neutrophils.

Pituitary GH stimulates the association of p125FAK with Jak2 via STAT3 and in vitro phosphorylation of p125FAK

Although the association between Jak2 and p125FAK has been reported, the possible association mechanism has not been confirmed (45). To investigate the association mechanism of p125FAK with Jak2, neutrophils were treated for 0–60 min with 100 ng/ml pituitary GH. Cells were then lysed, and equally aliquoted lysates were separately immunoprecipitated using anti-Jak2, STAT3, and
p125FAK Ab. Western blot analysis, as assessed by coimmunoprecipitation, showed a time-dependent association of p125FAK with Jak2 (Fig. 5A). Association between p125FAK and Jak2 was apparent at 15–30 min and diminished 60 min after pituitary GH stimulation. Subsequent immunoblotting with the immunoprecipitating Ab showed the presence of equal amounts of protein. Conversely, we observed the Jak2 and p125FAK association by immunoprecipitation with Jak2 and immunoblotting with p125FAK (Fig. 5B). The physical association of Jak2 and p125FAK was enhanced after stimulation with pituitary GH, but there was little constitutive association between these molecules in the controls not treated with pituitary GH. Because it is possible that Jak2 and p125FAK are indirectly linked by another protein and because STAT3 was also shown to be coimmunoprecipitated with Jak2 in neutrophils (Fig. 5B), we examined the possibility that STAT3 may mediate the association between Jak2 and p125FAK. Interestingly, as illustrated in Fig. 5, C and D, p125FAK was present in the STAT3 immunoprecipitates, and STAT3 was present in the p125FAK immunoprecipitates. The association between STAT3 and p125FAK was also increased after stimulation with pituitary GH.

To determine whether GH signal is involved in the phosphorylation of p125FAK, an in vitro kinase assay was performed using immunoprecipitated p125FAK obtained from the GH-stimulated neutrophils. Fig. 6A shows that the autophosphorylation of p125FAK is time dependent. It was shown that the autophosphorylation activity of p125FAK started to increase at 5 min and was maximal at 30 min. Then, the autophosphorylation activity of p125FAK was decreased, but persisted for 60 min (Fig. 6A). We further observed that genistein blocked the stimulatory effect of GH on the autophosphorylation of p125FAK (Fig. 6B).

**Discussion**

It is well established that GH is a physiological mediator of immune cell functions (15–19), and many of the actions of this stimululi are likely to be transduced through the Jak2 pathway (20, 21, 23). Although it has been reported that GH increases neutrophil adhesion, the possible mechanisms have not been examined (16). The present study demonstrates that pituitary GH stimulates neutrophil adhesion through the modulation of focal adhesion kinase (p125FAK), paxillin, and actin polymerization and further suggests

**FIGURE 4.** Focal localization of p125FAK, paxillin, and phosphotyrosine in pituitary GH-stimulated human neutrophils. Neutrophils were treated for 30 min with pituitary GH (100 ng/ml). Cells were then fixed with 4% paraformaldehyde for 15 min and stained for p125FAK (A and B), paxillin (D and E), and phosphotyrosine (G and H) using indirect double immunofluorescence labeling as described in Materials and Methods. C and F, Nomarski views for B and E; I, phase contrast view for H. Scale bar, 5 μm.

**FIGURE 5.** Jak2 associates with p125FAK via STAT3 in vivo in human neutrophils. A, Time dependence of the pituitary GH-stimulated association between p125FAK and Jak2. Neutrophils were treated with GH (100 ng/ml) for the indicated time periods. B, Jak2 association with p125FAK and STAT3 was regulated by pituitary GH. STAT3 was coimmunoprecipitated with p125FAK (C) and vice versa (D). Cell extracts were immunoprecipitated with p125FAK, and subsequently blots were probed with anti-Jak2 Ab as described in Materials and Methods. The same blots were then stripped and reprobed with anti-p125FAK Ab.
that the neutrophil adhesion pathway by pituitary GH is under Jak2-p125 FAK -paxillin tyrosine phosphorylation.

In the present study pituitary GH increased neutrophil adhesion to the plastic substratum. GH also triggered the tyrosine phosphorylation of Jak2 and STAT3 in neutrophils; this is consistent with previous studies of GH-stimulated tyrosine phosphorylation of Jak2 and STATs in the other cell types (20 –23). Our results revealed that the tyrosine phosphorylation of Jak2 occurs about 10 –15 min before p125FAK and paxillin tyrosine phosphorylation in neutrophils. There was a 10-min lag between the tyrosine phosphorylation of Jak2 and the p125 FAK and paxillin, which indicates those p125 FAK and paxillin phosphorylations occur downstream of Jak2. It suggests that GH-stimulated tyrosine phosphorylation of p125FAK and paxillin may occur through the Jak2 signaling event from the GH receptor and subsequently modulate actin polymerization in neutrophils. It is noteworthy to add that a small amount of constitutive tyrosine phosphorylation of Jak2 and p125 FAK was observed in neutrophils without any stimulation. In comparison with other experiments, the constitutive tyrosine phosphorylation of Jak2 and p125FAK in our system might be due to the difference in cell types (23, 27, 45). Inhibition of neutrophil adhesion and tyrosine phosphorylation of Jak2, p125 FAk, and paxillin by genistein implies that GH-induced neutrophil adhesion is related to the pattern of tyrosine phosphorylation of these signaling molecules. This observation is consistent with a previous report that hepatocyte growth factor-mediated tyrosine phosphorylation of p125FAK is inhibited by the tyrosine kinase inhibitor, herbimycin A, which also blocks spreading and the migratory response of oral squamous carcinoma cells (40).

The earliest and most important events in acute inflammation are the adhesion and emigration of neutrophils from the blood into the tissue. It has been noted that adherent inflammatory cells extend the pseudopodia-like process into underlying endothelial cells (2). To target cells to specific sites, neutrophils should become activated and adhere. Following adhesion, neutrophils undergo cell shape change before they transmigrate across the vessel wall and migrate along a chemotactic gradient (2, 6). The precise mechanism(s) for the adhesion signaling from neutrophil migration to firm adhesion remains largely unknown. Recent results have demonstrated that GH and prolactin stimulate p125FAK and paxillin tyrosine phosphorylation through the Jak2 pathway (37, 45). In accordance with previous studies, our findings demonstrates that a low dose of pituitary GH (50 ng/ml) induces a striking increase in tyrosine phosphorylation of p125FAK. Also, GH stimulated the tyrosine phosphorylation of paxillin, which is the focal adhesion component and putative p125FAK substrate. Several
growth factors have also been reported to increase the tyrosine phosphorylation of paxillin, presumably through the regulation of p125FAK in various kinds of cells (25, 26, 29, 46, 47). Our data showed that the tyrosine phosphorylation of p125FAK and paxillin exhibited a typical bell-shaped dose-response curve, in agreement with the pattern of adhesion and the tyrosine phosphorylation of Jak2. The disruptive effect of pituitary GH at high concentration on the tyrosine phosphorylation of p125FAK and paxillin in neutrophils is similar to the prolactin- or PDGF-induced tyrosine phosphorylation of p125FAK and paxillin in a breast carcinoma cell line or in Swiss 3T3 cells (25, 27).

The diverse signal transduction pathways of GH stimulation have also been studied in various cell types (15, 16, 45, 48). It was previously been reported that GH promotes association of the p85 subunit of phosphatidylinositol 3-kinase (PI-3 kinase) with insulin receptor substrate-1 and Jak2, and increases enzymatic activity of PI-3 kinase (48, 49). Zhu et al. have recently confirmed that p125FAK is associated with Jak2 after the stimulation of rGH in the CHO cell line (45). Further, it has been shown that the rGH signal can be transmitted to the actin cytoskeleton via Jak, p125FAK, and tensin, and the actin polymerization produced by GH stimulation occurs due to the PI-3 kinase activation (41). Moreover, it has been suggested that Jak2 is associated with p125FAK in neutrophils. We observed an apparent relationship between neutrophil adhesion and Jak2, and p125FAK could be mediated via STAT3. This association was significantly increased in neutrophils. In addition to promoting ruffle formation, we have determined that STAT3 plays a potential role as one of intracellular adaptor molecules in intracellular signaling (54). Al-

References


Acknowledgments

We thank Dr. Rajiv R. Ratan for help and comments on this study. Drs. Benjamin A. Kruskal and Brian Wilson are greatly appreciated for critical reading of the manuscript, and Vera Zachariassen is appreciated for preparation of the manuscript. Hwa-Jung Huh, Byung-Ki Kim, and Myung-Soo Lee are greatly acknowledged for their fine technical assistance.


