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IONIC EFFECTS ON GLUCOSE TRANSPORT AND METABOLISM BY ISOLATED MOUSE FAT CELLS INCUBATED WITH OR WITHOUT INSULIN

III. EFFECTS OF REPLACEMENT OF Na^+

JACQUES LETARTE AND ALBERT E. RENOLD
Institut de Biochimie Clinique, University of Geneva, Geneva (Switzerland)
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SUMMARY

1. The replacement of Na^+ in the medium leads to alterations in glucose metabolism by isolated mouse fat cells. In the absence of insulin, replacement of Na^+ by other cations leads to increased uptake and metabolism of glucose at all concentrations of glucose tested.

2. In the presence of insulin, replacement of Na^+ strikingly decreases glucose uptake and metabolism, thereby suggesting that insulin-stimulated glucose transport into fat cells is dependent on Na^+. Glucose uptake is not, however, directly proportional to the concentration of Na^+ in the incubation medium.

3. The decreased insulin-stimulated glucose uptake and metabolism resulting from Na^+ replacement in the medium is not due to irreversible structural damage, since insulin-stimulated cells preincubated in Na^+-free medium may regain near-normal ability to transport and metabolize glucose when transferred to medium of normal Na^+ concentration.

4. In the presence of normal Na^+ concentrations, the maximal velocity of glucose uptake and metabolism by insulin-stimulated fat cells is increased, while the apparent overall K_m of glucose uptake is unchanged.

5. It is postulated that insulin activates a carrier which binds and transports Na^+ along with glucose molecules. It is recognized that this concept must remain hypothetical for the time being.

INTRODUCTION

In the preceding two papers[1,2] we have already discussed our reasons for undertaking a detailed analysis of the relationship of insulin action to modifications in the ionic composition of the incubation medium, using isolated mouse fat cells as our test system. In the present report, we shall deal primarily with the effects of Na^+.

In many animal tissues Na^+ must be present for active sugar transport to take place[3,4]. Good evidence has been presented for the requirement for simultaneous

Address reprint requests: Institut de Biochimie Clinique, Sentier de la Roseraie, 1211, Geneva 4, Switzerland.

binding of Na^+ and sugar to a specific carrier before equilibration of sugar across certain plasma membranes may occur[5]. Although the concept was elaborated for active sugar transport across the intestinal mucosa or renal tubular cells[6,7], it is noteworthy that a similar relationship of sugar to ion transport has also been suggested for facilitated carrier-mediated diffusion in tissues such as muscle[8].

Some of the findings to be reported have been published previously in preliminary form[9,10].

MATERIALS AND METHODS

Unless otherwise stated, the animals, reagents, enzymes, insulin, buffer and incubation techniques were as described in the first paper of this series[1] and by Ho AND JEANRENAUD[11]. When Na^+ was to be replaced in the incubated medium, one of three different cations, *i.e.*, K^+, choline$^+$ or Tris$^+$, was used under otherwise identical experimental conditions. Only effects which were quantitatively and qualitatively similar for all three substituting cations were taken to result from the replacement of Na^+ rather than from the characteristics of the replacing cations themselves[12,13]. In all cases, the anionic composition of the medium as well as the gas phase were unchanged.

RESULTS

Complete replacement of Na^+ by three other cations induced marked changes in the metabolic activity of isolated fat cells, as shown in Table I. The changes differed in the absence or in the presence of insulin. In the absence of the hormone, replacement of Na^+ by Tris$^+$, choline$^+$ of K^+ led to increased glucose oxidation to CO_2 as well as to increased incorporation of glucose carbon into total lipids. In analyzing the data shown in Table I, the variation in baseline metabolic activities and in hormone-stimulated metabolic activities noted between different groups should

TABLE I

EFFECTS OF REPLACEMENT OF Na^+ ON D-[$^{14}C_6$]GLUCOSE METABOLISM BY ISOLATED FAT CELLS OF MICE

Glucose concn., 2.5 mM. Insulin concn., 1 munit/ml. Na^+ was totally replaced by K^+, choline$^+$ or Tris$^+$. The anionic composition of the buffer was unchanged. Results are expressed as μatoms glucose carbon incorporated into CO_2 or total lipids per g lipid wt. per 2 h of incubation. Each figure is the mean of 6 values ± S.E.

Medium Na^+ (mM)	*Replaced by*	*Control*		*Insulin*	
		CO_2	*Total lipids*	*CO_2*	*Total lipids*
144		2.43 ± 0.05	3.94 ± 0.01	9.86 ± 0.26	14.5 ± 0.41
0	Tris$^+$	4.04 ± 0.15	6.52 ± 0.06	4.56 ± 0.10	7.62 ± 0.24
144		3.88 ± 0.07	4.27 ± 0.23	16.1 ± 0.12	26.9 ± 0.46
0	Choline$^+$	5.96 ± 0.20	8.56 ± 0.07	6.92 ± 0.14	10.2 ± 0.25
144		1.57 ± 0.04	3.40 ± 0.13	11.8 ± 0.10	18.4 ± 0.40
0	K^+	3.69 ± 0.12	7.12 ± 0.14	8.1 ± 0.20	13.2 ± 0.40

not be taken into account, since, as has been previously discussed[2], the isolated fat cell preparation varies considerably from day to day, despite excellent reproducibility and precision within any one experiment, cell batch, or day.

The metabolic response to replacement of Na^+ by other cations is similar to that seen with the total replacement of K^+ in the absence of insulin[2]. On the other hand, a strikingly different response to the replacement of Na^+, when compared with the replacement of K^+, is seen when the modifications of the cationic environment are carried out in the presence of maximum insulin stimulation. While the absence of K^+ may result in a slight increase in the metabolism of insulin-stimulated adipose cells (an increase of 10 ± 3% over the insulin-stimulated activity in normal buffer, when measured in 10 different experiments performed over a period of 2 years), the replacement of Na^+ by K^+ markedly decreased insulin-stimulated glucose uptake and metabolism, as seen in Table I. The mean decrease observed in 29 experiments carried out over the same 2-year period was 39%, with a standard error of 5%, when the observations made with all three substituting cations were pooled.

The apparent dependence of maximally insulin-stimulated glucose uptake and metabolism on Na^+ was progressive, as shown in Table II for replacement by K^+ and in Table III for replacement by $Tris^+$ or $choline^+$. The major decrease in the insulin-stimulated uptake and metabolism occurred when residual Na^+ concentrations were 75–25 mM. Although this progressive decrease of the insulin-stimulated

TABLE II

EFFECT OF PROGRESSIVE Na^+ REPLACEMENT WITH K^+ ON D-[$^{14}C_6$]-GLUCOSE METABOLISM BY INSULIN-STIMULATED, ISOLATED FAT CELLS OF MICE

Insulin, 1 munit/ml. Expression of results as in Table I. Each figure is the mean of 6 values ± S.E.

Medium		CO_2	*Total lipids*
Na^+ (*mM*)	K^+ (*mM*)		
150	0	9.77 ± 0.11	16.6 ± 0.30
144	6	9.79 ± 0.22	17.4 ± 0.12
100	50	9.47 ± 0.09	16.1 ± 0.40
75	75	9.43 ± 0.22	15.7 ± 0.15
50	100	8.82 ± 0.07	14.9 ± 0.15
0	150	5.28 ± 0.28	12.2 ± 0.65

TABLE III

EFFECT OF PROGRESSIVE Na^+ REPLACEMENT WITH $Tris^+$ OR $choline^+$ ON D-[$^{14}C_6$]GLUCOSE METABOLISM BY INSULIN-STIMULATED, ISOLATED FAT CELLS OF MICE

Insulin, 1 munit/ml. Expression of results as in Table I. Each figure is the mean of 6 values ± S.E.

Medium Na^+ (*mM*)	*Replaced with* $Tris^+$		*Replaced with* $choline^+$	
	CO_2	*Total lipids*	CO_2	*Total lipids*
144	16.9 ± 0.33	25.8 ± 0.46	16.1 ± 0.12	26.9 ± 0.46
50	7.08 ± 0.26	10.2 ± 0.17	9.29 ± 0.19	13.7 ± 0.20
25	6.55 ± 0.22	9.07 ± 0.28	8.17 ± 0.20	12.0 ± 0.07
10	6.14 ± 0.36	8.32 ± 0.35	7.38 ± 0.17	11.2 ± 0.10
0	5.82 ± 0.10	8.49 ± 0.20	6.92 ± 0.14	10.2 ± 0.25

metabolic activity was repeatedly investigated, a stoichiometric type of relationship could not be shown.

The dependence upon Na^+ of glucose transport and metabolism was not only seen in cells maximally stimulated with insulin, but also at lower concentrations of the hormone when only mild or intermediate stimulation of glucose uptake and metabolism was achieved, as shown in Fig. 1. It is noteworthy that even at the concentration of 25 μunits insulin per ml, clearly within the physiological range, glucose metabolism to CO_2, fatty acids and glyceride glycerol was definitely greater in media with high Na^+ (133 mM) than in the low Na^+ media (25 mM), even though the low Na^+ media stimulated glucose metabolism in the total absence of added insulin, and this within the same experiment.

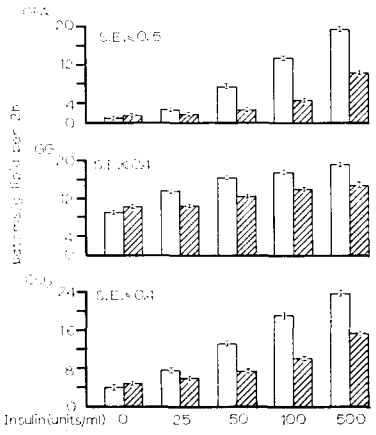

Fig. 1. Effect of replacement of Na^+ on D-[$^{14}C_6$]glucose metabolism by isolated mouse fat cells in the presence of increasing concns. of insulin. Glucose concn., 2.5 mM. The final concn. of Na^+ was 133 mM (open columns) or 25 mM (hatched columns). Na^+ was replaced by choline$^+$. Each figure is the mean of 6 values ± S.E. GFA = glyceride fatty acids; GG = glyceride glycerol.

TABLE IV

EFFECT OF REPLACEMENT OF Na^+ ON D-[$^{14}C_6$]GLUCOSE METABOLISM BY INSULIN-STIMULATED, ISOLATED FAT CELLS OF MICE IN THE PRESENCE OF INCREASING CONCENTRATIONS OF GLUCOSE

Insulin, 1 munit/ml. Expression of results are as in Table I. Na^+ replaced by choline$^+$. Each figure is the mean of 6 values ± S.E.

Medium glucose (mM)	*Medium Na^+, 144 mM*		*Medium Na^+, 50 mM*	
	CO_2	*Total lipids*	*CO_2*	*Total lipids*
1.25	4.67 ± 0.12	8.26 ± 0.18	3.21 ± 0.11	5.18 ± 0.09
1.67	5.98 ± 0.03	9.99 ± 0.19	4.25 ± 0.07	6.51 ± 0.25
2.5	7.18 ± 0.30	11.7 ± 0.30	5.09 ± 0.11	7.80 ± 0.17
5.0	10.4 ± 0.21	15.4 ± 0.32	6.51 ± 0.23	9.60 ± 0.17
10	12.7 ± 0.22	17.6 ± 0.30	7.40 ± 0.21	11.1 ± 0.31

All of the experiments reported so far were carried out in the presence of 2.5 mM glucose in the medium. Clearly, this is not a critical feature of the Na^+ replacement effect in insulin-stimulated fat cells, since, as shown in Table IV, the same depression of insulin-stimulated glucose metabolism was seen at all five glucose concentrations

(1.25–10 mM) tested. Moreover, taking the sum of glucose carbon metabolized to CO_2 and total lipids as an estimate of total glucose uptake, the results yielded two straight lines when plotted according to LINEWEAVER AND BURK[14] (Fig. 2). This suggests that in the presence of insulin, replacement of Na^+ decreases the apparent maximal velocity of glucose uptake while not significantly modifying the apparent overall K_m of the reactions involved. We are well aware of the tentative nature of this type of analysis applied to data in intact cells and based on the assumption that the metabolic products of glucose metabolism are a valid estimate of overall glucose uptake.

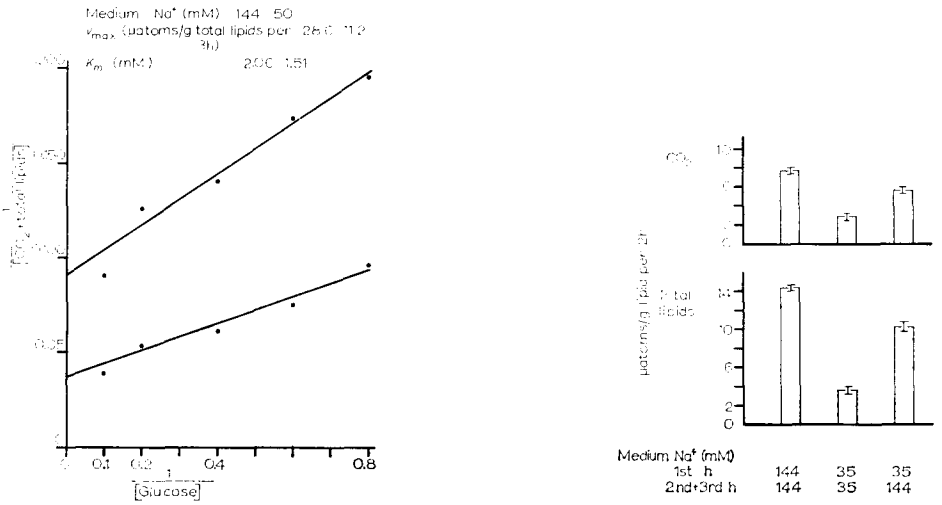

Fig. 2. Effect of replacement of Na^+ on D-[$^{14}C_6$]glucose metabolism by isolated mouse fat cells in the presence of insulin (1 munit/ml) and of increasing concns. of glucose in the medium. Na^+ was replaced by an equimolar amount of $Tris^+$. Incorporation of glucose carbon into CO_2 and total lipids were added, and used as an expression of the overall velocity of glucose uptake. Plot according to LINEWEAVER–BURK. Each point is the mean of 6 values.

Fig. 3. Reversibility of the effect of lack of Na^+ on D-[$^{14}C_6$]glucose metabolism by isolated mouse fat cells. Na^+ was replaced by $choline^+$. Cells were preincubated with buffer of normal or low Na^+ concn. as indicated, then transferred to a new medium with a normal or low concn. of Na^+. Glucose (2.5 mM) and insulin (1 munit/ml) were present throughout. Labeled glucose was added according to LINEWEAVER–BURK. Each point is the mean of 6 values.

The essential reversibility of the depressive effects of Na^+ replacement upon insulin-stimulated glucose uptake and metabolism by fat cells is shown in Fig. 3. For these experiments, cells were washed, suspended and incubated in the presence of insulin, 1 munit/ml, for 1 h in media containing either normal (144 mM) or low (35 mM) concentrations of Na^+. Glucose was present at the usual concentration of 2.5 mM, but contained no radioactive label. After this preincubation period of 1 h, the cells were transferred to fresh media which contained labeled glucose and either 144 or 35 mM Na^+, and were incubated for 2 h. It can be seen that insulin-stimulated glucose uptake and metabolism, initially depressed by Na^+ replacement, was stimulated toward normal when subsequently incubated in a medium of physiological Na^+ content. These observations render quite unlikely the possibility that the depressive effects of lack of Na^+ upon insulin-stimulated glucose uptake and metabolism by fat

cells are the result of nonspecific or structural damage to the plasma membrane or other structural entities of the cells. Furthermore, it should also be recalled that while Na^+ lack decreases glucose uptake and metabolism in the presence of insulin, it increases these functions in the absence of the hormone (Table I).

DISCUSSION AND CONCLUSION

The finding of considerable depression of insulin-stimulated glucose uptake and metabolism as a result of partial replacement of Na^+ in the medium appears to us to represent the principal observations made in this study. The observation is in agreement with the previous one by HAGEN *et al.*[15] in intact adipose tissue, but not with the results reported by RODBELL[16] in isolated fat cells from rats. Again, as in the previous report concerned with the effects of K^+ (ref. 2), we are unable to provide an explanation for the discrepancy between our findings and those of RODBELL, although we have examined a number of possible experimental variables to this end. Certainly, the difference is not simply due to a difference in the species from which the isolated fat cells were obtained. Accordingly, we are forced to assume that the difference is the result of as yet unrecognized variables in the techniques used for the isolation of fat cells. When dealing with a depressive effect, nonspecific damage or trauma must of course be thought of first. However, the clear-cut reversibility of the phenomenon as well as the qualitatively different response to Na^+ replacement in the absence or in the presence of insulin conclusively rule out this explanation, in our view.

Since we have encountered, in the experiments reported here as well as in the two previous papers, a number of relationships between cations and glucose uptake and metabolism, the existence of a possible link between glucose transport and the Na^+ pump, and therefore the (Na^+-K^+)-sensitive ATPase seemed a likely possibility. As recently reported by MODOLELL AND MOORE[17], we have also been able to measure an enzymatic activity in fat cells exhibiting many characteristics of the (Na^+-K^+)-sensitive ATPase and which was, furthermore, very likely membrane bound. The enzyme was clearly inhibited by ouabain, but its activity was not affected by insulin. Furthermore, whereas in the absence of insulin one may generalize that any condition known to interfere with the activity of the Na^+ pump results in the stimulation of glucose uptake and metabolism, as in the absence of K^+ or Na^+ or in the presence of ouabain, this generalization completely fails when it is extended to the insulin-stimulated state. Now, K^+ depletion has little or no effect, ouabain is ineffective, and the only effective cationic manipulation is the replacement of Na^+, which regularly and markedly decreases the insulin-stimulated glucose uptake and metabolism. We are forced to conclude that if the Na^+ pump is involved in glucose transport into fat cells, the mechanism of action must be an indirect one, at least in the presence of insulin.

In the previous paper of this series[2] we have presented our view that the results with ouabain and the manipulation of K^+ suggest that the carrier system for glucose might involve a complex formation of the carrier with glucose and also with Na^+ and/or K^+, the variant with all cationic binding sites occupied by only one monovalent cation being somewhat more active or mobile than that with some binding sites occupied by Na^+ and others by K^+. This view is schematized in the upper part of Fig. 4, which summarizes a tentative explanation for all of the results obtained in our

three studies. The assumption that the carrier exposed to a single species of monovalent cation is more active or effective for glucose transport than the carrier combined with both ionic species also would serve to explain the ouabain effect, since this glycoside is thought to prevent K^+ from binding to specific receptors in other tissues[18] and might do so here. Furthermore, the hypothetical mechanism suggested could lead to increased overall maximal velocity without change in apparent K_m, although it is recognized that the reverse could also be true under certain circumstances.

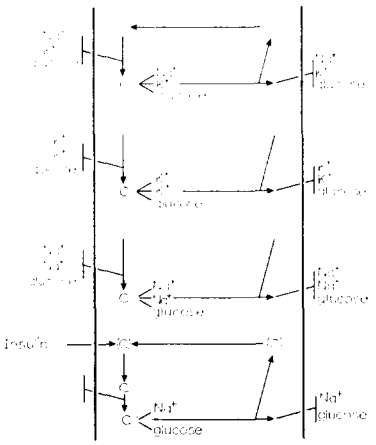

Fig. 4. Hypothetical carrier (C) model for the transport of glucose into isolated mouse fat cells. (C), hypothetical active carrier; (C) = hypothetical inactive carrier.

The tentative explanation proposed so far breaks down completely, however, in the presence of insulin. As postulated before by others[19–21] and as also suggested by our kinetic studies which demonstrated principally an increased maximal velocity of glucose transport into isolated fat cells in the presence of insulin, we are led to assume that insulin is likely to activate the otherwise inactive glucose carrier molecules, as shown in the lower part of Fig. 4. The carrier would have to be a "new" carrier, in the sense that it might be either a carrier similar to the previously discussed one (in the upper part of Fig. 4) but modified by insulin so as to provide more binding sites for glucose or to move faster through the membrane, or it might be a new carrier altogether, usually present in an inactive form and activated in the presence of insulin. The data obtained with isolated fat cells do not point to a change in the apparent affinity of the carrier for glucose in the presence of insulin, whereas such a change was previously shown in intact adipose tissue[22,23]. A most important feature of the new carrier (lower portion of Fig. 4) would be that of an obligatory requirement for Na^+ as well as for insulin. On the other hand, the insulin-stimulated carrier might well be active in the presence of Na^+, even without glucose, resulting in continued transport of Na^+ into the cell: this may serve to explain that the effect of insulin upon membrane resting electrical potential is the same, whether glucose is present in the incubation medium or not[24,25]. It is our present opinion that the tentative scheme shown in Fig. 4 suffices to explain all of the information accumulated to date. However, this in no way diminishes its tentative character. In particular, we continue to be fully aware of the principal difficulty in all of our studies, as already indicated

in the first paper of this series[1], that of the necessity to utilize an indirect measurement of glucose uptake rather than a direct one. Unfortunately, the isolated fat cell technique is not suitable for the precise measurement of either glucose uptake or intracellular concentrations of nonaccumulated substances[20].

The evidence pointing to the cell membrane as the primary site of insulin action has been recently and repeatedly reviewed[27–29], and it has been pointed out that a modification and extension of the original sugar-transport hypothesis of LEVINE AND GOLDSTEIN[30] has become mandatory. RODBELL[31] has recently proposed a unitary hypothesis for the mode of action of insulin upon the fat cell membrane, postulating as the primary event a structural change of the cell membrane, which would lead to altered affinity for different ions and to their altered relative distribution within the matrix of the cell membrane. This, in turn, might well affect all transport processes within the membrane, including carrier-mediated amino acid, glucose, and electrolyte transport, as well as the activity of adenyl cyclase. The tentative explanation which we have given in Fig. 4 could easily fit into the more general hypothesis proposed by RODBELL, and we rather favor this hypothesis. However, both our tentative explanation and the hypothesis of RODBELL are, as of the present, exceedingly difficult to verify experimentally, and much further work and improvement of available techniques will be required for the validation of either.

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