Rapid Publications
Studies on the Insulin Mediator
II. Separation of Two Antagonistic Biologically Active Materials from Fraction II

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SUMMARY

Insulin treatment significantly altered the elution profile of deproteinized muscle extracts chromatographed on Sephadex G-25 columns, particularly in fraction II, which contains the insulin mediator. Further purification of fraction II by high-voltage paper electrophoresis at pH 1.9 and 3.5 resulted in two active fractions. Fraction 1 → 4 stimulated the cyclic AMP-dependent protein kinase and inhibited glycogen synthase phosphoprotein phosphatase, and may be a novel substance. Fractions 1 → 6 and 3 → 6 inhibited the cyclic AMP-dependent protein kinase and stimulated glycogen synthase phosphatase. It is proposed that the insulin mediator is present in fractions 1 → 6 and 3 → 6. DIABETES 29:659–661, August 1980.

Recently, we demonstrated that a mediator generated by insulin is present in rat skeletal muscle extracts that inhibited the cyclic AMP-dependent protein kinase and stimulated glycogen synthase phosphoprotein phosphatase.[1] The same mediator was also shown to activate pyruvate dehydrogenase in adipocyte mitochondria.[2] The activation of pyruvate dehydrogenase might occur via increasing the free Ca^{2+} pool in the mitochondria[2] or through a direct stimulation of a calcium-sensitive phosphatase.

The effects of the mediator fraction on both glycogen synthase phosphoprotein phosphatase and pyruvate dehydrogenase are biphasic,[1,2] i.e., stimulatory at low concentrations and inhibitory at higher concentrations. In seeking an explanation for these biphasic effects, we have further purified this material by high-voltage paper electrophoresis. This paper describes the separation of a glycogen synthase phosphatase stimulator from a glycogen synthase phosphatase inhibitor. The fraction which inhibited glycogen synthase phosphoprotein phosphatase also stimulated the cyclic AMP-dependent kinase.

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MATERIALS AND METHODS

The treatment of animals with insulin and the preparation and fractionation of muscle extracts were performed as previously described.[1] In insulin-treated animals, the mean blood glucose concentration was 114 mg/dl (versus 167 mg/dl for control) and glycogen synthase-I was 32% (versus 24% for control). Subfractions of fraction II were pooled, lyophilized, and redissolved in 5 ml of 50 mM formic acid. For high-voltage paper electrophoresis, 0.5 ml of concentrated fraction II was applied as a narrow band on a sheet of Whatman 3 MM filter paper (46 × 57 cm). Electrophoresis was carried out in 5% formic acid (pH 1.9) at 5 mA/cm for 40 min with alanine and lysine as markers. Zones which contained active materials were cut out and eluted with 50 mM formic acid containing 0.1 mM EDTA and 0.01 mM cysteine. Eluates were lyophilized to dryness and redissolved in 2 ml of 50 mM formic acid. Fractions 1 and 3 from the first electrophoresis run were further purified by a second electrophoresis in acetic acid: pyridine buffer (pH 3.5) at 6 mA/cm for 35 min. Again, zones containing active material were cut out, eluted, and concentrated to a final volume of 2 ml.

Assays for cyclic AMP-dependent protein kinase and glycogen synthase phosphoprotein phosphatase were performed as previously described.[1] All assayed fractions were first lyophilized to remove formic acid and then redissolved in the assay mixture immediately before assay.

RESULTS AND DISCUSSION

The handling of animals, muscle preparation, and purification of the insulin mediator steps 1 through 4 (Table 1) were done as previously described.[1] In step 3, endogenous ATP, ADP, and AMP were identified with markers and removed. The remaining material was eluted, lyophilized and chromatographed on Sephadex G-25 as previously described.[1] We have already shown that the insulin mediator, as defined

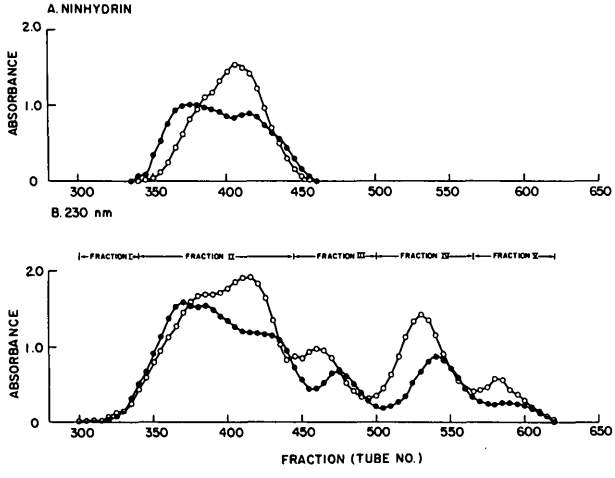
TABLE 1
Purification of the insulin mediator

Step	Procedure
1	Frozen powdered muscle.
2	Heat deproteinized.
3	Paper chromatography.
4	Sephadex G-25 chromatography of nucleotide "free" fraction.
5	High-voltage electrophoresis at pH 1.9 of fraction II.
6	High-voltage electrophoresis at pH 3.5 of fractions 1 and 3.

by its ability to inhibit the cyclic AMP-dependent protein kinase, to activate the glycogen synthase phosphoprotein phosphatase, and to activate pyruvate dehydrogenase,[2] is present predominantly in fraction II of the Sephadex G-25 column chromatogram.[1] We have now observed that, when Sephadex G-25 chromatograms of control and insulin-treated muscle extracts are directly compared, (Figure 1) there is a consistently observed alteration in the 230 nm absorption and ninhydrin profiles of fraction II under conditions in which there is no significant difference in the total amount of material present in fraction II.* The altered profile of the insulin-pretreated extract was better maintained when the elution buffer contained 0.1 mM EDTA and 0.01 mM cysteine. These results indicate that there is a redistribution of ninhydrin-positive materials after insulin pretreatment, and sulfhydryl groups might play an important role in stabilizing the insulinized state during chromatography.

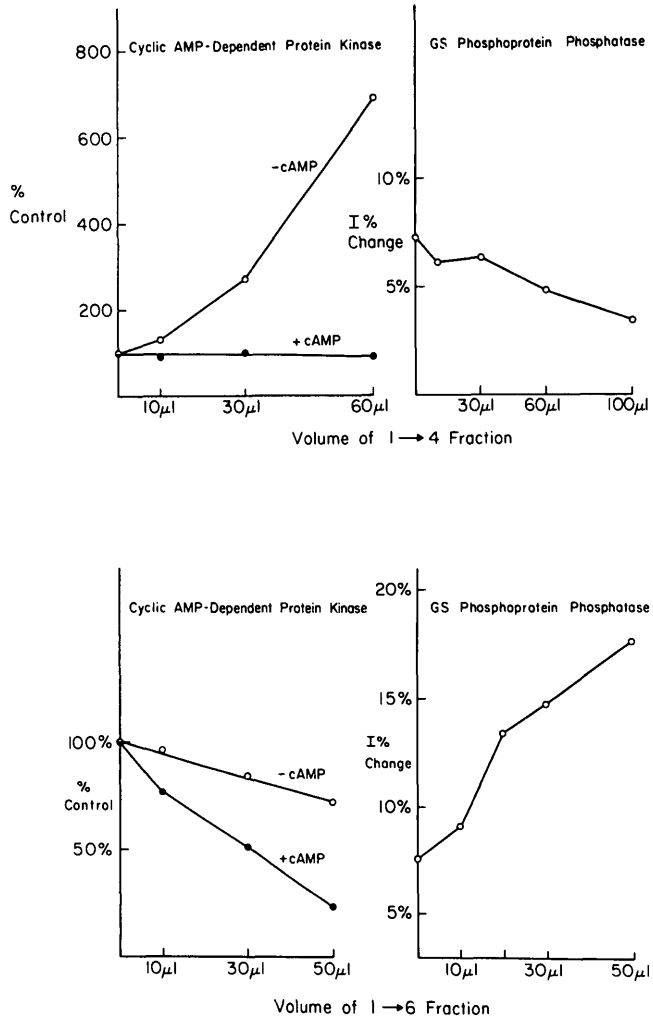
Further purification of fraction II by high-voltage paper electrophoresis at pH 1.9 (step 5, Table 1) resulted in 4 fractions which were identified on the basis of ninhydrin reactivity and fluorescence detection. These fractions were cut out as bands, eluted from the paper, lyophilized as previously described[1] and tested for their effect on both the cyclic AMP-dependent protein kinase and phosphoprotein phosphatase activities. Fraction 1, close to the anode, markedly inhibited phosphoprotein phosphatase activity and stimulated cyclic AMP-dependent protein kinase. Fraction 3, closer to the cathode, but removed from fraction 1 by 7 cm, stimulated phosphoprotein phosphatase and inhibited the cyclic AMP-dependent protein kinase (data not shown). Both fractions 1 and 3 were eluted from the paper, lyophilized as previously described[1] and refractionated by a second high-voltage paper electrophoresis at pH 3.5 (Table 1, step 6). Seven fractions were identified by ninhydrin reactiv-

FIGURE 1. Sephadex G-25 chromatograms of both deproteinized control and insulin-treated muscle extracts. Materials eluted from Whatman 3 MM filter paper sheets were lyophilized and redissolved in 5 ml of 50 mM formic acid and applied to a Sephadex G-25 column (5 × 85 cm). The column was eluted with 50 mM formic acid containing 0.1 mM EDTA and 0.01 mM cysteine with a flow rate of 15 sec/drop and 50 drops per fraction. (○), control muscle extract from 61 grams of original muscle; (●), insulin-treated extract from 55 grams of original muscle. This result has been observed consistently in 3 experiments.



* The differences between control and insulin chromatograms in the total peak amounts of fraction III, IV, and V are most likely due to incomplete removal of nucleotides in the preceding paper chromatography step and are not related to the insulin treatment.

FIGURE 2. Effect of fractions 1 → 4 and 1 → 6 on cyclic AMP-dependent protein kinase and glycogen synthase phosphoprotein phosphatase. The control activities of protein kinase in the absence and presence of cyclic AMP were 0.06 and 1.5 nmol/min/mg protein, respectively. The final concentration of cyclic AMP is 2.5 μM, when present.



ity and fluorescence detection, cut out as bands, eluted, and lyophilized as previously described.[1] Fractions 1 through 3 were from the anodal portion and fractions 4 through 7 were from the cathodal portion of paper. Fraction 1 → 4 (first number indicates the fraction number from pH 1.9, the second from pH 3.5) markedly stimulated cyclic AMP-dependent protein kinase in the absence of cyclic AMP, with no effect on the protein kinase activity in the presence of cyclic AMP (Figure 2). When tested on glycogen synthase phosphoprotein phosphatase, this fraction inhibited phosphatase activity in a dose-dependent manner. The inhibition of phosphatase by fraction 1 → 4 is probably not due to the presence of phosphoprotein phosphatase inhibitors as described by others[3–8] which, because of their higher molecular weight, would be separated from fraction II in the Sephadex G-25 column fractionation. Since cyclic AMP moved toward the anode under our electrophoretic conditions (pH 3.5), fraction 1 → 4 may contain a novel substance(s) having effects on both cyclic AMP-dependent protein kinase and glycogen synthase phosphatase opposite from the mediator. Fraction 1 → 6 inhibited the cyclic AMP-dependent protein kinase to a greater extent in the presence than in the absence of cyclic AMP, and stimulated the phosphoprotein phosphatase (Figure 2). This indicated that the insulin mediator was present in this fraction. Fraction 3 → 6 also stimulated phosphatase activity, but to a lesser extent than fraction 1 → 6 (data not shown), indicating that it also contained the insulin mediator. Of interest was the fact that while fraction 3 (pH 1.9) had a stimulatory effect on the glycogen synthase phosphatase, fraction 1 (pH 1.9) had an inhibitory effect. However, at pH 3.5 a material (1 → 6) was separable from the phosphatase inhibitor (1 → 4) of fraction 1 that does stimulate the phosphatase. One possible explanation under current investigation is that the phosphatase activator is chemically related to the protein kinase activator. Although we have not studied the effect of insulin on the formation of the protein kinase activator, we suggest that the presence of a protein kinase activator and a phosphoprotein phosphatase inhibitor may help explain the fact that insulin has been shown to increase certain intracellular protein phosphorylations[9–12] along with its predominant pattern of decreasing protein phosphorylation.[13–15]

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