A High-Fat Diet Influences Insulin-Stimulated Posttransport Muscle Glucose Metabolism in Rats

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Because of a failure to detect significant quantities of intracellular glucose, it has been generally accepted that transport rather than phosphorylation is the rate-limiting process of muscle glucose metabolism under most (but not all) physiological conditions. Here, we have measured tissue free levels of the glucose analog 2-deoxy-D-glucose (2DG) in red quadriceps muscle of rats fed a high-fat diet (59% of energy from fat) for 3 weeks, to identify the barrier to insulin-stimulated glucose uptake previously seen in such animals. Measurements were performed on pentobarbital-anesthetized rats following exogenous infusion of radiolabeled 2DG. A glucose clamp was used to maintain plasma insulin at high physiological levels (~120 mU/L). Three other treatment groups representing normal insulin action (chow-fed), extreme glucose uptake (maximal insulin stimulation + hyperglycemia), and insulin resistance with elevated free intracellular glucose (epinephrine infusion) were also studied for comparison. In chow-fed animals, no muscle free 2DG was detected, confirming transport as the rate-limiting process. In fat-fed animals, a significant elevation in muscle free 2DG was observed ($P < .01$ *v* chow-fed controls). The elevation was similar in magnitude to that in epinephrine-infused rats, and implied a limitation of insulin action at a posttransport step. This result was confirmed with a more complex modeling analysis. We conclude that posttransport steps influence insulin-stimulated in vivo muscle glucose metabolism in long-term high-fat–fed rats.

MUSCLE PLAYS A PIVOTAL ROLE in glucose homeostasis, since this tissue is responsible for the bulk of whole-body insulin-mediated glucose disposal.[1,2] Identification of the mechanisms that control muscle glucose metabolism is therefore central to an understanding of insulin-resistant states such as obesity and non–insulin-dependent diabetes mellitus.

Although there is no doubt of the importance of glucose transport in the regulation of muscle glucose metabolism,[3,4] there is now considerable evidence that posttransport events also play a significant role.[5]

Because of low glucose-6-phosphatase activity in muscle,[6] intracellular phosphorylation is functionally an irreversible process under normal circumstances. Thus, the overall rate of glucose utilization can only be modulated directly by the hexokinase reaction or preceding metabolic steps. Nevertheless, since hexokinase is subject to product inhibition,[7] downstream metabolic events could also indirectly inhibit the phosphorylation process. Consequently, there is continued interest in determining the relative importance of the transport and phosphorylation processes in overall muscle glucose uptake over a range of physiological conditions.[8-11]

The primary purpose of this study was to identify the rate-limiting step (transport *v* phosphorylation) in a model of diet-induced insulin resistance: the long-term high-fat–fed rat. It is possible that modifications to the glucose transport system[12] or posttransport metabolism such as the glucose–fatty acid cycle[13] contribute to the insulin resistance of this model.

We used a variation of a classic technique[14] in which an accumulation of free intracellular glucose was used to detect a shift (from transport toward phosphorylation) in the rate-limiting step. Here, tissue content of an exogenously infused radiolabeled glucose analog, 2-deoxy-D-glucose (2DG), was measured rather than the authentic substrate. Under normal circumstances, 2DG undergoes negligible metabolism following phosphorylation, and is therefore trapped in the tissues following uptake. Consequently, it is commonly used to study glucose metabolism.[15,16]

As well as interpreting muscle free 2DG levels directly, additional metabolic information was derived from the experimental data using a variation of our published modeling analysis.[17] Because modifications to this established technique were involved, we have restudied, as controls, those treatment groups in which elevated muscle free 2DG levels were previously reported.

To facilitate the rapid tissue sampling necessary to preserve the labile free hexose, only anesthetized rats were studied. Previously, we found that in anesthetized rats white muscle yields relatively noisy results, and in the present context it is metabolically inactive.[17] Here, we concentrated solely on red skeletal muscle.

MATERIALS AND METHODS

Overview

All studies were approved by the Animal Experimentation Ethics Committee of the Garvan Institute/St Vincent's Hospital, following guidelines of the National Health and Medical Research Council of Australia.

All rats were studied during a hyperinsulinemic glucose clamp.[18] In the final stage of experiments, infusions of radiolabeled 2DG were used to create approximately constant plasma concentrations of tracer, at which time muscle samples were excised for assay of tracer content. Under ideal conditions, the tissue to plasma ratio of 2DG activity is a direct index of the relative effect of transport and phosphorylation processes. However, for short infusions, nonequilibrium may be a confounding factor, and for excessively long experiments, dephosphorylation processes could hinder a simple interpretation of results. Because of the latter consideration, 2DG infusion time was restricted to 20 minutes in our experiments. This short tracer exposure time is less than half that traditionally used by our group[16] and others.[15] To compensate for nonequilibrium effects, two 2DG tracers (with different radiolabels and different infusion regimens) were infused simultaneously. These primed and unprimed infusions were designed to produce plasma

Submitted January 16, 1997; accepted February 13, 1997.

Supported by the National Health and Medical Research Council (Australia).

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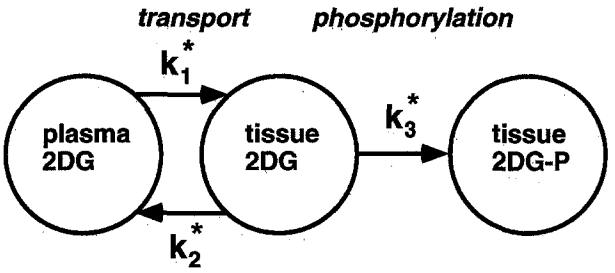

Fig 1. A model of tissue 2DG metabolism. Hexose is transported from the plasma into the intracellular space, where it is phosphorylated. The rate constants k_1^* and k_2^* refer to cellular influx and efflux, respectively; k_3^* describes the phosphorylation of intracellular 2DG.

profiles that approached a plateau in a balanced fashion from above and below, respectively. Any nonequilibrium errors would therefore tend to cancel in mean data.

The experimental data were also analyzed in terms of the simple kinetic model of 2DG metabolism shown in Fig 1. As well as yielding additional metabolic information, the analysis allowed for a more rigorous assessment of nonequilibrium effects. Although the individual rate constants completely describe the system of Fig 1, two groups of parameters conveniently summarize modeling results[17]: (1) The rate constant, $K^* = k_1^* k_3^* / (k_2^* + k_3^*)$ characterizes the overall steady-state conversion of plasma 2DG to intracellular 2DG-6-P; and (2) The ratio, $f_1^* = K^*/k_1^* = k_3^*/(k_2^* + k_3^*)$, quantitatively describes the relative influence that transport and phosphorylation processes exert on the overall rate of hexose utilization. It has a theoretical range of values from zero (phosphorylation rate-limiting) to one (transport rate-limiting). It was previously introduced[17] to analyze muscle results, which can include cases in which transport is completely rate-limiting. (Under these circumstances, it is mathematically cumbersome to express results as a set of individual rate constants, since k_3^* is effectively infinite.)

Animal Preparation

Adult male Wistar rats were maintained from birth in a temperature-controlled (22° ± 1°C) environment with a 12-hour light:dark cycle (lights on at 6 AM) with free access to rodent chow and water. At a body weight of about 250 g, rats were transferred to individual cages for a period of 3 weeks, where most continued to be fed laboratory chow ad libitum. However, one group was fed fixed rations of a high-fat diet, with total energy content (350 kJ/d) matching the mean intake of the chow-fed rats. The caloric composition of the chow diet was 16% fat, 26% protein, and 58% carbohydrate. The corresponding composition for the high-fat diet was 59% fat, 21% protein, and 20% carbohydrate.[19]

One week before study, the jugular artery and carotid vein of each rat were cannulated under ketamine hydrochloride (90 mg/kg)/xylazine (10 mg/kg) anesthesia. After recovery, rats were returned to the cages, and the previous feeding regimen was resumed. At the time of study, rats weighed approximately 350 g (range, 320 to 380).

Short-Term Study

A hyperinsulinemic glucose clamp procedure[18] was used in all studies. All rats were studied under pentobarbital anesthesia after a 5-hour fast. Anesthesia was induced by intravenous (IV) injection of sodium pentobarbitone (Nembutal 40 mg/kg; Abbott Laboratories, Sydney, Australia) and maintained throughout the study period by IV infusion (30 mg · kg^{-1} · h^{-1}). Throughout the experiment, body temperature was monitored by a rectal probe and maintained in the range of 37° to 38°C by an electric heating pad.

Immediately after induction of anesthesia, a fixed-rate insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) IV infusion (0.25 or 2.0 U · kg^{-1} · h^{-1}) was initiated. A variable-rate glucose infusion adjusted on the basis of frequent blood samples was used thereafter to maintain plasma glucose close to a target value (7 or 15 mmol). In one group of animals, epinephrine (David Bull Laboratories, Victoria, Australia) was also infused (9 μg/h).

After approximately 75 minutes, a metabolic steady state was achieved, and tracer administration consisting of simultaneous infusions (each ~1.5×10^6 dpm/min) of 2-deoxy-D-[2,6-^{3}H]glucose (Amersham, Amersham, UK) and 2-deoxy-D-[^{14}C(U)]glucose (NEN, Boston, MA) were commenced. The tritiated infusion was primed by a bolus equivalent to 25 minutes of infusion.

Tracer infusions were maintained for 20 minutes when rats were killed by Nembutal overdose (60 mg). Immediately before death, the quadriceps muscles were exposed and freeze-clamped in situ using aluminum tongs precooled in liquid nitrogen. A sample of red quadriceps muscle was excised without thawing. It was stored at −70°C until analysis.

All infusions were administered through the venous line; blood samples were taken from the arterial line. Blood (200 μL) was sampled at approximately 10-minute intervals during the stabilization period and at 1, 2, 5, 10, and 15 minutes following initiation of tracer infusion. A final (400 μL) sample was taken at 20 minutes. Plasma was separated by centrifugation, analyzed for glucose (YSI 23 AM; YSI, Yellow Springs, OH), and then stored at −20°C for later analysis. Red blood cells from pretracer samples were suspended in saline and returned to the experimental animal.

Sample Analysis

Muscle samples were initially powdered in a ball mill (Dismembrator; Braun, Melsungen, Germany) precooled in liquid nitrogen; thereafter, plasma and muscle samples were processed in an identical fashion. Samples were deproteinized in 4% (wt/vol) perchloric acid on ice and centrifuged. Supernatants were neutralized with potassium carbonate and freeze-dried to remove excess water.

Anion exchange (AG 1-X8; Bio-Rad, Hercules, CA) was used to separate reconstituted samples by charge. Nonionized and ionized species were associated with free and phosphorylated 2DG, respectively, after correcting for the proportion of the nonionized fraction that could not be phosphorylated by yeast hexokinase. (Samples were incubated for 1 hour at 37°C in buffer containing excess hexokinase, glucose-6-phosphate dehydrogenase, and Tris 100 mmol/L, $MgCl_2$ 100 mmol/L, ATP 40 mmol/L, and NADP 25 mmol/L). Concentrations of ^{3}H and ^{14}C species were determined by liquid scintillation spectrometry (scintillant: Picoflour 40; Packard, Downers Grove, IL; counter: Beckman LS800; Beckman, Irvine, CA).

Insulin content of the final plasma sample was determined by radioimmunoassay.[20] Epinephrine levels were not measured, but the infusion rate used (9 μg · h^{-1}) has been previously shown[21] to elevate plasma epinephrine to high physiological levels (~5 nmol · L^{-1}).

Treatment Groups

Four groups of rats were studied. The minimum group size was six (Table 1).

Table 1. Treatment Groups

Group	No.	Diet	Insulin Infusion Rate (U · kg^{-1} · h^{-1})	Target Plasma Glucose (mmol/L)	Epinephrine Infusion Rate (μg/h)
Control	9	Chow	0.25	7	0
Maximal	7	Chow	2.00	15	0
Epinephrine	6	Chow	0.25	7	9
Fat-fed	6	High-fat	0.25	7	0

Mathematical Analysis

A direct estimate of the equilibrium distribution volume of free 2DG was obtained from the tissue to plasma concentration ratio averaged across both tracers. Tissue levels were corrected for extracellular contamination using a previously measured[17] value of 8.3% for the extracellular space.

The assumptions and rationale of the modeling analysis have been described previously.[17] Here, two nonlinear regression analyses were sequentially performed to analyze data from each rat. Firstly, plasma tracer concentrations were fitted to an assumed profile. For each infusion regimen, the form of the fitted equations corresponded to the double-exponential decay observed following bolus tracer administration.[22] Plasma time courses for both tracers were fitted simultaneously using the same set of four parameters. Next, the rate constants k_1^*, k_2^*, and k_3^* were estimated by fitting the solutions[17] of the system equations describing the model of Fig 1 to measured tissue free and phosphorylated 2DG concentrations. Here, the fitted plasma curves were used as the driving functions. During the regression, the ratio k_2^*/k_1^* was constrained to a previously determined value of 3.4.[17]

For both regression analyses, parameters were calculated using a least-squares iterative procedure, incorporating a Marquardt-Levenberg optimization scheme.[23]

This regression analysis yields biased results if estimates of tissue free 2DG (after correction for extracellular contamination) are negative. Under these circumstances only, model parameters were estimated instead from the limiting behavior ($k_3^* \rightarrow \infty$) of the system equations.[17] This procedure is statistically conservative, it produced less significant differences in f_1^* between treatment and control groups.

In these calculations, previously measured[17] values for the ratio k_2^*/k_1^* and the extracellular space were assumed. They were based on the tissue distribution volume of exogenously administered 3-*O*-methylglucose and L-glucose, respectively. Additional studies (data not shown) have confirmed that fat feeding does not affect either of these parameters.

Statistical Analysis

Results are presented as the mean ± SEM. Data were analyzed by ANOVA using commercially available software (superANOVA; Abacus Concepts, Berkeley, CA). For each experimental variable, only the planned contrasts between treatment groups and the control group were considered. A Dunnett correction for multiple comparisons was applied. P less than .05 was regarded as statistically significant.

RESULTS

Table 2 summarizes the experimental conditions under which measurements were made. Plasma insulin, plasma glucose, and glucose infusion rate for the maximal group (Table 1) were significantly different from control values. The corresponding parameters for the other treatment groups were statistically indistinguishable from the control values.

Table 2. Clamp Parameters

Treatment	Plasma Insulin (mU/L)	Plasma Glucose (mmol/L)	GIR (mg · min^{-1} · kg^{-1})
Control	110 ± 13	6.8 ± 0.1	9.3 ± 0.8
Maximal	2,227 ± 114*	14.7 ± 0.7*	49.1 ± 3.4*
Epinephrine	120 ± 7	6.4 ± 0.4	4.4 ± 2.8
Fat-fed	123 ± 20	6.9 ± 0.1	4.0 ± 1.1

NOTE. Plasma insulin was assayed from the final sample. Plasma glucose was averaged over the 20-minute tracer infusion period. GIR is from the final 45 minutes of the clamp.

Abbreviation: GIR, glucose infusion rate.

*Significantly different from control ($P < .001$).

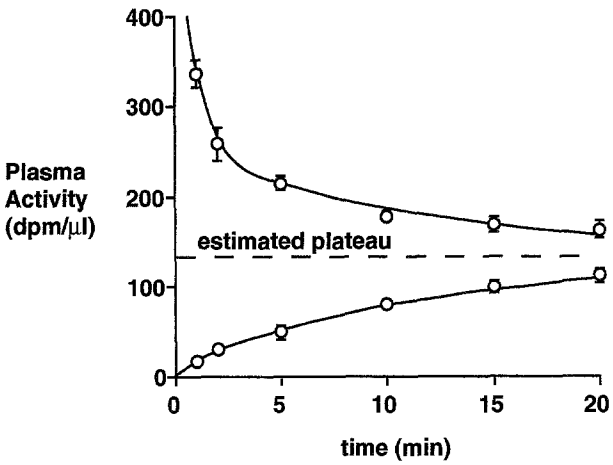

Fig 2. Plasma response curves to a primed and unprimed infusion of 2DG. Data are the mean values for the control group. The curves and plateau shown were derived from a regression analysis of mean data. Data were normalized to an infusion rate of 10^6 dpm/min and a body weight of 350 g.

Plasma response curves to simultaneous primed and unprimed infusions of radiolabeled 2DG for the control group are shown in Fig 2. Similar profiles were seen for the three treatment groups. These results confirm that a primary experimental design criterion, a balanced approach to steady state from both above and below, was achieved.

Distribution volumes of free 2DG in red quadriceps muscle are shown in Fig 3. Values shown are the mean values of the tissue to plasma concentration ratio for both 2DG species. All three treatment ratios differ from the control ($P < .01$). The value for the control group is statistically indistinguishable from zero (t test, $P = .2$). Because of the balanced experimental design, the mean distribution ratio would be expected to closely approximate the equilibrium value. Ratios calculated using data from the individual 2DG tracers were similar to those of Fig 3 but, as would be expected, less precise. Nevertheless, all contrasts of ratios between treatment and control groups for individual 2DG tracers were still significant ($P < .05$).

Values for f_1^*, our rate-limiting step indicator,[17] are included in Table 3. Movements of this model-derived parameter are

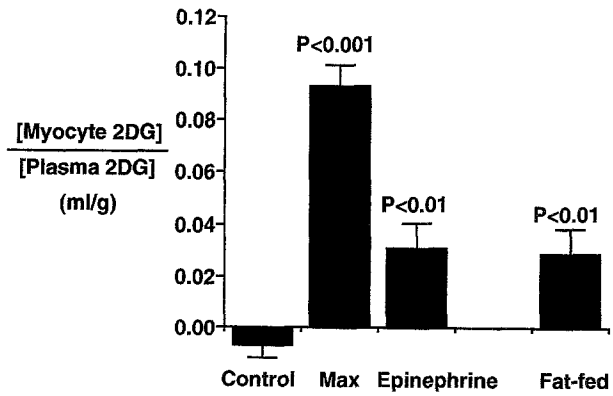

Fig 3. Ratio of intracellular free 2DG content in red quadriceps muscle (per g) to plasma (per mL). Values are the mean of the tissue to plasma ratio for the 2 tracers.

Table 3. Model-Derived Indices of Hexose Utilization in Red Quadriceps Muscle

Treatment	f_t^*	K* (ml · min^{-1} · g^{-1})	R_g (μmol · 100 g^{-1} · min^{-1})
Control	1.02 ± 0.02	0.0120 ± 0.0028	8.0 ± 1.8
Maximal	0.65 ± 0.03†	0.0268 ± 0.0038†	49.6 ± 6.3†
Epinephrine	0.88 ± 0.04*	0.0087 ± 0.0013	6.0 ± 1.0
Fat-fed	0.90 ± 0.04*	0.0138 ± 0.0019	10.3 ± 1.5

NOTE. f_t^* is an index of the influence of transport on overall 2DG uptake. A value of 1 occurs when transport is completely rate-limiting. Smaller values imply that phosphorylation impedes uptake to some extent. K* is the capacity of each gram of tissue to clear 2DG from the plasma. R_g estimates steady-state tissue glucose utilization; it includes a correction for the different affinities of hexokinase for 2DG and glucose.[17]

*$P < .02$, †$P < .001$: significantly different from control.

consistent with the distribution ratio data shown in Fig 3. All three treatment groups exhibited a decrease ($P < .02$) in f_t^* compared with the control, consistent with transport having slightly less of an influence on overall 2DG uptake under these circumstances. Also shown in Table 3 are indices of glucose utilization in red quadriceps. Maximal insulin stimulation/hyperglycemia was the only treatment considered here that could be shown statistically to modify net glucose uptake ($P < .001$). Whether expressed as clearance of 2DG (K*) or absolute glucose uptake (R_g), no difference was detected between either the epinephrine group or fat-fed group and control.

Values for k_1^*, the rate constant describing transport influx, closely followed those for K* (data not shown). Of the three treatment groups, only the maximal group differed significantly from the control ($P < .001$). Therefore, for this group, the decrease of f_t^* and the increased 2DG distribution ratio (compared with control) can be attributed to an overload of the phosphorylation process due to substantially increased influx of hexose. For the epinephrine and fat-fed groups, the change in these parameters is likely to be a direct consequence of the reduced capacity of the muscle to phosphorylate hexose.

DISCUSSION

In this study, we have demonstrated that a high-fat diet generates a significant posttransport barrier to glucose metabolism in red skeletal muscle. This conclusion is based on an observed elevation of tissue free 2DG resulting from a change in the relative velocity of transport and phosphorylation processes. The possibility that the elevation was solely due to a departure from steady state was excluded by a kinetic modeling study. The size of the change in free 2DG levels induced by the high-fat diet was similar to that observed when blood epinephrine was elevated to the high physiological range. Changes were relatively small, but were clearly statistically significant. Irrespective of which index was used (tissue to plasma distribution ratio or model-derived f_t^*), fat-fed animals were significantly different from controls ($P < .02$) even after correction for multiple comparisons.

Free glucose (or free 2DG) is difficult to measure in vivo. Anoxia is a potent promoter of hexose phosphorylation, with most free glucose depleted via this route within seconds of disruption of the blood supply.[24] Consequently, we (like most other investigators in this area) have of necessity studied anesthetized animals to facilitate rapid tissue sampling. Although this strategy has practical advantages, it is not without drawbacks. We have previously shown that anesthesia suppresses insulin-mediated glucose uptake into skeletal muscle[25] and disturbs liver glucose metabolism.[26] We were therefore aware that an interaction between anesthetic and other treatment effects might occur. In previous studies with conscious rats, fat feeding was observed to decrease insulin-stimulated glucose uptake in red skeletal muscle by about 40%.[27] However, no such effect was evident in the pentobarbital-anesthetized rats studied here. It is therefore possible that there are some similar (but nonadditive) effects of fat feeding and pentobarbitone. However, it should also be borne in mind that at physiological insulin levels muscle glucose uptake is low in anesthetized rats, and is more difficult to quantify.

The various indices of glucose metabolism presented here have different sensitivities to changes in the rate of phosphorylation. The phosphorylation process has only a small effect on the overall rate of hexose uptake under conditions, such as those studied here, where transport is (close to) rate-limiting. In contrast, a strong (inverse) relationship exists between the 2DG tissue to plasma activity ratio and the rate of phosphorylation under the same conditions. It is therefore not surprising that changes in the 2DG distribution ratio (and the closely related parameter f_t^*) did not translate into experimentally detectable changes in 2DG uptake. However, it is reasonable to expect that an impaired phosphorylation process would have a larger effect in the conscious state when glucose flux into muscle is not depressed by anesthesia.

Previously, several mechanisms have been postulated for diet-induced insulin resistance. These include modification to the glucose transport system through indirect effects, such as a possible impairment of function caused by an alteration in membrane fluidity.[28] Direct effects on transporter function have also been proposed,[12] but recent studies have produced conflicting results.[29-31] Other explanations based on an inhibition of posttransport glucose disposal by metabolites of lipid fuels (eg, the glucose–fatty acid cycle[13]) have also been suggested. Although acute systemic elevation of circulating fatty acid levels has previously been shown to inhibit phosphorylation in heart,[32] it has not been established whether increased dietary fat intake would lead to a similar effect in skeletal muscle. The present study would strongly support such a proposition.

Our results are consistent with the known involvement of hexokinase in the response of muscle metabolism to insulin. Recently, it has been demonstrated that the expression[33] and activity[34] of hexokinase II (the dominant isoform found in skeletal muscle) are regulated by insulin. Moreover, studies with transgenic mice[35] suggest that these changes contribute functionally to the insulin-induced increase of muscle glucose uptake. Thus, the barrier to posttransport glucose uptake might be expected to contribute to at least some classes of insulin resistance. Recent modeling analyses of tracer data from forearm perfusion[11] and positron-emission tomographic[10] studies suggest that this is the case for the insulin resistance

associated with non–insulin-dependent diabetes mellitus. However, confirmation of these results using direct methods of the type described here would be experimentally difficult.

Anesthetic agents are known to nonspecifically perturb cell membrane function[36] and thus alter the behavior of proteins, such as glucose transporters, embedded in the lipid matrix. It is therefore possible that the barrier to muscle glucose metabolism at the transport step is exaggerated in the anesthetized state. In general, this would make an impairment of the phosphorylation process more difficult to detect, while any modulation of the transport process could easily be masked by the anesthesia. We therefore cannot preclude the possibility that effects of fat feeding also include modifications to the glucose transport process.

In conclusion, we have demonstrated a significant posttransport limitation to in vivo insulin-stimulated muscle glucose uptake in chronically high-fat–fed rats. In a broader context, we regard the generally held view that glucose uptake is determined solely by transport in most common physiological states as not firmly established.

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