Vol. 214, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 5, 1995 Pages 69-74

FLUID FLOW AND OSMOTIC STRESS INDUCE TYROSINE PHOSPHORYLATION OF AN ENDOTHELIAL CELL 128 KDa SURFACE GLYCOPROTEIN

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Received July 21, 1995

To investigate the flow sensing and the early signaling events in vascular endothelial cells (ECs), we surveyed changes in phosphotyrosine levels of glycoproteins using immunoblot analyses. Cultured bovine arterial ECs were exposed to steady laminar flow by using a cone-plate type flow apparatus and glycoprotein fractions were partially purified by lectin affinity column chromatography. A 128 kDa protein band in the *Ricinus-communis-agglutinin*-bound fraction showed a rapid and consistent augmentation of tyrosine phosphorylation by flow. Cell surface domain-restricted biotinylation revealed that the 128 kDa glycoprotein has extracellular domain(s). Tyrosine phosphorylation of the 128 kDa protein was also observed in ECs subjected to hyper- or hypo-osmotic shock but not in ECs stimulated by Ca^{2+} mobilizing agents.

The vascular endothelium is constantly exposed to hemodynamic forces of blood flow. *In vivo* and *in vitro* studies have revealed that flow induces a number of morphological, physiological and biochemical changes in ECs (1-6, see 7 for review) and suggested that these cells can sense the direction as well as the magnitude of fluid shear stress (8). Recent studies have demonstrated that rapid increase of flow rate activates trimeric G proteins (9, 10), induces Ca^{2+} mobilization (11-13) and increases K^+-conductance (14, 15) in ECs. However, little is known about the molecular basis for flow sensing by these cells. To investigate possible involvement of protein tyrosine phosphorylation in EC flow sensing and subsequent early signaling events, we surveyed changes in phosphotyrosine levels of cell surface proteins. Here we report that a 128 kDa cell surface transmembrane glycoprotein was rapidly tyrosine-phosphorylated in ECs exposed to flow. This protein was also tyrosine-phsophorylated by hyper- and hypo-osmotic shocks. These results suggest that the tyrosine phosphorylation of the 128 kDa membrane glycoprotein is a step in the common signaling pathway activated by various mechanical stimuli.

MATERIALS AND METHODS

Flow experiment: Bovine arterial ECs (BAECs) were obtained from a piece of the carotid artery, cultured and identified as described previously (16). Briefly, BAECs were cultured in 1 : 1 mixture of Dulbecco's Modified Eagle medium and Ham's F12 containing 10 mM Hepes

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0006-291X/95 $12.00

buffer, pH 7.4 (DF medium) supplemented with 10% fetal bovine serum (FBS) on 60 mm plastic culture dishes (MS-11600, Sumitomo Bakelite Co., Ltd., Tokyo). Confluent monolayers were starved for 10 hr with 1% FBS in DF medium and incubated for 1 hr in 2 ml of flow medium before they were set in a flow apparatus. The flow medium was Hanks' medium containing 0.3% bovine serum albumin and 2% polyvinylpyrrolidone (360 K average molecular weight, Sigma), which was used to increase medium viscosity (5 cP at 37°C). To expose BAECs to steady laminar flow, a cone-plate type viscometer (RE-100L, Toki Sangyo Co., Ltd., Tokyo) equipped with a rotating cone (0.8°, 24 mm radius) and a sample cup shaved to accommodate snugly a 60 mm tissue culture dish was used. Fifty rpm (18~20 dyn/cm^2, 360 s^{-1}) was used for the flow condition and 1 rpm (0.4 dyn/cm^2 , 7 s^{-1}) was used as the control condition. Since this viscometer-based flow apparatus measures the torque exerted on the rotating cone, fluid shear stress and shear rate on the dish surface can be determined if the viscosity of the fluid is known and if the dish surface is flat. Each dish was selected for flatness of the culturing surface. The preincubation and the flow experiment were done at 37°C in humidified air.

Osmotic shock and chemical stimulation: BAEC monolayers in 60 mm dishes were preincubated for 1 hr in 2 ml of serum-free DF medium. Then 2 ml of one of the test media were added and cells were incubated for 2 min. The test media were serum-free DF medium (control), the same medium containing 600 mM sucrose (hyperosmotic), and 10 mM Hepes buffer, pH 7.4 (hyposmotic). For chemical stimulation, 2 ml of serum-free medium containing 2 x concentrated stimulant was added.

Analysis of protein tyrosine phosphorylation: BAECs exposed to a mechanical or chemical stimulus were washed 3 times with ice-cold phosphate buffered saline (PBS) containing 1 mM Na_3VO_4. They were lysed and homogenized in 1 ml of ice-cold lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/ml phenylphosphate, 10 μM genistein, 2 μM erbstatin analogue, and 0.4% Triton X-100, pH 7.4. After centrifugation at 15,000 x g for 20 min, the supernatant was incubated with *Ricinus communis agglutinin* I (RCA_{120}) conjugated agarose beads (Seikagaku Corp., Tokyo) at 4°C for 30 min and washed extensively with the lysis buffer. The bound material was recovered in SDS-PAGE sample buffer without β-mercaptoethanol and electrophoresed on a 6% gel. Protein bands were electrophoretically transferred onto a PVDF membrane and probed with a monoclonal anti-phosphotyrosine (anti-PY) antibody (4G10, Upstate Biotechnology Inc.). Biotinylated anti-mouse Ig (Zymed) and [^{125}I]streptavidin (Amersham) were used to visualize and quantify bound antibodies by a Bioimage analyzer (BAS2000, Fuji Film). The linearity of our quantification was confirmed by including in each membrane at least 3 different dilutions of the same sample. Immunoblot analyses were also done using anti-JAK1 (Upstate Biotechnology Inc.), anti-pp120 (Transduction Lab.), anti-$p125^{FAK}$ (Transduction Lab.), anti-JAK2 and anti-gp130 (17). The latter two antibodies were kind gifts from Drs. Tetsuya Taga and Kanji Yoshida (Institute of Molecular and Cellular Biology, Osaka University) and Prof. Tadamitsu Kishimoto (Faculty of medicine, Osaka University).

Cell surface labeling: Plasma membrane domain-restricted biotinylation was carried out by the method of Brandi et al. (18) using NHS-SS-biotin (Pierce). Labeled BAECs were lysed in the phenylphosphate-free lysis buffer and the RCA_{120}-bound material was released by 0.2 M lactose in the same buffer. A half of the eluted sample was immunoprecipitated with anti-PY and protein A Sepharose beads (Pharmacia), and the bound material was eluted by 10 mM phenylphosphate. After SDS-PAGE under the non-reducing condition, gel bands were transferred to a PVDF membrane, which was then probed with [^{125}I]streptavidin and autoradiographed. The other half of the sample was applied on an avidin column (Pierce), washed with 0.5 M NaCl in the lysis buffer. The bound material was recovered in the lysis buffer containing 5% β-mercaptoethanol. After SDS-PAGE under the reducing condition, the sample was probed by immunoblotting with anti-PY.

RESULTS AND DISCUSSION

We first attempted to study fluid flow effects on tyrosine phosphorylation in BAECs by immunoblot analyses with anti-PY on the whole cell extract or the anti-PY immunoprecipitable material in the extract. However, no significant, flow-dependent changes were detected due to constitutively high levels of tyrosine phosphorylation in the cell. Since molecules related to mechanical stress sensing may be in or associated with the plasma membrane, we used lectin affinity as the first step to obtain a preparation enriched for such proteins. Several kinds of lectins were tested, and we found that an RCA_{120}-bound glycoprotein with an apparent molecular mass of 128 kDa (GP128) in 6% SDS-PAGE under the non-reducing condition showed a rapid and consistent flow-dependent increase in tyrosine phosphorylation (Fig.1, lanes 1-5). While BAECs exposed to less than 4 dyn/cm^2 (20 rpm) of fluid shear stress for 7 min had the basal level of tyrosine phosphorylated GP128, those exposed to this or higher flow levels for also 7 min had higher phosphorylation levels of this protein. It appeared that the threshold for this response was 4 dyn/cm^2 or 80 s^{-1}. When flow was fixed at a level above the threshold (50 rpm for Fig.1, lanes 6-10), the phosphorylation increase became detectable within 5 min, and often as early as 1 min. After 7 min, the average increase was 1.6 times (SD=0.2, n=11, $p<0.001$) the level of the control cells exposed to 1 rpm (Fig. 5). Since the amount of GP128, assessed by Coomassie blue staining intensity, was the same in samples with or without flow stimulation (data not shown) or in samples with or without osmotic shock (Fig. 2), the observed change appeared to reflect the true change in the tyrosine phosphorylation state.

Since there is a number of known phosphotyrosine containing polypeptides with their molecular weights close to 128 kDa, such as pp125^{FAK}, pp120, JAK1 and JAK2, we have tested by immunoblotting if GP128 can be recognized by antibodies against any of these

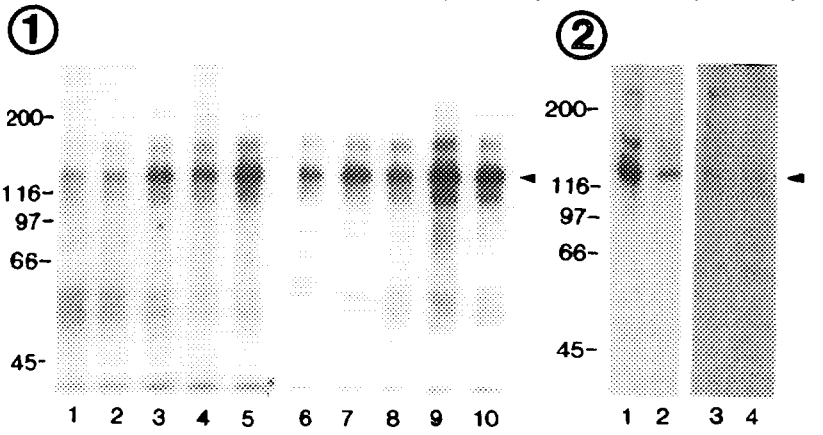

Fig. 1. Flow-induced tyrosine phosphorylation of GP128 (*arrowhead*). BAECs were exposed to various levels of flow rate (2, 10, 20, 50 and 100 rpm) for 7 min (lanes 1-5, respectively) or to a constant flow rate at 50 rpm for 0, 1, 2, 5 and 10 min (lanes 6-10, respectively). Immunoblot analyses of RCA_{120}-bound fractions of the whole BAEC lysate using anti-PY.

Fig. 2. Hyposmotic shock-induced tyrosine phosphorylation of GP128 (*arrowhead*). BAECs were exposed to hypotonic medium (lanes 1, 3) or to isotonic medium (lanes 2, 4) for 2 min. Each lane was loaded with 5 μg of the RCA_{120}-bound material and probed with anti-PY and autoradiographed (lanes 1, 2) or stained with Coomassie blue (lanes 3, 4).

proteins. When tested against the whole BAEC extract, these antibodies except anti-JAK2 (the quantity of this protein was presumably insufficient in the sample) bound to their corresponding antigens. However, they reacted with no components, including the anti-PY labeled 128 kDa band, in the RCA_{120} bound material (data not shown), indicating non-identity of GP128 with any of the 4 proteins. Interleukin-6 signal transducer (gp130) is an RCA_{120}-binding, tyrosine phosphorylated transmembrane glycoprotein (19). Recent studies have shown that the gp130 signaling pathway plays a role in cardiomyocyte hypertrophy (20, 21), suggesting possible involvement of gp130 in the mechanism of cellular response to mechanical stimuli. Our immunoblot analysis revealed that gp130 was present in the RCA_{120}-bound fraction of BAECs extracts but was distinct from GP128 by two criteria. First, gp130 ran with a slightly higher apparent molecular weight (134 kDa) in our SDS-PAGE, and second, partial amino acid sequences from internal peptides of purified GP128 showed no homology to the published sequences of human (22) or mouse (23) gp130.

Because it is possible that GP128 is a cytoplasmic protein associated with a transmembrane protein, cell surface biotinylation was done on BAECs. Anti-PY immunoprecipitated material in the RCA_{120}-bound fraction contained a 128 kDa component that had an avidin binding activity, and the biotinylated material after being washed in a high salt solution still contained a band at about 130 kDa recognized by anti-PY (Fig. 3). These results are consistent with the idea that GP128 is a transmembrane cell surface glycoprotein. As Fig. 3

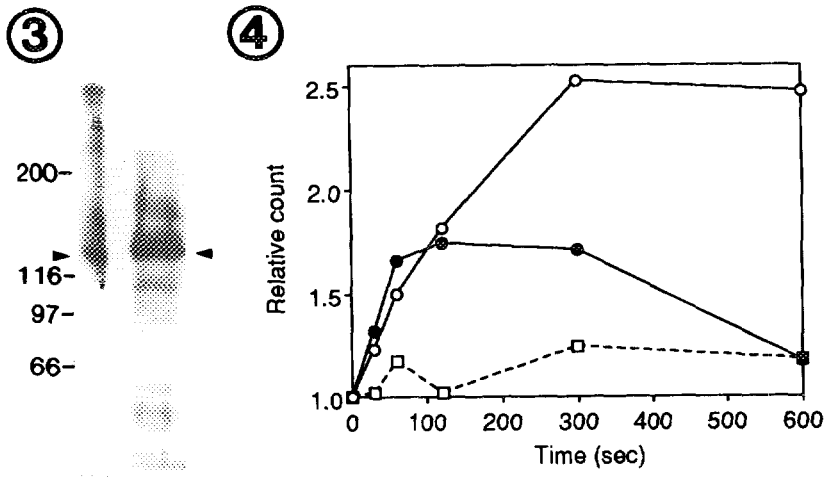

Fig. 3. Cell surface biotinylation of GP128 (*arrowhead*). The RCA_{120}-bound fraction of BAECs labeled with NHS-SS-biotin was immunoprecipitated with anti-PY and probed with [^{125}I]streptavidin (*left lane*), or loaded on an avidin column, washed with 0.5 M NaCl, eluted with β-mercaptoethanol and probed with anti-PY (*right lane*).

Fig. 4. Time course of GP128 tyrosine phosphorylation induced by osmotic shocks. BAECs were exposed to hypotonic medium (*closed circles*), hypertonic medium (*open circles*), or isotonic medium (*open squares*). The RCA_{120}-bound material in the cell extract made at each time point was analyzed by immunoblotting with anti-PY and radioactivity associated with the 128 kDa band was quantified. Relative counts against the count at time 0 are plotted. The data are the means of 3 experiments.

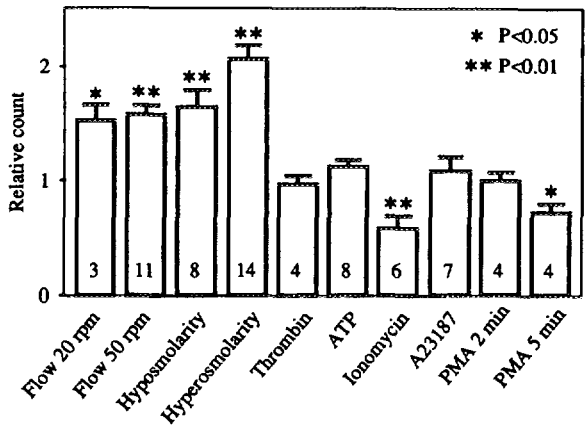

Fig. 5. Effects of mechanical stresses and Ca^{2+} mobilizing reagents on GP128 tyrosine phosphorylation. BAECs were exposed to flow (20 or 50 rpm for 5 min), osmotic shocks, 1 U/ml thrombin, 20 μM ATP, 5 μM ionomycin, 10 μM calcium ionophore A23187, or 100 nM PMA (2 or 5 min). Cells were treated for 2 min except for flow and PMA stimulation. For quantification see the legend for Fig. 4. Relative counts against the control cells (Mean± SE) are shown. The number of experiments is indicated at the bottom of each bar.

shows, GP128 has slower electrophoretic mobility under the reducing condition than the non-reducing condition, suggesting the presence of intermolecular S-S bonds.

Tyrosine phosphorylation of GP128 increased also by hyper- and hypo-osmotic shocks (Figs. 4, 5). The increase became apparent within 30 sec, reaching the maximum level in 2~5 min. The level was sustained at least for 10 min under the hypertonic condition, while the increase was transient under the hypotonic condition (Fig. 4). Increased tyrosine phosphorylation in the similar molecular weight range was reported earlier in cells of a human intestine cell line under hyposmotic condition (24).

Fluid flow is known to induce phosphoinositide turnover (25) followed by Ca^{2+} mobilization (11-13) and PKC activation (26) in ECs. To investigate the relationship between the GP128 tyrosine phosphorylation and these signaling events, effects of thrombin, ATP, ionomycin, Ca^{2+} ionophore A23187, and phorbol 12-myristate 13-acetate (PMA) on the phosphorylation level were examined. One unit/ml thrombin or 20 μM ATP increased cytoplasmic free Ca^{2+} by 100~400 nM in our fura-2 loaded BAECs (data not shown). As shown in Fig. 5, none of the reagents augmented the GP128 tyrosine phosphorylation level and ionomycin and PMA had rather inhibitory effects. These results indicate that the tyrosine phosphorylation of GP128 is not mediated by Ca^{2+} mobilization or PKC activation. While our data cannot completely rule out the possibility that GP128 tyrosine phosphorylation is a step within a known cell signaling pathway, this phosphorylation may indicate the presence of a new pathway activated by flow and osmotic shocks. It appears, therefore, BAECs have multiple mechanisms for flow sensing and signal transduction.

Acknowledgments: This work was supported by a research grant from the Ministry of Health and Welfare of Japan (6B-1), Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture and a research fund from the Kanehara Memorial Foundation, and Special Coordination Funds for Promoting Science and Technology from Science and Technology Agency of Japan.

REFERENCES

1. Dewey, CF, Jr., Bussolari, SR, Gimbrone, MA, Jr., and Davies, PF. *J Biomech Engng* **103**: 177-185 (1981).
2. White, GE, Gimbrone, MA, Jr., and Fujiwara, K. *J Cell Biol* **97**: 416-424 (1983).
3. Frangos, JA, Eskin, SG, McIntire, LV, and Ives, CL. *Science* **227**: 1477-1479 (1985).
4. Jo, H, Dull, RO, Hollis, TM, and Trbell, JM. *Am J Physiol* **260**: H1992-H1996 (1991).
5. Koller, A, and Kaley, G. *Am J Physiol* **260**: H862-H868 (1991).
6. Resnick, N, Collins, T, Atkinson, W, Bonthron, DT, Dewey, CF, Jr., and Gimbrone, MA, Jr. *Proc Nat Acad Sci USA* **90**: 4591-4595 (1993).
7. Malek, AM and Izumo, S. *J Hypertension* **12**:989-999 (1994).
8. Masuda, M, and Fujiwara, K. *Front Med Biol Eng* **5**: 79-87 (1993).
9. Ohno, M, Gibbons, GH, Dzau, VJ, and Cooke, JP. *Circulation* **88**: 193-197 (1993).
10. Kuchan, MJ, Jo, H, and Frangos, JA. *Am J Physiol* **267**: C753-C758 (1994).
11. Mo, M, Eskin, SG, and Shilling, WP. *Am J Physiol* **260**: H1698-1707 (1991).
12. Schwarz, G, Callenwaert, G, Droogmans, G, and Nilius, B. *J Physiol* **458**: 527-538 (1992).
13. Ando, J, Ohtuka, A, Korenaga, R, Kawamura, T, and Kamiya, A. *Biochem Biophys Res Comm* 190: 716-723 (1993).
14. Olesen, SP, Calpham, DE, and Davies, PF. *Nature* **331**: 168-170 (1988).
15. Schilling, WP, Mo, M, and Eskin, SG. *Exptl Cell Res* **198**: 31-35 (1992).
16. Masuda, M, and Fujiwara, K. *Biol Cell* **77**: 237-245 (1993).
17. Yoshida, K, Chambers, I, Nichols, J, Smith, A, Saito, M, Yasukawa, Shoyab. M, Taga, T, and Kishimoto, T. *Mechanisms Develop* **45**: 163-171 (1994).
18. Brandli, AW, Parton, RG, and Simons, K. *J Cell Biol* **111**: 2909-2921 (1990).
19. Murakami, M, Narazaki, M, Hibi, M, Yawata, H, Yasukawa, K, Hamaguchi, M, Taga, T and Kishimoto, T. *Proc Nat Acad Sci USA* **88**:11349-11353 (1991).
20. Pennica, D, King, KL, Shaw, KJ, Luis, E, Rullamas, J, Luoh, S-M, Darbnne, WC, Knutzon, DS, Yen, R, Chien, KR, Baker, JB, and Wood, WI. *Proc Nat Acad Sci USA* **92**: 1142-1146 (1995).
21. Hirota, H, Yoshida, K, Kishimoto, T, and Taga, T. *Proc Nat Acad Sci USA* **92**: 4862-4866 (1995).
22. Hibi, M, Murakami, M, Saito, M, Hirano, T, Taga, T, and Kishimoto, T. *Cell* **63**: 1149-1157 (1990).
23. Saito, M, Yoshida, K, Hibi, M, Taga, T and Kishimoto, T. *J Immunol* **148**: 4066-4071 (1992).
24. Tilly, BC, Berghe, N, Tertoolen, LGJ, Edixhoven, MJ, and Jonge, HR. *J Biol Chem* **268**: 19919-19922 (1993).
25. Prasad, ARS, Logan, SA, Nerem, RM, Schwartz, CJ and Sprague, EA. *Circ Res* **72**: 827- 836 (1993).
26. Malek, AM, Greene, AC and Izumo, S. *Proc Nat Acad Sci USA* **90**: 5999-6003 (1993).