Apparent thermogenic effect of injected glucagon is not due to a direct effect on brown fat cells

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Dicker, Andrea, J In Zhao, Barbara Cannon, and Jan Nedergaard. Apparent thermogenic effect of injected glucagon is not due to a direct effect on brown fat cells. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1674—R1682. 1998.—To examine the significance of brown adipose tissue for the thermogenic response to glucagon, we injected glucagon intraperitoneally into rats (that have glucagon-sensitive brown fat cells) and into hamsters (that have glucagon-insensitive brown fat cells). Although a thermogenic response to glucagon injection was apparently observed in rats, this response was not augmented by cold acclimation and was not dose dependent. Similar observations were made in hamsters. The thermogenic response could be fully blocked by prior injection of the β-adrenergic blocker propranolol. Thus no direct thermogenic response to injected glucagon could be demonstrated, and the thermogenic response observed was fully due to vehicle injection. However, glucagon injection was able to unmask mitochondrial [3H]GDP binding. As expected, isolated brown fat cells from rats and mice responded thermogenically to glucagon but brown fat cells from hamsters were unresponsive. The EC50 of the rat brown fat cells was high (5 nM); these cells also responded thermogenically to glucagon. However, glucagon injection could be observed; this may be related to differences in glucagon preparations. Brown fat cells from certain species are, however, glucagon sensitive. It is uncertain whether glucagon is the endogenous agonist for these receptors, but the presence of the glucagon-responsive receptor indicates alternative means to norepinephrine for stimulation of brown adipose tissue thermogenesis and, probably, of recruitment.

nonshivering thermogenesis; cold acclimation; guanosine diphosphate binding capacity; propranolol; secretin

INJECTION OF GLUCAGON PREPARATIONS into intact animals was demonstrated some 40 years ago to lead to a thermogenic response (10, 11). Several subsequent studies (see below) have confirmed this effect, and the existence of a “glucagon thermogenesis” has become generally accepted (25). However, the physiological relevance and the anatomic localization of the induced thermogenesis still remain equivocal.

In mammals, brown adipose tissue has been proposed to be the site of the thermogenic action of injected glucagon preparations (20, 28). This concept is based on observations of an increase in the temperature of the interscapular brown adipose tissue pad (9), an increased blood flow to the tissue (42) during infusion of glucagon preparations, and an increased thermogenic response to injection of glucagon preparations in cold-acclimated animals (14, 15) as well as on the ability of brown adipose tissue cells or tissue fragments to respond thermogenically to glucagon preparations in vitro (6, 22, 23, 26, 30, 32).

However, confoundingly, a thermogenic effect of injected glucagon preparations has been reported in different mammalian species, even at ages at which brown adipose tissue is not expected to be prominent (e.g., in adult dogs (41) and adult humans (7)) and even in species not normally accepted as possessing brown adipose tissue, such as pigs (24). Thus the participation of brown adipose tissue in the observed thermogenic response is not firmly established.

To elucidate the degree to which brown adipose tissue is responsible for the thermogenic response to glucagon injections, we decided to use a previous observed (36) species difference, namely, that isolated brown fat cells from rats respond thermogenically to glucagon (23, 30, 32), whereas cells from hamsters do not (16). Thus, if brown adipose tissue is mainly or solely responsible for the thermogenic response to injected glucagon in the intact animal, a marked difference in the response should be observable between these two species.

However, unexpectedly, our investigations implied that present-day glucagon preparations are not thermogenic when injected into intact rats or hamsters.

METHODS

Animals and In Vivo Experiments

For the studies of thermogenesis in intact animals, female rats of the Sprague-Dawley strain with an initial weight of 150 g were obtained from a local supplier (Eklunds, Stockholm, Sweden). Certain experiments, as stated, were performed with male Wistar rats (B & K, Stockholm, Sweden). The rats were divided into two groups: warm acclimated (28°C) and cold acclimated (5°C). The acclimation periods were 4–5 wk.

The rats were kept in single cages, with a 6:18-h light-dark photoperiod. All animals had free access to food (rat and mouse standard diet, B & K) and tap water.

Hamsters were from the local colony of Stockholm University. The animals used were >10 wk of age and of either sex. They were caged singly and maintained under the same photoperiod as the rats. They were acclimated to either 24°C (warm acclimated) or 5°C (cold acclimated) for 4–5 wk. They had free access to food (sunflower seed-based diet, with rabbit pellets, dried carrots, and oats) and tap water.

The oxygen consumption of the rats and hamsters was measured in unanesthetized animals in an open-circuit system as described in detail previously (13). One warm-acclimated and one cold-acclimated rat were studied on each day in alternating sequence. For the measurements, the rat or the hamster was placed in a Plexiglas metabolic chamber with a volume of 5 liters. This chamber was placed inside a temperature-controlled incubator. The temperature was maintained at 27–29°C. Air was drawn through the system, and
the outgoing dried air was analyzed with a paramagnetic oxygen analyzer. The oxygen concentration of the outgoing air was recorded by a pen recorder and is presented here as the decrease in percentage units from that of the ingoing air (20.94%). For comparisons between animals, the metabolic rate was expressed in ml O$_2$·min$^{-1}$·kg$^{0.75}$·1.$^{-1}$

The resting metabolic rate was defined as the lowest rate of oxygen consumption observed for a period of at least 10 min during the habituation period of 2 h in the chamber before the injections. The animals were injected intraperitoneally with glucagon (1 mg/kg body wt if not otherwise stated; see Peptide Hormones) and/or norepinephrine [1 mg (−)-arterenol bitartrate (Sigma, St. Louis, MO)/ml 0.9% NaCl; 1 mg/kg body wt], and an increase in metabolic rate followed, as detailed in the legends of Figs. 1–5.

Mitochondrial [3H]GDP Binding Capacity

For estimation of the [3H]GDP binding capacity of brown adipose tissue mitochondria, rats were acclimated to 5°C as described in Animals and In Vivo Experiments. After the acclimation period, four cold-acclimated rats were transferred on each occasion to 28°C overnight (15 h). The following morning, glucagon, norepinephrine, saline, or nothing was injected; the rats remained at 28°C. Thirty minutes after the injections, the rats were CO$_2$ anesthetized and killed by decapitation. The brown adipose tissue was dissected out and homogenized in 20 ml of 250 mM sucrose. Brown fat mitochondria were prepared as described previously (34). Briefly, the homogenate was centrifuged at 8,500 g for 10 min, the pellet was resuspended in 250 mM sucrose and centrifuged at 800 g for 10 min, and the resulting supernatant was centrifuged at 8,500 g. The resulting pellet was resuspended in 250 mM sucrose containing 2% fatty acid-free BSA. After centrifugation at 8,500 g for 10 min, the pellet was washed with 250 mM sucrose and recentrifuged at 8,500 g for 10 min. The resulting pellet was resuspended in a small volume of 250 mM sucrose and stored on ice. Protein concentration was determined by the fluorescamine technique with Fluram (Roche).

The [3H]GDP binding capacity of the brown fat mitochondria was estimated as described previously (34). Briefly, the mitochondria were incubated at room temperature for 10 min at a concentration of 0.5 mg mitochondrial protein in 0.5 ml medium consisting of 100 mM [14C]sucrose, 10 µM [3H]GDP, 20 mM TES (pH 7.1), 1 mM EDTA, and 5 µM rotenone. The [14C]sucrose was used as a marker for the extramitochondrial volume. A 0.4-ml aliquot of the incubation mixture was filtered under vacuum through a Sartorius cellulose nitrate filter with a pore size of 0.45 µm, and the filters were dried and finally dissolved in scintillation medium before the radioactivity was counted. Specific [3H]GDP binding was that observed in excess of the [3H]GDP amount expected from the [14C]sucrose marker.

Brown Fat Cells and In Vitro Thermogenesis

Brown fat cells were prepared from rats, mice, and hamsters living under normal animal house conditions at 20–22°C, principally by the collagenase method described earlier, with minor modifications (45).

The rate of oxygen consumption of the isolated brown fat cells was followed in an oxygen electrode chamber, principally as described earlier (45). The cells were suspended in a Krebs-Ringer bicarbonate buffer with added 10 mM glucose, 10 mM fructose, and 2% (or 4%) fatty acid-free BSA, as detailed elsewhere (45).

Peptide Hormones

If not otherwise stated, the glucagon used was the product Glucagon (Novo Nordisk, Bagsvaerd, Denmark). This is a substance for the clinic, prepared for injection with two ampules to be combined, one containing glucagon HCl corresponding to 1 mg (11U) glucagon plus 107 mg lactose, and the other containing 1.3 ml purified water (referred to as aqua); the glucagon found in this product in the present experiments was prepared from pig pancreas. Where indicated, glucagon from Sigma, prepared from pig pancreas, or synthetic glucagon (also from Sigma), was used.

All other peptides were from Sigma, including vasoactive intestinal polypeptide (VIP; synthetic, porcine sequence), gastric inhibitory polypeptide (GIP; synthetic, human sequence), glucagon-like peptide-1 (GLP-1; human), secretin (synthetic, human sequence), growth hormone releasing factor (GHRF; synthetic, bovine sequence or rat sequence, as indicated). They were all dissolved in water.

RESULTS

Apparent Thermogenic Effects of Glucagon Injection Into Intact Animals

Apparent thermogenesis in intact rats. Isolated brown fat cells from rats have earlier been demonstrated to respond thermogenically to glucagon (6, 23, 30). We therefore first investigated the thermogenic response to glucagon injections in intact rats. As seen in Fig. 1A, in rats that had been housed at thermoneutrality, a single injection of glucagon apparently induced a thermogenic response (i.e., an increase in the rate of oxygen consumption). The response to this glucagon injection was at least as large as the response to the classical inducer of thermogenesis, norepinephrine, which was tested here in the same animal after the metabolic rate had returned to basal levels. In a compilation of five experiments performed in this way (Fig. 2A), it is seen that this relationship was generally observed.

When the same type of experiment was repeated with a cold-acclimated rat (Figs. 1B and 2A), a response to glucagon was seen that was not higher than that observed in warm-acclimated rats. Because an increased response to glucagon in cold-acclimated rats had earlier been observed (14), this absence of effect of acclimation to cold was unexpected. The response to norepinephrine, however, showed the expected effect of recruitment (21) by being much higher in the cold-acclimated than in the warm-acclimated rats (Figs. 1B and 2A).

Because the absence of an effect of cold acclimation on the glucagon response was unexpected, we tried different variations of the experimental protocol. These included performing the same type of experiments on Wistar rats (as used in Ref. 14), which, however, gave results similar to those with Sprague-Dawley rats (an ∼90% increase over resting metabolic rate in warm-acclimated rats and a ∼70% increase in cold-acclimated rats; thus again no potentiation of response to glucagon injection due to cold acclimation). We also investigated the response in rats decacclimated acutely (overnight) from cold acclimation, as performed in Ref. 29; again, the response in these rats was not larger.
than that in the corresponding controls (not shown). We also injected a glucagon solution made up in saline from the purchased powder (Sigma) instead of the injection solution of glucagon routinely used here, and again we found similar results.

Thus, independently of experimental method, animal, etc., we were unable to observe the augmented response to glucagon in cold-acclimated animals that would be expected if brown adipose tissue was a main mediator of the thermogenic response to glucagon.

Glucagon-induced unmasking in rats.

From the above data, there was no direct indication that glucagon influenced brown adipose tissue thermogenesis. In an effort to demonstrate some influence of glucagon on the tissue in vivo, we examined whether glucagon could bring about the apparent activation of brown adipose tissue mitochondria termed "unmasking." This phenomenon, the physiological background of which is still unresolved, is observable as an increased [3H]GDP binding capacity in brown fat mitochondria not caused by an increase in the amount of uncoupling protein 1 (12, 34). Such an unmasking can be induced by agents or conditions stimulating the tissue when it is in an inactive state. We therefore tested whether glucagon could induce this unmasking phenomenon. As seen in Fig. 3, in rats returned overnight to thermoneutrality (and thus not requiring any brown adipose tissue thermogenesis), a saline injection had only a weak effect on the number of [3H]GDP binding sites, whereas a norepinephrine injection, as expected, led to a marked unmasking of [3H]GDP binding sites. A glucagon injection also led to a statistically significant unmasking, which was, however, apparently smaller than that to norepinephrine. Thus, clearly, there was an effect of

Fig. 1. Effect of injection of glucagon and norepinephrine (NE) on rate of oxygen consumption (thermogenesis) of intact rats and hamsters. A: warm-acclimated rat. B: cold-acclimated rat. C: warm-acclimated hamster. D: cold-acclimated hamster. A–D represent individual tracings during oxygen consumption measurements in the metabolic chamber described in METHODS. Animals had been habituated to the metabolic chamber for 1–2 h before start of tracings shown here. Where indicated, they were injected with glucagon (1 mg/kg body wt) or with NE (1 mg bitartrate/kg body wt). Body weights of animals were 403 g (A), 265 g (B), 119 g (C), and 101 g (D); indicated breaks in recordings were 36 min in C and 32 min in D. Y-axes are direct measurements of oxygen content in outgoing air, expressed as decrease in oxygen tension vs. normal air, and are thus not corrected for body size (this is done in the compilation in Fig. 2). Apparent decreases in oxygen consumption at time of injections are artifacts due to opening of chamber during injections. Time scale in A applies to all figures.

Fig. 2. Effect of glucagon and NE injections in warm-acclimated and cold-acclimated rats (A) and hamsters (B). A and B are based on experiments like those exemplified in Fig. 1. Data are means ± SE from 5 warm-acclimated rats, 5 cold-acclimated rats, 4 warm-acclimated hamsters, and 4 cold-acclimated hamsters. Resting metabolic rate (R) was defined as the lowest rate of oxygen consumption observed for a period of at least 10 min during a period of −2 h before indicated injections. G and N, highest metabolic rate after injection of glucagon or NE, respectively. Mean weight ± SE of warm-acclimated rats was 274 ± 5 g, of cold-acclimated rats, 261 ± 5 g, of warm-acclimated hamsters, 122 ± 7 g, and of cold-acclimated hamsters, 96 ± 7 g. ns, P > 0.05, *P < 0.05 and **P < 0.01, effect of agent (G or N) vs. R (Student’s paired t-test). NS, P > 0.05, ##P < 0.05 and ###P < 0.01, effect of acclimation to cold (Student’s unpaired t-test).
glucagon injection on a parameter of brown adipose tissue activity, demonstrating both that the glucagon injection was technically successful and that it affected brown adipose tissue in some way, despite the absence of effect of cold acclimation on the magnitude of the apparent thermogenic response to glucagon. However, even these experiments did not necessarily indicate that glucagon interacted directly with the brown adipose tissue in the rats.

Apparent thermogenesis in intact hamsters. Isolated brown fat cells from hamsters have earlier been demonstrated not to be able to respond thermogenically to glucagon (16). Despite this, results very similar to those obtained with intact rats (Figs. 1, A and B, and 2A) were obtained with warm- and cold-acclimated hamsters (Figs. 1, C and D, and 2B). There was a small thermogenic response to glucagon injection (which, due to the higher variability of the response, failed to reach statistical significance), but this response was not augmented by cold acclimation, in contrast to the response to norepinephrine that showed the expected augmentation due to acclimation to cold (13). Thus neither the ability or inability of isolated brown fat cells to respond thermogenically to glucagon nor the recruitment state of brown adipose tissue influenced the response observed in intact animals.

Dose-response relationship for the glucagon effect on thermogenesis. Although 1 mg/kg body wt is the standard dose of glucagon earlier used to demonstrate a thermogenic effect of glucagon in intact animals, this may not be an optimal dose, and this could be the reason that no augmentation of the thermogenic effect due to cold acclimation could be observed. However, as seen in Fig. 4, in both rats and hamsters, glucagon concentrations between 0.3 and 10 mg/kg body wt were unable to influence the metabolic rate to a greater extent than the standard concentration of 1 mg/kg used, nor was there any remarkable alteration in the kinetics of the response as an effect of glucagon dosage (not shown). However, what is particularly evident from the data is that injection of the vehicle in which glucagon was dissolved elevated metabolism to the same extent as did any of the glucagon concentrations. This indicated that the response observed may be a stress response, rather than a true response to the glucagon in the injection, and we were unable to detect any thermogenic response to glucagon in excess of that induced by the injection of the vehicle.

Effect of β-adrenergic blockade. A stress response is most likely mediated via the sympathetic nervous system and norepinephrine release. If such a norepinephrine release takes place in brown adipose tissue, the norepinephrine could interact with β-receptors and thus stimulate thermogenesis indirectly. To test this possible explanation for the apparent thermogenic effect of injection of glucagon preparations, we tested the effect of injection of a β-adrenergic antagonist before glucagon injection. Because thermogenesis in brown fat cells from both hamsters (45) and rats (44) is stimulated solely through β3-receptors, and because β3-receptors are less sensitive to propranolol than are β1/β2-receptors (1), a relatively high dose of β-blocker (20 mg propranolol/kg body wt) was used.

In Fig. 5, it is first demonstrated (Fig. 5A) that two successive injections of glucagon resulted in two thermogenic responses of similar magnitude. In Fig. 5B, propranolol was injected after the end of the first response to glucagon. The injection of propranolol was...
in itself without effect, but it fully eliminated the thermogenic response to the ensuing second injection of glucagon. Thus the apparent glucagon effect was clearly mediated via a β-adrenergic pathway.

Furthermore, exactly the same type of responses were seen with solvent (aqua) alone: each injection caused a similar thermogenic response (Fig. 5C) that could be fully eliminated by prior injection of propranolol (Fig. 5D).

Experiments similar to those in Fig. 5 were also performed with warm-acclimated rats (not shown), giving similar results.

The conclusion from the in vivo experiments is thus that the apparent glucagon-induced thermogenesis observed in rats and hamsters (Figs. 1 and 2) was mainly a result of an injection stress and was thus catecholamine mediated. No unequivocal thermogenic response to glucagon in itself could be discerned, and there is no inherent reason to conclude that the thermogenesis induced emanated entirely from brown adipose tissue.

Effects of Glucagon on Isolated Brown Adipocytes

The implication from the present experiments was that glucagon in itself was without effect on thermogenesis and that the apparent effects were due to the release of endogenous norepinephrine, even in the rat. The results may thus call into question whether the earlier reports on the ability of rat brown fat cells to directly respond thermogenically to glucagon are valid with present-day glucagon preparations. We therefore investigated whether brown fat cells prepared from rats, mice, and hamsters possessed this ability.

Rat brown fat cells. In brown fat cells isolated from rats that had been maintained at room temperature, the addition of the solvent for glucagon (aqua) was in itself without effect on thermogenesis, but the addition of norepinephrine, as expected, led to a large increase in thermogenesis (Fig. 6A, left trace). As seen, glucagon (here at a dose of 10 µM) was indeed able to induce a marked stimulation of oxygen consumption, fully up to the level obtained with norepinephrine (Fig. 6A, right trace), and an ensuing addition of norepinephrine did not lead to a further increase in thermogenesis. Thus the rat brown fat cells seemed clearly responsive to glucagon, in agreement with earlier observations (23, 30). Also, a preparation of synthetic glucagon (Sigma) had a similar thermogenic response (not shown). Because the maximal responses to glucagon and norepinephrine were not additive, the responses seemed to use the same final effector pathways. It is likely that this pathway is the one mediated by cAMP (17), but this has not been studied directly here.

A dose-response curve for the thermogenic effect of glucagon (and of norepinephrine, for comparison) in these cells is seen in Fig. 7A. Maximum stimulation of oxygen consumption was seen at ~100 nM glucagon.
and the magnitude of the response was practically identical to that seen with norepinephrine.

The EC_{50} for glucagon obtained here was 5 nM. This value is lower than most earlier reported EC_{50} values from studies of thermogenesis in brown adipose tissue fragments and in isolated brown fat cells (=100 nM) (23, 30) and similar to that observed in studies of stimulation of lipolysis in brown adipose tissue fragments and cells (22, 23).

However, because the plasma levels for glucagon correspond to only ~50 pM (28) in both warm- and cold-acclimated rats, transiently increasing to ~400 pM during acute cold exposure (31), a thermogenic response could not, according to these results, be induced by circulating plasma glucagon. There are, however, two reports (6, 32) from one group indicating an EC_{50} value of ~10 pM for glucagon stimulation of thermogenesis in isolated rat brown fat cells, i.e., a 1,000-fold higher sensitivity than that reported here. We have no explanation for the immense difference in EC_{50} values between glucagon sensitivities in different laboratories but it may be noted that with sensitivities of ~10 pM, the brown fat cells would theoretically be constantly virtually fully activated by circulating glucagon, and there would be no need for, or effect of, sympathetic stimulation of thermogenesis.

On the basis of the in vivo results presented here, the possibility that glucagon was mediating its effect indirectly even in vitro had to be considered. In the in vitro situation, this could be due to sympathetic nerve vesicles occasionally adhering to adipocytes (33). If glucagon were suggested to have such an effect, or for unknown reasons should interact directly with the β-receptors, this would render the stimulated respiration sensitive to propranolol. However, as seen in Fig. 7B, the glucagon-stimulated respiration was completely insensitive to propranolol, whereas the norepinephrine-stimulated respiration was fully inhibited by propranolol at the expected concentrations for interaction with the β-receptors. Thus the glucagon response in rat brown fat cells is not mediated via β-receptors and/or the release of norepinephrine from adhering nerve vesicles, etc.

Mouse brown fat cells. Brown fat cells were also isolated from mice, and the glucagon responsiveness of these cells was investigated. Also in this species, glucagon had a thermogenic action on the brown fat cells (Fig. 6B), and the maximal level reached was similar to that reached by norepinephrine stimulation. However, the EC_{50} was apparently even higher than in the rat, i.e., close to 1 µM.

Hamster brown fat cells. In Fig. 6C, two oxygen electrode traces are shown, demonstrating the absence of stimulation by glucagon (at different concentrations) of oxygen consumption in brown fat cells isolated from the Syrian hamster; this is also in agreement with earlier observations (16). The absence of effect of glucagon in this species is not due to a species difference concerning the hormone itself, because, according to Swiss-Prot, the glucagon molecule is identical in rat, mouse, and hamster (and pig and man).

Thermogenic Effects of Other Glucagon Family Members

The experiments presented here clearly indicate that glucagon can stimulate thermogenesis in isolated rat brown fat cells. The concentrations of glucagon needed to bring about this action were, however, in our hands, clearly supraphysiological, thus calling into question the physiological role of the hormone as a thermogenic agent in these cells. It may therefore be suggested that glucagon is not the endogenous activator of the responses seen here but that other peptides interact with the same receptor, perhaps with higher affinity. Therefore, in an attempt to identify a hormone that reacted with a higher affinity, a series of experiments was
performed with other peptide hormones in the glucagon superfamily.

When tested in a concentration range from 100 pM to (0.1 or) 1 µM, the peptides VIP, GIP, GLP-1, and GHRF (rat or bovine) did not lead to clear and consistent increases in oxygen consumption. However, secretin gave a marked stimulation of respiration, achieving the same maximal response as glucagon or norepinephrine (Fig. 8). The EC$_{50}$ value for secretin was 22 nM; thus although also being a full agonist, secretin had an even lower affinity than had glucagon. It would thus seem that none of the tested hormones in the glucagon family were better agonists than glucagon. To test whether any of the other hormones mentioned were receptor ligands (without having a stimulatory effect) for the receptor mediating the response to glucagon or secretin, we examined whether they could inhibit the thermogenic response to glucagon or secretin (at equimolar concentrations of 100 nM). This was not the case (not shown); nor did they influence the response to 100 nM norepinephrine, nor were the maximal responses to glucagon or secretin, glucagon, and norepinephrine additive (not shown); nor did they influence the response to 100 nM. This was not the case (not shown).

Through the glucagon receptor, mediating the response to glucagon or secretin, we examined whether they could inhibit the thermogenic response to glucagon or secretin (at equimolar concentrations of 100 nM). This was not the case (not shown); nor did they influence the response to 100 nM norepinephrine, nor were the maximal responses to secretin, glucagon, and norepinephrine additive (not shown). Thus glucagon remains the member of the glucagon superfamily that interacts most avidly with this receptor.

**DISCUSSION**

In the present investigation, we were unable to induce thermogenesis in intact animals in vivo by injection of glucagon, in contrast to results from some earlier investigations. A small effect observed on mitochondria isolated after glucagon injection indicated that the glucagon signal directly or indirectly reached brown adipose tissue, but no thermogenic effect could be ascribed to interaction of glucagon with brown adipose tissue in vivo. However, isolated brown fat cells from rats were thermogenically responsive to glucagon, a response not mediated through $\beta$-receptors. This response was species specific in that brown fat cells from hamsters were not responsive. On the basis of these observations, questions may be raised as to whether a thermogenic response to glucagon exists in vivo, whether this has any physiological function, and whether glucagon itself is the endogenous ligand for this nonadrenergic response.

**Why Was No Thermogenic Effect Observable In Vivo?**

Because it has become generally accepted that glucagon has a thermogenic effect when injected into intact animals (25) and because such an effect has been observed by several authors, especially between 1957 and 1990 (see introduction), the absence seen here of a thermogenic effect that could be ascribed to the glucagon content of the injections was unexpected. We find it unlikely that our results are due to a technical limitation in our system; the responses to norepinephrine injections observed in the same animals on the same occasions were fully as expected. Neither do we feel that the glucagon solutions used were unsuitable; the glucagon product mainly used here is produced for injection into humans, was prepared for injection into rats, and was thus verified to be functional. Furthermore, another source of glucagon (as a powder, from Sigma) was also without demonstrable thermogenic effect in vivo.

It may therefore be discussed why thermogenic responses to injected glucagon preparations have been observed earlier. In this context, it may be pointed out that until recently, all glucagon preparations were products purified from pig pancreas, and it has been reported that certain such earlier preparations did contain impurities (27, 37). Thus our suggestion is that earlier observations of a thermogenic effect of injection of glucagon preparations were due to the presence of other factors in these preparations, factors that either interacted with the glucagon receptors (in certain animals) or that led to the release of endogenous norepinephrine, which, in its turn, stimulated brown adipose tissue thermogenesis. It is, of course, impossible today to investigate to what degree contaminants were present in the preparations of glucagon used in the 1960s through 1980s, and the suggestion forwarded here for the discrepancies between certain earlier and the present results can therefore not be experimentally tested. However, until it has been shown with present-day preparations, which are probably of a higher purity than those used earlier, or with synthetic glucagon, that a dose-dependent thermogenic response can be induced in any mammal in vivo, the existence of a glucagon-induced thermogenesis in mammals may be considered unproven.

Because Glucagon is Able to Induce Thermogenesis in Isolated Brown Fat Cells, Why Does Injected Glucagon Not Induce Thermogenesis in Intact Animals?

As reported here, norepinephrine and glucagon are nearly equipotent in inducing thermogenesis in isolated rat brown fat cells, with an EC$_{50}$ of 1–10 nM. Thus it would be anticipated that by injecting the substances
into intact rats, we should observe similar thermogenic responses, but as demonstrated in the present study, this was not the case: norepinephrine was thermogenic, but glucagon was not. A simple explanation is, of course, that the classic injection dose of 1 mg/kg body wt corresponds to 3 µmol/kg for norepinephrine and only to 0.3 µmol for glucagon. Ten milligrams glucagon per kilogram is thus an equimolar dose, but even this concentration did not elicit thermogenesis to a greater extent than did solvent (Fig. 4). This could perhaps be explained by the pharmacokinetics of glucagon: the plasma half-life has been estimated to be only 2 min (3), and this may make it impossible by injection to reach the plasma concentrations in the high nanomolar range needed. It should, of course, be technically possible to increase circulating glucagon levels sufficiently to induce a brown fat-mediated thermogenic response in rats and mice (but not in hamsters). This could probably be done, e.g., by infusing instead of injecting the compound (9, 42). However, even concerning these responses, it cannot be fully eliminated that the response is not secondary to released endogenous norepinephrine. This has routinely been tested with simultaneous injection of propranolol, which was reported to be without inhibitory effect, at least in newborn rabbits (20). However, these experiments were performed long before it was realized that brown adipose tissue thermogenesis is induced via β3-adrenergic receptors that are much less propranolol sensitive than the β1-receptors (1), and the propranolol doses used may not have been sufficiently high to inhibit these receptors.

However, even if glucagon in itself does induce thermogenesis when infused at a sufficiently high level, it is a semantic question whether the thermogenic outcome of this type of experiment should be considered evidence that the hormone glucagon is thermogenic, because the glucagon levels needed are presumably 1,000-fold supraphysiological.

Other Glucagon Effects

To some extent, the present observations of the absence of a bona fide thermogenic response to injected glucagon may necessitate reevaluation also of other acute effects of glucagon injection, especially such effects that, like thermogenesis, are probably mediated via an increase in cAMP levels. These probably include effects that, like thermogenesis, are probably mediated by the brown fat cells. Because any agent that chronically increases cAMP (3, 39), a known inducer of brown fat recruitment (3, 39), may be sensitive to glucagon in the picomolar range (23). Such types of glucagon effects may be mediated via a glucagon receptor coupled to inositol 1,4,5-trisphosphate production and Ca2+ release (8, 40), but this has not as yet been demonstrated in brown fat cells.

Why Are There Low-Affinity Glucagon Receptors on Rat Brown Fat Cells?

The mere observation that hamster brown fat cells are apparently devoid of cAMP-thermogenesis-coupled glucagon receptors implies that it is unlikely that this type of glucagon receptor has a general central role in the control of thermogenesis. It is, however, equally unlikely that other animals (rats, mice) are equipped with receptors devoid of physiological function.

A possibility is that the receptors responding thermogenically to glucagon (on the basis of their low affinity for glucagon, it is perhaps doubtful to call them glucagon receptors) respond endogenously to substances other than glucagon. We have here examined different members of the glucagon superfamily but have been unable to ascertain that any of these had a higher affinity than glucagon itself. If the endogenous ligand were to reach the receptor not via the blood but be released from nerve endings, much higher levels of ligand could, however, be achieved close to the receptor. Indeed, the presence of some members of the glucagon superfamily has been indicated in immunohistochemical studies of brown adipose tissue (18), but no functional roles for these compounds have as yet been identified.

We would, however, like to point out that stimulation of these low-affinity glucagon receptors closely induces brown fat cell thermogenesis in some species (as demonstrated here) and also probably brown adipose tissue recruitment. It is unknown whether human brown adipose tissue possesses this type of glucagon receptor, but it is likely that it does, because glucagon stimulates lipolysis in human white adipose tissue (38). Thus finding a means of activation of these receptors could still be a challenge for the development of drugs to stimulate brown adipose tissue and thus probably to ameliorate obesity.

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