Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 64-69

Structural and Functional Abnormalities in the Adipocyte Plasma Membrane from db/db Mouse, and the Effect on the Abnormalities of Oral Treatment with AS-6

Tomoyoshi Hosokawa, Kunio Ando and Gakuzo Tamura

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Yayoi-1-1, Bunkyo-ku, Tokyo 113, JAPAN

Received September 7, 1984

Summary: The protein bands of adipocyte plasma membranes from the genetically obese diabetic mice C57BL/KsJ db/db (db/db mice) showed slight but significant changes compared with their lean littermates. The treatment for 1 week with a new antidiabetic agent, AS-6, caused the changes to revert toward the condition in the lean littermates. In the absence of insulin, the plasma membrane and mitochondria mixture (P3 fraction) of the lean littermates densely labeled 55000 and 57000 dalton protein bands by phosphorylating with (α-^{32}P)-ATP, whereas the labeling was less in the P3 from AS-6 treated and untreated db/db mice. Insulin inhibited phosphorylation of these bands in P3 from the lean littermates and untreated db/db mice, while the hormone enhanced the labeling in AS-6 treated db/db mice compared with the basal conditon without insulin. Ca^{2+} greatly enhanced the labeling in all three groups, whereas Mg^{2+} mimicked the insulin action diminishing the labeling of these bands in the lean and untreated db/db groups. However, Mg^{2+} enhanced the phosphorylation in the P3 from AS-6 treated db/db mice compared with the basal condition.

It is increasingly evident from current studies that insulin binding to its receptors causes the phosphorylation/dephosphorylation of some cellular proteins (1-5). The finding that insulin stimulates the phosphorylation of the ß-subunit of its own receptors which thus acquires protein kinase activity (6-10) supports this thesis. However, most of these studies deal with materials from normal animals, and lack evidence showing how diabetes modifies this phosphorylation/dephosphorylation processes. The genetically obese diabetic mouse, C57BL/KsJ db/db (db/db mouse), is an excellent model of human obese diabetes (11). The db/db mouse is hyperinsulinemic and highly resistant to insulin (12). We have shown that an ascochlorin derivative, AS-6, reduces

Abbreviation ;SDS-PAGE sodium dodecysulfate polyacrylamide gel electrophoresis.

0006-291X/84 $1.50

the insulin-resistance of db/db mice in the animal, and at the tissue and cellular levels (13-16) and ameliorates the diabetic syndrome.

In the present study we evaulated the structural and functional differences at the subcellular level between the db/db mice and their lean littermates, and examined how oral treatment with AS-6 affects the difference.

MATERIALS and METHODS

The animals: Male db/db and their lean littermates, 12 weeks old, were kindly supplied by the Research Laboratories, Chugai Pharmaceuticasl CO. The db/db mice were randomly allocated to 2 groups soon after arrival and the treatment was initiated simultaneously with the allocation.

AS-6 treatment: The treatment was carried out as reported previously (16).

Plasma membrane of adipocytes: After treatment for 7 days, all of the mice were sacrificed and the epididydmal adipose tissues were removed. The tissues from each group were combined, cut into 3 mm pieces and digested with collagenase (17). The adipocytes were passed through 250 μm mesh, and washed twice with 10 volumes of homogenization buffer (18). The cell suspension was homogenized with a teflon homogenizer, and the homogenates were fractionated by differential centrifugation. The resuling P3 fraction (the mixture of plasma membrane and mitochondria) was purified by discontinuous Ficol density gradient centrifugation. Proteins were determined by the method of Lowry et al (19).

Phosphorylation of P3 fraction (20): The P3 fraction (56 μg as proteins) was added to 50 μM $MgCl_2$, 50 μM $CaCl_2$, 5 mM NaCl, 0.2 mM KCl, and 50 mM Tris buffer (pH 7.4) with or without insulin (1 mU/ml), and the reaction was initiated by the addition of 50 μci (δ-^{32}P)-ATP at 37 C. The total volume was 1 ml. After 2 min the reaction was terminated by the additon of ice cold 5% trichloroacetic acid (TCA), and the mixture was filtered. The filter was washed with 5% TCA, dried and the radioactivity was counted.

Autoradiography of SDS-PAGE: The P3 fractions from the three groups were incubated with the mixture of 25 μci (δ^{32}P)-ATP, 50 μM $MgCl_2$, 50 μM $CaCl_2$, 5 mM NaCl, 0.2 mM KCl, and 50 mM Tris buffer (pH 7.4) in a total volume of 1 ml at 37 C for 5 min in the presence or absence of insulin (1 mU/ml), $CaCl_2$ (20 mM), or $MgCl_2$ (20mM). The reaction was terminated by the addition of 3N perchloric acid, and the mixtures were centrifuged. The precipitates were washed twice with 3N perchloric acid, and redissolved in stacking gel buffer (pH 6.8) containing 10% mercaptoethanol by heating in 100 C for 3 min. The aliquots were subjecterd to SDS-PAGE, and after drying, the labeled bands were detected by autoradiography using Kodak SD-10 X-ray film (21).

RESULTS AND DISCUSSION

SDS-PAGE pattern of membrane proteins: As shown in Fig 1, the proteins in the purified plasma membrane from the three experimental groups were nearly identical, with small exceptions indicated by arrows. These differences between the lean littermates and untreated db/db mice are significant however, since AS-6 treatment restored the disappeared protein bands at the A region and deleted the B band. Cuatrecasas et al. reported that the SDS-PAGE pattern of plasma membrane from genetically obese rats apparently differed from that

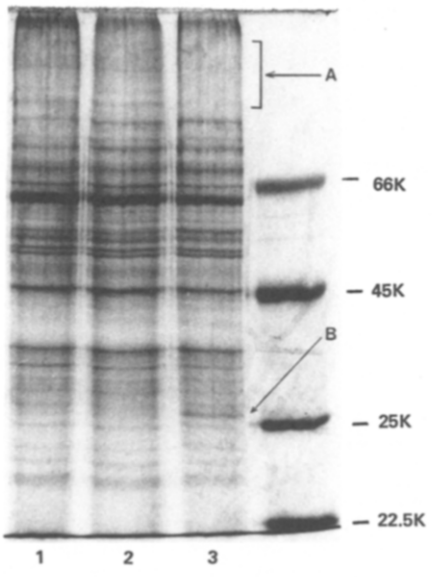

Figure 1. SDS polyacrylamide gel electrophoretic patterns of the plasma membranes from AS-6 treated and untreated db/db mice, and their lean littermates.

The purified plasma membranes (70 µg as proteins) obtained from AS-6 treated and untreated db/db mice (n = 7, each) ,and their lean littermates (n = 16) were treated with the Tris buffer (62.5 mM, pH 6.8) containing 40% mercapto-ethanol, 20% sucrose and 4% sodium dodecylsulfate in boiling water bath for 3 min, and subjected to SDS-PAGE. The proteins were detected by staining with Coommasie blue.
Lane 1 is the lean littermates, lane 2 AS-6 treated db/db mice, and lane 3 the control db/db mice. Note the differences indicated by the arrows.
Bovine serum albumin (66 K), ovalbumin (45 K), chymotrypsinogen (25 K), and trypsin inhibitor (22.5 K) were used as the standards.

of normal rats (22). However, Kahn et al. (23) have shown that the patterns of hepatocyte membranes both from ob/ob mice and their lean littermates were indistinguishable from each other. The difference in findings may represent differences in the mechanism of insulin resistance in various states or may reflect methodological difference and will require further study to elucidate.

Incorporation of ^{32}P from (γ-^{32}P)-ATP into the P3 fraction: In the absence of insulin ^{32}P incorporation into TCA-precipitable fraction of P3 was not different on the mg protein basis (Table 1). When calculated on g tissue basis, however, more ^{32}P was incorporated in the lean littermates and the AS-6 treated groups than in the untreated db/db controls. The increase simply reflects a larger protein content in the former groups.

Table 1. Effect of insulin on incorporation of phosphate into TCA insoluble fraction of adipocyte P3 fraction from AS-6 treated and untreated db/db, and their lean littermate mice

Groups	^{32}P Incorporation				P
	Insulin (-)		Insulin (+)		Insulin(-)
	cpm/mg protein	cpm/g tissue	cpm/mg protein	cpm/g tissue	vs.(+)
	x 10^6	x 10^5	x 10^6	x 10^5	
db/db Controls	3.314 ± 0.363	1.965 ± 0.215	2.137 ± 0.136	1.267 ± 0.081	- 35.5%, P < 0.05
AS-6 treated	3.840 ± 0.301	2.692 ± 0.211*	5.210 ± 0.608**	3.652 ± 0.426**	+ 35.6%, P < 0.05
db/db mice	± 15.9%	± 37.0%	+ 143.8%	+ 188.2%	
Lean littermate	3.448 ± 0.319	3.076 ± 0.284**	3.929 ± 0.119**	3.504 ± 0.107**	+ 14.0%, ns.
controls	+ 4.0%	+56.5%	+83.8%	+ 176.5%	

The figures represent the mean ± SE. (n = 4) * P <0.05 and ** P <0.01, significance by Student t-test. Male db/db mice 12 weeks old were randomly allocated to 2 groups, and one group (n = 5) was fed a diet admixture of 0.1% AS-6. The other group (n = 6) together with their lean littermates (n = 22) were fed the control diet. After 1 week the mice were sacrificed and the epididymal adipose tissues were removed. The adipocytes prepared according to the method of Rodbell (17) were homogenized in the homogenization buffer, and the P3 fractions were separated by differential centrifugation. The incubation was carried out as described in the text, and ^{32}P incorporation into TCA insoluble fraction was determined. Note the difference in the incorporation between the insulin present and absent conditions (1 mU/ml). P indicates the statistical significance between the insulin present and absent conditions.

Insulin significantly diminished ^{32}P incorporation (- 35.5%, below the basal condition, P <0.05) in the untreated db/db group but had no effect on the incorporation in the lean littermates (+ 14.0 %, ns.) compared with that without insulin. In contrast, insulin significantly enhanced the incorporation (+ 34.7%, above the basal condition, P <0.05) in the AS-6 treated db/db mice. Therefore, insulin enlarged the difference in ^{32}P incorporation between the untreated db/db controls and the other two groups; when compared with the untreated db/db controls on mg protein basis, the addition of insulin increased the labeling 1.8 fold in the lean littermates and 2.4 fold in the AS-6 treated db/db mice.

Autoradiography of SDS-PAGE: Under the basal condition without insulin, seven protein bands were labeled in the P3 of the lean littermate; two (55K and 57K dalton) were thick and the others thin (Fig.2. lane 1). The labeling of the 55K and 57K bands was much less in both db/db groups (AS-6 treated lane 2, and untreated lane 3) than in the lean littermates, and the addition of insulin apparently diminished the density of these bands in the lean littermates (lane

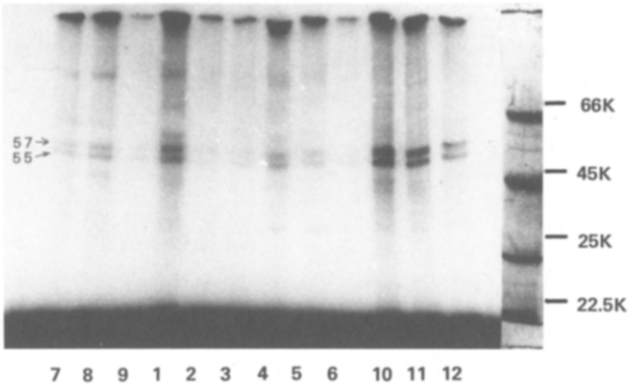

Figure 2. Radioautograms of SDS-PAGE patterns of the P3 fractions labeled with (γ-^{32}P)-ATP from AS-6 treated and untreated db/db mice, and their lean littermates.

The lanes 1-3 are the patterns in the absence of insulin, the lanes 4-6 those in the presence of insulin (1 mU/ml), the lanes 7-9 those in the presence of an additional Mg^{2+} (20 mM), and the lanes 10-12 those in the presence of an additional Ca^{2+} (20 mM). Each left lane is the lean littermates, the middle lane AS-6 treated db/db mice, and the right lane the db/db controls. Note the difference in the density of the 55000 and 5700 dalton bands.

4) and the db/db control (lane 6). In contrast, the bands were dense with AS-6 treatment in the presence of insulin compared with the basal condition, though the densities were less than in the lean littermates (lane 5). This indicates that the treatment modifies insulin action enhancing the labeling of these protein bands.

The additon of Ca^{2+} afforded an effect oposite to insulin on the phosphorylation of these bands. The labeling was much enhanced in all groups suggesting that this phosphorylation is Ca^{2+} dependent (lanes 10-12); the order of density was the greatest in the lean littermate group (lane 10) followed by the AS-6 treated db/db group (lane 11), and the labeling was significantly less in the db/db control (lane 12).

Mg^{2+} showed an effect similar to insulin on the labeling of the P3 of lean littermate and db/db controls (lanes 7 and 9), while the cation again enhanced the phosphorylation in the AS-6 treated group, and the density became comparable to in the lane 4. This suggests that the membrane of db/db mice hampers the efflux of divalent cations and the treatment with AS-6 partially corrects this defect.

This is the first comparative study at the subcellular level using the db/db mice and their lean littermates. Compared with the lean littermates, the db/db mice are apparently abnormal at the subcellular level both structurally and functionally, although the exact mechanism of insulin resistance at this level remains to be elucidated. As our previous studies of these animals at the tissue and cellular levels, oral treatment with the ascochlorin derivative AS-6 caused the structural abnormalities in the plasma membrane of db/db mice to revert toward the condition in lean littermates. This reversion is associated with a partial recovery from the functional abnormalities at the subcellular level. It is quite likely that a reduction in insulin resistance by AS-6 treatment is related to the strucutural and functional restorations at the subcellular level demonstrated in this study.

REFERENCES

1. Denton, R.M., Coore, H.G., Martin, B.R.,and Randle, P.J.(1971) Nature New Biology 231, 115-116.
2. Sheorain, V.S., Khatra, B.S.,and Sonderling, T.R.(1982) J.Biol.Chem. 257, 3462-3470.
3. Alexander, M.C., Palmer, J.L., Pointer, R.H., Kowaloff, E.M., Koumjian, L.L.,and Avruch,J.(1982) J. Biol. Chem. 257, 2049-2055.
4. Brownsey, R.W.,and Denton, R.M.(1982) Biochem. J. 202, 77-86.
5. Smith, C.J., Wejkisnora, P.J., Warner, J.R., Rubin, C.S.,and Rosen, O.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2725-2729.
6. Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, N.W.,and Osathamondh, H.(1982) J. Biol. Chem. 257, 15162-166.
7. Jacobs, S., Sahyoun, N.E., Saltiel, A.R.,and Cuatrecasas, P.(1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6211-13.
8. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L.,and Kahn, C.R.(1983) Proc. Natl. Acad. Sci. U.S.A. 80, 2137-2141.
9. Petrizzolli, L., Herrera, R.,and Rosen, O.M.(1983) Proc. Natl. Acad. Sci. U.S.A. 81, 3332-36.
10. Rees-Jones, R.W., Hedo, J.A., Zick, Y., and Roth, J.(1983) Biochem. Biophys. Res. Commun. 116, 417-422.
11. Hummel, K.P., Dickie, M.M., and Coleman, D.L.(1966) Science 153, 1227-28.
12. Coleman, D.L., and Hummel, K.P.(1974) Diabetologia 10, 607-610.
13. Hosokawa,T., Ando,K., and Tamura, G.(1983) Jpn.J.Atherosclerosis. 11,396-404.
14. Hosokawa, T., Ando, K.,and Tamura, G.(1984) Diabetes in press.
15. Hosokawa, T., Ando, K.,and Tamura, G.(1984) Biochem. Biophys. Res. Commun. Manuscript under inspection.
16. Hosokawa, T., Ando, K.,and Tamura, G.(1982) Agr.Biol.Chem. 46. 2865-69.
17. Rodbell, M.(1964) J. Biol. Chem. 239, 375-80.
18. Jarett, L.(1974) Methods in Enzymol. 31. 60-71.
19. Lowry, O.H., Rosebrough, N.F., Farr, A.L.,and Randoll, R.J.(1951) J.Biol.Chem. 193, 265-275.
20. Seals, J.R., McDonald, J.M.,and Jarett, L.(1978) Biochem. Biophys. Res. Commun. 83, 1365-1372.
21. Seals, J.R., McDonald, J.M.,and Jarett, L.(1979) J.Biol.Chem. 254, 6997-7001.
22. Chang, K.J., Mareus, N.A.,and Cuatrecasas, P.(1974) J.Biol.Chem. 249, 6854-65.
23. Kahn, C.R., Neville, D.M.,and Roth, J.(1973) J.Biol.Chem. 248, 244-50.