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PROTEIN PHOSPHORYLATION AND ACTIVATION OF PLATELETS BY WHEAT GERM AGGLUTININ

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SUMMARY: The incorporation of $^{32}P_i$ into the 47 and 20 kDa polypeptides in platelets activated by wheat germ agglutinin (WGA) was studied. The pattern of enhanced phosphorylation produced by the lectin was comparable to that by thrombin. The 47 kDa polypeptide was phosphorylated at both serine and threonine while the 20 kDa protein was mainly labeled at serine residues. However, the ratio of phosphoserine to phosphothreonine in the 47 kDa polypeptide in WGA-activated platelets was higher than thrombin-stimulated platelets. Addition of N-acetylglucosamine at different times blocked platelet activation by WGA. There was a concomitant modification in the phosphorylation of the 47 kDa protein. These data suggest that the phosphorylation of the 47 kDa polypeptide may modulate the WGA-receptor mediated activation of platelets. Our studies also demonstrate that activation of platelets by different stimuli may lead to differential phosphorylation of different amino acid residues in the same protein.

Platelet stimulation by different agonists is accompanied with enhanced $^{32}P_i$ incorporation into two endogenous polypeptides of 47 and 20 kDa respectively (1-4). Activation of protein kinase C may be responsible for the increased phosphorylation of the 47 kDa protein (5), whereas the 20 kDa polypeptide, identified as the myosin light chain, is phosphorylated by Ca^{2+} and calmodulin-dependent protein kinase (6, 7). Recently, the 47 kDa polypeptide has been shown to have multiple phosphorylation sites (8). However, the function of this polypeptide remains unknown. It may also be phosphorylated by protein kinases, other than protein kinase C. When platelets are stimulated by ADP, the 20 kDa polypeptide is rapidly phosphorylated which may be related to the shape change in platelets (9) whereas platelets activated with platelet activating factor (PAF) undergoes shape change in parallel with the phosphorylation of 47 kDa protein (10). Therefore, the activation of platelets by different stimuli may not follow the same mechanism or kinetics. In this

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study, the kinetic characteristics and the nature of the phosphorylated amino acids of these two polypeptides in platelets stimulated by WGA and thrombin are described. This lectin is known to activate platelets like many physiological agents but its action may be readily blocked at different times with the appropriate sugar. The state of phosphorylation of the 47 kDa protein under these conditions may provide important information on the mechanism of platelet activation.

MATERIALS AND METHODS

Platelets - Blood was collected from healthy volunteers in plastic syringes containing 0.1 volume of 3.8% trisodium citrate as the anticoagulant. Platelets were washed and resuspended in 25 mM Tris-HCl buffer, pH 7.4 containing 0.125 M NaCl, 0.1% bovine serum albumin and 0.1% glucose (11). Platelets thus prepared were used in aggregation studies or incubated at 37° for 1 hr with $^{32}P_i$ (1.0 mCi/ml, ICN Radiochemical) for studying the kinetics of phosphorylation upon stimulation. Platelet aggregation experiments were carried out as described previously with bovine thrombin (Parke Davis) or WGA (US Biochemicals) (11).

Gel Electrophoresis and Autoradiography - Samples of stimulated and unstimulated $^{32}P_i$-labeled platelets were boiled in SDS-sample buffer (0.064 M Tris-HCl, pH 6.1/5% 2-mercaptoethanol/10% glycerol/3% SDS) for two minutes and subjected to 12% or 15% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (12). The gel was stained in 0.1% coomassie brilliant blue in acetic acid and methanol, destained, dried and subjected to autoradiography (Kodak XAR-5). The autoradiographs were routinely scanned by a Gelman DC-16 gel scanner. The area under the peak was integrated manually.

Phosphoamino Acid Analysis of Proteins - In order to determine the nature of phosphoamino acids, the SDS-polyacrylamide gel was stained in coomassie blue, treated with 1 N NaOH according to the method of Cheng et al. (13) and then processed for autoradiography. In another experiment, $^{32}P_i$-labeled proteins were separated on a 12% gel by electrophoresis. The 47 kDa polypeptide band was localized by autoradiography. The protein was eluted from the excised gel with 50 mM NH_4HCO_3/0.1% SDS/10 mM 2-mercaptoethanol and was precipitated with 10% TCA with 100 µg of bovine serum albumin as a carrier. The pelleted protein was hydrolyzed with 6N HCl for two hours and subjected to one-dimensional high voltage electrophoresis (Shandon High Voltage Electrophoresis Apparatus), at pH 3.5 for 45 min (14). The separated phosphoamino acids were then exposed to X-ray films.

RESULTS AND DISCUSSION

Effect of N-acetylglucosamine on the phosphrylation induced by WGA - Platelets stimulated with WGA showed enhanced phosphorylation of 47 and 20 kDa proteins. The effect on the phosphorylation was comparable to that of thrombin (Figure 1).

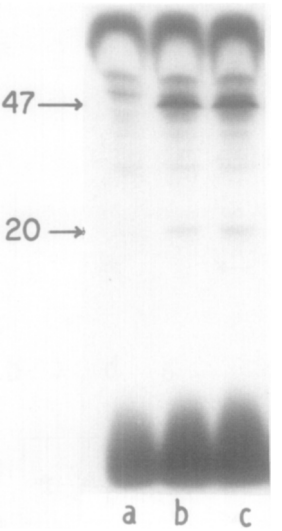

Fig. 1. Phosphorylation of proteins in platelets activated with WGA (2.6 μM) and thrombin (8.3 nm). The proteins were separated by SDS-PAGE. The gel was stained, destained,dried and subjected to autoradiography. Lane a: control platelets; Lane b: platelets exposed to WGA; lane c: platelets exposed to thrombin. The molecular weight markers used were carbonic anhydrase (31,000), ovalbumin (43,000), bovine serum albumin (68,000) and phosphorylase b (94,000).

Since phosphorylation of proteins may modulate the activation of platelets, it was of interest to explore the kinetics of phosphorylation of 47 and 20 kDa proteins and their possible correlation with the activation of platelets by WGA. The stimulation of platelets by WGA can be inhibited by N-acetylglucosamine (4). Aggregation of platelets was arrested by adding excess N-acetylglucosamine at different times after the addition of WGA (Figure 2A). The sugar was more effective in blocking aggregation when added at early time points. It is noteworthy that concomitantly the phosphorylation of the 47 kDa protein in the WGA-stimulated cells was inhibited under the same conditions. The inhibition of phosphate incorporation was time-dependent (Figure 2B). With the addition of N-acetylglucosamine the inhibition of both phosphorylation and aggregation could be stopped even at five seconds after the stimulation with WGA. The quantitation of the 47 kDa phosphorylated protein revealed that the half-maximal incorporation of $^{32}P_i$ into this polypeptide as determine by autoradiography was achieved in approximately 10 s which could be inhibited by the subsequent addition of N-acetylglucosamine (Figure 2C). However, it is

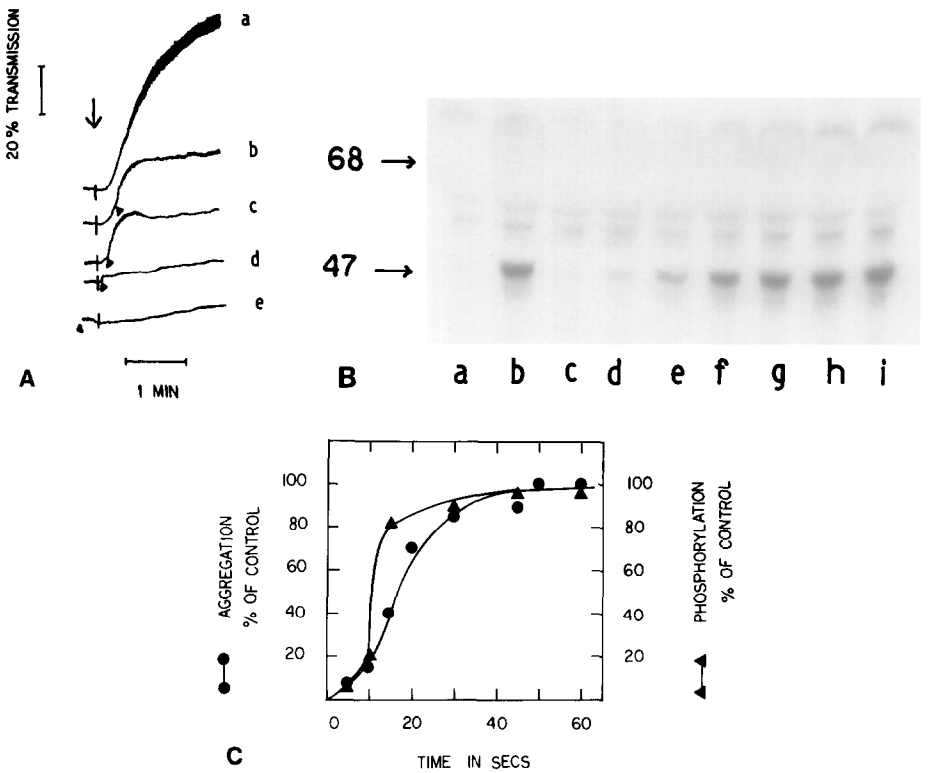

Fig. 2. Inhibition of platelet aggregation and 47 kDa protein phosphorylation. Platelets (3 x 10^8/ml) were exposed to 2.6 μM of WGA at zero time (arrow) and then N-acetylglucosamine (2 mM final con.) was added at definite time intervals A. Tracing a: Platelets activated by WGA; tracing b to d: platelets exposed to WGA and then N-acetylglucosamine was added at 20, 10 and 5 sec respectively; tracing e: platelets exposed to N-acetylglucosamine prior to WGA addition. B. Phosphorylation pattern of duplicate samples as used in the aggregation experiments (Fig. 2A). After a total time of 1 min, SDS buffer was added to each sample. Lane a: control platelets, lane b: platelets exposed to WGA only, lane c: platelets exposed to N-acetylglucosamine only; lane d to i: platelets exposed to WGA and then N-acetylglucosamine was added at 5, 10, 15, 30, 45 and 60 sec. C. Relationship between platelet aggregation and the 47 kDa protein phosphorylation as determined above in Fig. 2A and 2B. ●-● shows aggregation of platelets as percent of control. Control refers to the aggregation of platelets at 1 min with 2.6 μM of WGA. ▲-▲ shows phosphorylation of the 47 kDa protein as percent of that produced at one min with 2.6 μM of WGA. The abcissa shows the time of addition of N-acetylglucosamine.

interesting to note that the blockage of half-maximal aggregation by the same sugar was possible approximately at 15 s. These data suggest that the phosphorylation of the 47 kDa protein precedes aggregation when platelets are stimulated by WGA. However, the phosphorylation of the 20 kDa protein in the presence of WGA followed a biphasic pattern (Figure 3). The maximum incorporation of $^{32}P_i$ into this protein was achieved within 10 seconds. There was a gradual dephosphorylation which continued up to 30 seconds follow-

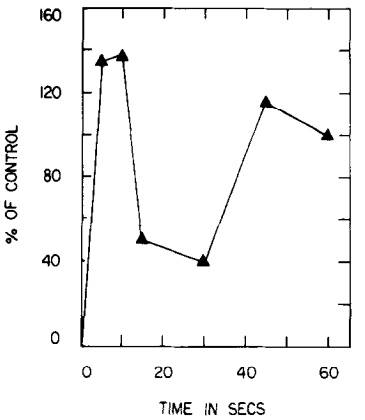

Fig. 3. Quantitation of phosphorylation (▲-▲) of 20 kDa protein at different times. The experimental procedure is described in the legend of Figure 2B.

ed by enhanced phosphorylation of this protein. This pattern was consistent from experiment to experiment. Similar results were reported previously for the platelet myosin light chain phosphorylation in the presence of ADP (9). According to these authors, the shape change of platelets and phosphorylation of 20 kDa protein parallel each other. It is possible that the enhanced phosphorylation of the myosin light chain in the second phase could be related to the aggregation of the platelets.

Analysis of Phosphorylated Amino Acids - In order to determine the nature of the phosphorylated amino acids, the SDS-polyacrylamide gels were treated with 1 N NaOH. Since the phosphate-ester linkages to tyrosine and threonine, but not serine, are stable at high pH (14, 15), this led to the release of radioactive phosphate from only serine residues. Under these conditions, the phosphorylated 20 kDa protein was completely removed indicating that only the serine residues are phosphorylated in this polypeptide whereas the radioactivity in the 47 kDa polypeptide persisted under these conditions (Fig. 1 and 4A). Thus, the remaining phosphorylated amino acid residues in this protein are either threonine or tyrosine or both (Figure 4A). In order to determine the ratio of phosphoserine to phosphothreonine/phosphotyrosine in the presence of WGA or thrombin, the 47 kDa polypeptide was excised from the gel and the protein was eluted (14). The acid hydrolysate of the polypeptide was subjected to high voltage electrophoresis (Figure 4B). The

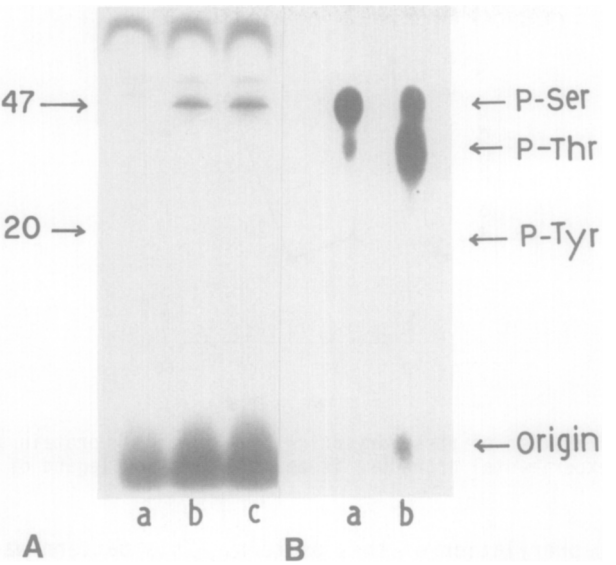

Fig. 4 A. Analysis of phosphorylated amino acids. Platelets were stimulated with WGA or thrombin and the phosphorylated proteins were separated by electrophoresis. A. The gel was stained in coomassie blue and then incubated at 40°C with 1 N NaOH for 1 hr. The gel was destained, dried and subjected to autoradiography. The control for this pattern is Fig. 1. B. Phosphorylated proteins from activated platelets were analyzed by SDS-PAGE. The 47 kDa protein was localized by autoradiography. The protein was eluted from the excised gel with 50 mM NH_4HCO_3/0.1% SDS and then precipitated with 100 µg BSA as a carrier. The proteins were hydrolyzed in the presence of 6N HCl and the hydrolysate was subjected to high-voltage electrophoresis followed by autoradiography. Lane a: 47 kDa protein from WGA-activated platelets. Lane b: 47 kDa protein from thrombin-activated platelets. The standards used were 10 µg of each phosphoserine, phosphothreonine and phosphotyrosine.

mechanism of phosphorylation of the 47 kDa protein by WGA and thrombin appears to be different. While WGA-stimulated platelets showed higher phosphorylation at serine residues, the thrombin-stimulated platelets showed more phosphorylated threonine. No phosphotyrosine could be detected with either stimulant.

Previously, we have shown that WGA is a true activator of platelets (4). With the use of the appropriate sugar, here we have shown that platelet aggregation by WGA and phosphorylation of the 47 kDa polypeptide vary in a coordinate manner. It is also important to note that phosphorylation of the protein precedes aggregation. These observations imply that phosphorylation of the 47 kDa protein may be involved in platelet activation by WGA. Another important point is that the stimulation of platelets by different activators

may follow different mechanisms (9, 10). The ratio of the phosphorylated amino acids in platelets activated by WGA and by thrombin was significantly different.

Thus, the biochemical mechanism of actions of different agonists on platelets including roles played by different protein kinases can be different.

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