Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1434-1440

INSULIN BINDING TO ERYTHROBLASTIC LEUKEMIC CELLS IS DECREASED BY INSULIN AND INCREASED BY AMINO ACIDS

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Received September 3,1980

SUMMARY

Erythroblastic leukemic cells incubated in media supplemented with high amino acid concentrations subsequently bound 55.5% more [^{125}I]insulin than cells incubated in low amino acid media. Cycloheximide (1 μg/ml) abolished this effect. Incubation of cells with physiological (100 μU/ml) doses of insulin in low amino acid media decreased insulin binding by 34.7%. In high amino acid media the same dose of insulin decreased insulin binding by 33% compared to supplemented media without insulin but increased binding by 22.5% compared to basal media without insulin. The data suggest that amino acids or specific amino acids may exert a regulatory influence on insulin receptor homeostasis.

Specific insulin receptors have now been demonstrated on a wide variety of cell membranes and subcellular membranes (for review, see 1). Numerous authors have reported alterations in insulin receptor affinity or number in an ever-increasing array of diverse physiological and pathological states (2-10); of these, the plasma insulin concentration has been most convincingly shown to display an inverse relationship to insulin binding (4,5,6,9,11,12). In humans, decreased insulin binding (down-regulation) has been shown within five hours of euglycemic hyperinsulinemia by Insel et al in erythrocytes (13).

We report here studies carried out on the EBL[2] cell, a chemically induced tumor cell (14) which has been used as a model of a normal erythroblast (15,16,17). We have previously demonstrated that EBL cells exhibit specific insulin receptors which are coupled to biological responses (17) and rapidly internalize insulin (18). Prompted by the work of Schworr and Mortimore on

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0006-291X/80/191434-07$01.00/0

the inhibitory effects of certain amino acids on lysosomal activity (19) we postulated that amino acids may affect the turnover of membrane proteins and specifically that of the insulin receptor. To test this hypothesis we studied the effect of insulin and amino acids on insulin binding by EBL cells. The studies below indicate that pre-exposure to insulin leads to rapid "down-regulation" of insulin binding; in contrast pre-exposure to amino acids leads to apparent "up-regulation."

MATERIALS AND METHODS

EBL cells. Cells were passaged and harvested from hepatic sinusoids of Long-Evans rat pups as previously described (14,15). In view of the deleterious effects of ammonium chloride lysis (22), this step was omitted resulting in up to 5% contamination with erythrocytes. However, such contamination with erythrocytes is not reflected in erroneous insulin binding data (17). Following isolation, cells were washed twice in Ringer's solution (15) and counted by Coulter counter. Cell viability was never less than 95% by Trypan blue dye exclusion (21).

Primary Culture. Basal media was Eagles Media with Earles Balanced Salt Solution without glutamine (Gibco) supplemented with 0.5% bovine serum albumin (Fraction V, Armour) and Penicillin (50U/ml)/Streptomycin (50 μg/ml) (Gibco). Glutamine (1.778 mM) was added to give 5X normal rat plasma concentrations (22). The exact amino acid concentrations were, in mM: arg 0.59, val 0.39, ile 0.396, leu 0.396, phe 0.194, lys 0.397, his 0.219, trp 0.049, thr 0.403, cystine 0.999, met 0.101, gln 1.778 and tyr 0.119.

Supplemented media was basal media supplemented with all amino acids present in rat plasma to yield 4X or higher physiological concentrations. Final amino acid content of this medium was, in mM: asp 0.154, arg 1.47, pro 0.792, gly 1.76, ala 1.98, val 1.27, ile 0.836, leu 1.144, phe 0.546, lys 2.157, his 0.571, trp 0.357, ser 1.237, thr 1.723, glu 0.88, cystine 0.324, met 0.329, gln 8.892, asn 0.308 and tyr 1.071. All media were warmed to 37°C and gassed with 95% O_2/5% CO_2 for 10 min. prior to adjustment of pH to 7.4. Osmolarity of basal media was 275 mOsmoles/L and of supplemented media 291 mOsmoles/L representing a 5% difference. Light and electron microscopy showed no visible effects of the different media on cell morphology, which was well preserved.

6.25X10 cells/ml were incubated in 16ml of media in 50ml tissue culture flasks in triplicate for one hour at 37°C in 95% O_2/5% CO_2. Following incubation, cells were harvested by gentle agitation of flasks, transfer to plastic centrifuge tubes and centrifugation at 230X g at 4°C for 5 min. Flasks were rinsed with 10 ml ice cold phosphate buffered saline which was added to the cell pellet and, after gentle resuspension, recentrifuged. The pellet was washed once more with 10 ml phosphate buffered saline and following centrifugation, resuspended in buffer 'G' (23) to yield a final concentration of 1x10 cells/ml. Cell viability at this stage was never less than 90% by Trypan blue dye exclusion.

Insulin trapping, undissociated binding and degradation. For experiments where insulin was added during incubation, parallel flasks were incubated with [^{131}I]albumin to estimate media trapping and [^{125}I]insulin to estimate residual bound insulin. Trapping of [^{131}I]albumin was estimated at 0.063%. Addition of 240 pg [^{125}I]insulin/ml revealed binding of 0.5-1.0% with tracer alone and 0.3% in the presence of 100 μU/ml unlabelled insulin. These figures are insufficient to account for the degree of down-regulation observed. (EBL

Table 1 Effect of amino acids and insulin on specific binding of ^{125}I-insulin at tracer concentrations to 4X10^7 EBL cells.

	Basal Media	Supplemented Media
Control	100 ± 4.3 (N = 26)	155.5 ± 10.4 (N = 26) a
Insulin (100μU/ml)	65.3 ± 6.9 (N = 9) b	122.5 ± 12.7 (N = 9) c d

Cells were incubated at 37°C for one hour prior to binding assay as described in Methods. N = number of observations. Effects of perturbants in individual experiments were significant. However due to variability in absolute binding between cells from different litters, data have been normalized to percent of basal media control values in each experiment. (Mean basal media control specific binding: 4.95%. Range: 2.05 - 11.55%). The variability reflects differences in maturation of the tumor cells and morbidity of the host animal. Significance of differences between means was tested by Student's t test.

a. Significant increase v. basal media control: $p < 0.001$
b. Significant decrease v. basal media control: $p < 0.001$
c. Significant increase v. basal media + Insulin (100μU/ml): $p < 0.01$
d. Not significant v. basal or supplemented media control

receptor concentration is 22,3000 per cell (17) therefore residual bound insulin occupies 0.85% of the total number of receptors per cell). Degradation of trace [^{125}I]insulin (120 pg/ml) in the media during one hour at 37°C amounted to 19% or less as assessed by solubility in 15% trichloroacetic acid. There was no difference in degradation in basal and supplemented media during incubation or in subsequent binding assays.

Insulin Binding Assay. Porcine insulin was generously donated by Dr. Ronald Chance of Eli Lilly (Indianapolis, Inc.). [^{125}I]insulin was prepared by a modification of the lactoperoxidase method of Marchalonis (24) and purified before use as previously described (17). Binding assays were performed with trace concentrations of [^{125}I]insulin (40 pg) as described by Gambhir et al (23) except that incubation was at 37°C for 5 min. where obtains binding equilibrium with minimal insulin degradation for EBL cells. (17). Bound [^{125}I]insulin was separated from free by centrifugation through dibutyl phthlate. Non-specific binding (percent bound in the presence of 10^5 ng/ml unlabeled insulin) was subtracted from the percent of the tracer bound to 4X10 cells to yield percent maximum specific binding. Cell viabilities after binding assays were never less than 85%. Non-specific binding was the same after incubation in the different media; it accounted for approximately 40% of total binding after incubation in basal and 30% in supplemented media.

RESULTS

Incubation of EBL cells for 1 hour at 37C° in supplemented media resulted in a 55.5% increase in maximum specific binding (N = 26, $p < 0.001$) (Table 1). Incubation with 100 μU/ml insulin, (Table 1), compared to basal media control,

Table 2. Effect of cycloheximide (1 μg/ml) on amino acid effect on specific ^{125}I-insulin binding at tracer concentrations to 4 X 10^7 EBL cells.

	Basal Media	Supplemented Media
Control	7.5 ± 0.35	8.67 ± 0.54 [a]
Cycloheximide	6.2 ± 0.65 [b]	6.7 ± 0.26 [c d]

Experiments were run as described in Methods. Data are means of percent specific binding ± SEM of 3 observations. Significance of difference between means was tested by Student's t test.
a. Significant increase v. basal media control: $p < 0.05$
b. No significant decrease v. basal media control
c. Significant decrease v. supplemented media control: $p < 0.05$
d. No significant decrease v. basal media.

resulted in a 34.7% "down-regulation" in basal media ($p < 0.001$) but a 22.5% "up-regulation" in supplemented media ($p < 0.01$). The latter "up-regulation" was significant compared to values in basal media with insulin ($p < 0.01$). Preliminary Scatchard analyses are consistent with changes predominantly in insulin receptor number.

The amino acid effect on insulin binding was preserved when the binding assay was prolonged to 30 minutes at 37°C with Bacitracin 5mg/ml which rendered [^{125}I]insulin degradation undetectable. Under these conditions percent maximum specific binding of cells incubated in basal media was 6.08± 0.57% and in supplemented media 13.66±1.24% ($N = 3$, $p < .01$). Addition of basal or supplemented media directly to the binding buffer had no effect on maximum specific binding (data not shown). Cycloheximide (1 μg/ml) present during incubation had no effect on maximum specific binding in basal media but reduced binding in supplemented media (Table 2). Cycloheximide at a dose of 1 μg/ml inhibits ^{3}H-leucine incorporation into protein in EBL cells to 2.3% of control values within 1 hour in basal media ($N = 3$, $p < 0.001$).

DISCUSSION

We have demonstrated rapid in vitro "down-regulation" of insulin binding to EBL cells in the presence of physiological concentrations of insulin (100 μU/ml). Results of parallel estimations of trapped insulin in media and

residual bound insulin after incubation are incompatible with such decreased binding being merely an artifact of insulin contamination. "Down-regulation" in EBL cells occurred within one hour, a much shorter time interval than that required by other cell types in vitro or in vivo (25).

We have also demonstrated rapid in vitro "up-regulation" in response to increased amino acid concentrations in the media. The inhibition of this effect by cycloheximide suggests a requirement for protein synthesis. However caution is required in interpretation of cycloheximide data as it has also been shown to inhibit protein degradation (26). Thus we cannot exclude the possibility that the amino acid effect is mediated by inhibition of insulin receptor degradation. Furthermore, it may be that the observed amino acid effects apply not only to insulin receptors but also to other membrane proteins.

That amino acids, or specific amino acids, should modulate metabolic events is not without precedent. Work from this laboratory (27,28) and others (29,30) has shown the effect of branched chain amino acids, notably leucine, on protein synthesis and degradation in muscle. Schworr and Mortimore reported inhibition of protein degradation by glutamine in liver (19), and Pain et al. inhibition of initiation complex formation in amino acid depleted Ehrlich Ascites Tumor Cells (31). Specific amino acids stimulate the synthesis of phosphatidylcholine by the kidney, suggesting that amino acids may coordinate the synthesis of protein and phospholipid destined to form plasma membrane (32).

Although our data does not allow us to distinguish between an amino acid effect mediated by general availability of structural units for protein synthesis or a specific effector molecule(s), two points need emphasis. The basal medium is capable of supporting EBL cells in permanent culture (33), with a doubling time of 12 hrs. Secondly, cycloheximide inhibited the increased insulin binding seen in supplemented media but had no effect on binding in basal media. The latter suggests that receptor turnover in the

absence of the ligand is too slow to be assessed by our methods within 1 hr. The above observations would suggest however that amino acids are not influencing insulin binding simply through availability of structural units for protein synthesis.

Our observation that physiological doses of insulin mediate "down-regulation" in low amino acid concentrations but that increased amino acid concentrations override such "down-regulatory" effects suggests that such counter-regulation may have physiological relevance. It is, of course, difficult to speculate on potential physiological significance in a tumor cell. Nevertheless, control of insulin receptor number or affinity by the amino acid milieu seems reasonable. Circulating amino acid concentrations are decreased in the presence of increased circulating insulin, mediated in part by decreased amino acid release from muscle (34). Thus, a fall in plasma amino acids may contribute to the rapid down-regulation of insulin receptor affinity (13) reported in euglycemic hyperinsulinemia in man. While it is clear that insulin's many effects in vitro on different cells have been shown in amino acid free media and in vivo under conditions of relatively low circulating amino acids, the fine tuning of the insulin response by the amino acid milieu may not have been appreciated. Additionally this phenomenon could apply selectively to a greater or lesser degree to certain cell types, which could allow appropriate preferential insulin responses to occur in different tissues.

Acknowledgement

The authors are grateful to Dr. Sam Spicer for histological studies and to Mrs. Barbara Whitlock for secretarial help. This work was supported in part by Grants AM-02001 from NIAMDD, and the Medical University of South Carolina Biomedical Research Appropriation 1979-1980.

REFERENCES

1. Kahn, C. (1976) J. Cell. Biol. 70:216-286.
2. Wachslicht-Rodbard, J., Gross, H.A., Rodbard, D., Ebert, M.H. and Roth, J. (1979) N. Eng. J. Med. 300:882-889.
3. Kahn, C.R., Goldfine, I.D., Neville, D.M. Jr. and DeMeyts, P. (1978) Endocrinology 103:1054-1066.

4. Bar, R.S., Gorden, P., Roth, J., Kahn, C.R. and DeMeyts, P. (1976) J. Clin. Invest. 58:1123-1135.
5. Harrison, L.C., Martin, F.I.R., and Melick, R.A. (1976) J. Clin. Invest. 58:1435-1441.
6. Olefsky, J.M. (1976) J. Clin. Invest. 58:1450-1460.
7. Kahn, C.R., Flier, J.S., Bar, R.S., Archer, J.A., Gorden, P., Martin, M.M., and Roth, J. (1976) N. Engl. J. Med. 294:739-745.
8. Thomopoulos, P., Roth, J., Lovelace, E. and Pastan, I. (1976) Cell. 8: 417-423.
9. Vigneri, R., Pliam, N.B., Cohen, D.C., Pezzino, V., Wong, K.Y. and Goldfine, I.D. (1978) J. Biol. Chem. 253:8192-8197.
10. DeMeyts, P., Roth, J., Neville, D.M. Jr., Gavin, J.R. III and Lesniak, M.A. (1973) Biochem. Biophys. Res. Commun. 55:154-161.
11. Roth, J., Kahn, C.R., Lesniak, M.A., Gorden, P., DeMeyts, P., Megyesi, K., Neville, D.M. Jr., Gavin, J.R. III, Soll, A.H., Freychet, P., Goldfine, I.D., Bar, R.S. and Archer, J.A. (1976) R.O. Greep, editor. Recent Prog. Horm. Res. 31:95-139.
12. Freychet, P. (1976) Diabetologia 12:83-100.
13. Insel, J.R., Kolterman, O.G., Saekow, M. and Olefsky, J.M. (1980) Diabetes 29:132-139.
14. Wise, W.C. (1974) J. Natl. Cancer Instit. 52:611-612.
15. Wise, W.C.(1976) J. Cell. Physiol. 87:199-212.
16. Hempling, H.G., and Wise, W.C.(1975) J. Cell. Physiol. 85:195-208.
17. Galbraith, R.A., Wise, W.C. and Buse, M.G. (1980) Diabetes 29:571-578.
18. Galbraith, R.A., Spicer, S.S., Wise, W.C. and Buse, M.G. (1980) Abstracts, Endocrine Society 62nd Annual Meeting, p. 158.
19. Schworr, C.M. and Mortimore, G.E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:3169-3172.
20. Seglen, P.O. and Reith, A. (1976) Exp. Cell. Res. 100:276-280.
21. Hughes, C. (1973) in Tissue Culture. P.F. Kruse and M.K. Patterson. eds. Academic Press, New York p. 406.
22. Mallette, L.E., Exton, J.H. and Park, C.R. (1969) J. Biol. Chem. 244: 5713-5723.
23. Gambhir, K.K., Archer, J.A. and Carter, L. (1977) Clin. Chem. 23: 1590-1595.
24. Marchalonis, J.J. (1969) Biochem. J. 113:299-305.
25. Kahn, C.R. (1980) Metabolism 29:455-466.
26. Pariza, M.W., Butcher, F.R., Kletzien, R.F., Becker, J.R. and Potter, V.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:4511-4515.
27. Buse, M.G. and Reid, S.S. (1975) J. Clin. Invest. 56:1250-1261.
28. Buse, M.G. and Weigand, D.A. (1977) Biochim. Biophys. Acta 475:81-89.
29. Fulks, R.M., Li, J.B. and Goldberg, A.L. (1975) J. Biol. Chem. 250: 290-298.
30. Li, J.B. and Jefferson, L.S. (1978) Biochim. Biophys. Acta 544:351-359.
31. Pain, V.M., Lewis, J.A., Huvos, P., Henshaw, E.C. and Clemens, M.J. (1980) J. Biol. Chem. 255:1486-1491.
32. Havener, L.J. and Toback, F.G. (1980) J. Clin. Invest. 65:741-745.
33. Kluge, N., Ostertag, W., Sugiyama, T., Arndt-Jovin, D., Steinheider, G., Furusawa, M. and Dube, S.K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:1237-1240.
34. Zinneman, H.H., Nutall, F.Q. and Goetz, F.C. (1966) Diabetes 15:5-8.