Pathogenetic Aspects of the Obese-Hyperglycemic Syndrome in Mice (Genotype *obob*):

II. Extrapancreatic factors*

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Summary. The cause of the pronounced insulin resistance of the hereditary obese-hyperglycemic syndrome in mice (*obob*) is so far unknown. To evaluate whether growth hormone (GH) is of pathogenetic significance in this context the *in vivo* incorporation of sulfate into costal cartilage was measured in obese-hyperglycemic mice and their lean littermates at various ages. It was found that a) GH administration raised the sulfation activity of the costal cartilage in both types of mice; b) the hormone further increased the insulin resistance of the obese-hyperglycemic animals; c) the sulfate incorporation of the obese mice was considerably elevated after 1 month of age and remained high until the age of about 10 months. In 17 months old animals it had returned to a level similar to that of the lean littermates. In addition, the epiphyseal width of the tibia was increased in the obese mice. Since it was not clear whether the increased rate of sulfate incorporation was due to an elevated endogenous GH activity and/or the high serum insulin levels prevailing in the obese mice at this period of life a study of the pituitary morphology was carried out. The lack of obvious structural or quantitative differences between the pituitary alpha cells of the lean and obese mice did not exclude the possibility that the elevated sulfation activity was caused by the high serum insulin levels rather than an increased circulating GH activity. — Glucokinase activity, characterized by a high K_m value, was found in the livers of both lean and obese mice. A significant elevation of this enzyme activity, but not of the hexokinase activity, in the obese mice strongly suggests that the insulin resistance does not extend to the liver cells in the obese-hyperglycemic syndrome.

Aspects de la pathogénèse du syndrome obésité-hyperglycémie chez la souris (génotype *obob*).: II. Facteurs extra-pancréatiques

Résumé. La cause de la résistance prononcée à l'insuline caractérisant le syndrome obèse-hyperglycémique de la souris *obob* n'est pas encore connue. Cette étude a été entreprise dans le but d'évaluer le rôle pathogénique éventuel de l'hormone de croissance (GH), mesurant *in vivo* le taux d'incorporation de sulfate dans le cartilage costal chez les souris normales et obèses hyperglycémiques de même nichées et à des âges différents. Les résultats suivants ont été obtenus: a) L'administration de GH augmente l'incorporation de sulfate dans le cartilage costal des deux types de souris. b) La présence de GH augmente la résistance à l'insuline des animaux obèses-hyperglycémiques. c) L'incorporation de sulfate est considérablement augmentée chez la souris obèse âgée de plus d'un mois et reste élevée jusqu'à environ 10 mois. Chez des animaux de 17 mois, elle est ramenée à un taux

semblable à celui des souris normales d'une même nichée. En plus, l'épiphyse du tibia était plus large chez la souris obèse. Une étude morphologique de l'hypophyse a été faite ensuite puisqu'il n'était pas établi que les taux élevés d'incorporation de sulfate soient dus à une activité endogène accrue de la GH et/ou à l'insulinémie élevée observée chez la souris obèse en cette période de la vie. L'absence de différences structurelles ou quantitatives entre les cellules α hypophysaires des souris normales et obèses n'exclut pas l'éventualité que l'activité de sulfation accrue soit due à l'insulinémie élevée plutôt qu'à une augmentation de l'activité de l'hormone de croissance. Une activité de la glycokinase caractérisée par un K_m élevé a été trouvée dans le foie des souris normales et obèses. Chez la souris obèse, une élévation significative de cette activité enzymatique, — alors que l'activité de l'hexokinase n'est pas augmentée — suggère que les cellules hépatiques ne sont pas impliquées par la résistance à l'insuline décrite dans le syndrome obèse-hyperglycémique.

Zur Pathogenese des obes-hyperglykämischen Syndroms der Maus (Genotyp *obob*) II. Extrapankreatische Faktoren

Zusammenfassung. Die Ursache der Insulinresistenz der *obob* Maus ist noch immer unbekannt. Um festzustellen, ob dem Wachstumshormon eventuell pathogenetische Bedeutung zukomme, wurde die Inkorporation von Sulfat in den Rippenknorpel von *obob* Mäusen und normalgewichtigen Kontrolltieren verschiedener Altersklassen gemessen. Die Injektion von Wachstumshormon erhöht die Inkorporation von Sulfat bei allen Tieren. Bei den fettstüchtigen nahm nach der Injektion außerhalb der Insulinresistenz zu. Die spontane Inkorporation von Sulfat in die Rippenknorpel war bei den *obob* Tieren nach dem ersten und ungefähr bis zum zehnten Lebensmonat signifikant erhöht. Bei 17 Monate alten Tieren wurden den bei Normaltieren gemessenen entsprechende Werte gefunden. Weiterhin war die Breite der Tibia-Epiphyse der obesen Maus größer. Da es auf Grund dieser Resultate nicht möglich war, zu unterscheiden, ob die erhöhte Inkorporation tatsächlich auf erhöhte Konzentrationen endogenen Wachstumshormons und/oder auf die gleichzeitig bestehende Hyperinsulinämie zurückzuführen sei, wurden die Hypophysen der Tiere histologisch untersucht. Dabei konnten zwischen den obes-hyperglykämischen und den normalen Tieren weder strukturelle Unterschiede noch solche in der Zahl der A-Zellen festgestellt werden. Es ist deshalb vorderhand unmöglich, die Hyperinsulinämie als Ursache der bei den *obob* Mäusen festgestellten Erhöhung der Sulfatinkorporation auszuschließen. Bei Kontroll- und *obob*-Mäusen war die Glucokinaseaktivität, nicht aber diejenige der Hexokinase erhöht. Die Aktivitätssteigerung der Glucokinase war bei den *obob* Mäusen deutlich ausgeprägter und es wird deshalb angenommen, daß die Leberzellen der *obob* Tiere nicht insulinresistent sind.

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A salient feature of the hereditary obese-hyperglycemic syndrome in mice (*obob*) is a pronounced insulin resistance, which gives rise to increased levels of blood glucose and serum insulin [14, 3, 23]. So far there is no generally accepted theory as to the cause of the insulin resistance. The demonstration of a defective *in vivo* muscular response following the administration of insulin led to the suggestion of a primary intrinsic insulin resistance of the muscle cell [21]. However, this hypothesis does not fit with the more recent observation that in the obese animals the diabetogenic influence is apparently active only during a limited period of life [23]. The onset of the obese-hyperglycemic syndrome in young animals during a period of active growth, and, in particular, the disappearance with age of many abnormalities suggested the possibility that circulating humoral agents causing insulin resistance should also be considered. Growth hormone (GH) may be of significance in this context, since this hormone is known to cause marked insulin resistance both in man and animals. In the present study a bioassay method sensitive to GH and based upon the *in vivo* incorporation of sulfate into mouse costal cartilage has been applied to both lean and obese-hyperglycemic animals of different ages. In addition, data on the epiphyseal width of the tibia are presented as well as an evaluation of the pituitary morphology. Some preliminary results on the kinetics and activities of liver glucokinase in these animals are also included.

Material and Methods

Animals. Obese-hyperglycemic mice and their lean littermates were obtained from the non-inbred stock maintained at the Histological Department, University of Uppsala. The colony was established in 1959 and originates from breeding-couples obtained from the R. B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A. After weaning at 21 days of age the animals were reared on a uniform diet consisting of commercially available mouse chow [23] supplemented with vitamins (Protovit®, Roche) once every second week. If not otherwise stated male mice with free access to food and water were used.

Blood sampling. Blood was obtained by puncture of the orbital vein plexus with a thin-walled glass tube. It was collected in dry test tubes without anticoagulants, the serum was separated by centrifugation, and, when not analysed immediately, was stored at -18°C .

Chemical assays. Glucose determinations were performed with the glucose oxidase method on 5 μl serum samples, as described by Hjelm & deVerdier [9]. Serum insulin was measured by the double antibody radioimmunoassay of Hales and Randle [6] using mouse insulin (23 IU/mg) as a standard and the insulin- ^{125}I immunoassay kit supplied by the Radiochemical Centre, Amersham, England. The hexokinase and glucokinase activities in the livers of female mice, 4.5–7 months old, were measured in the supernatants

of liver homogenates, which had been centrifuged at $105000 \times g$ for 1 h. The assays were carried out essentially according to Salas *et al.* [17] by following spectrophotometrically the formation of NADH at room temperature (24°C) in a coupled kinase-glucose-6-phosphate dehydrogenase reaction sequence. The results are expressed as micromoles of glucose phosphorylated per minute per g liver protein. K_m -values for glucokinase activities were computed using the Lineweaver-Burk plot. Protein determinations were performed on triplicate samples of the liver supernatant as described by Lowry *et al.* [11] with human serum of known protein concentration as a standard.

Bioassays. In order to estimate the GH sensitive sulfate incorporation activity into cartilage the method developed by Herbai [7] was applied. This double isotope technique permits quantitative estimation of the rate of *in vivo* incorporation of sulfate into an anatomically defined part of the costal cartilage of the mouse; corrections being made for the different sizes of the sulfate pools of the obese and lean mice. The results have been expressed as ng of sulfate incorporated per 5 pairs of ribs per 30 min. The effects of two different GH preparations on the sulfate incorporation activity of obese and lean mice were evaluated. The animals received one daily i. p. injection of either ovine (NIH-GH-S9, 1968) or bovine (NIH-GH-B13, 1968) GH dissolved in isotonic saline adjusted to pH 9.5 with 0.1 N NaOH.

As a complementary study the proximal epiphyseal widths of the left tibia were measured in groups of mice of different ages. The epiphyseal lines were visualized by treatment of the sagittally hemisectioned bone in a silver nitrate solution as described by Evans *et al.* [5]. The mean value for the width of each epiphyseal line was calculated from 10 measurements with a calibrated ocular screw micrometer at a magnification of $35 \times$. Care was taken to treat the bones as uniformly as possible to avoid errors due to variations in tissue shrinkage.

Morphological studies. For light microscopic studies of the pituitaries 8 obese mice and 8 lean littermates, 5 months old, were decapitated and the glands rapidly excised and fixed for 3–4 h in Zenker-formal solution. After careful removal of adherent tissue fragments each pituitary gland was blotted and weighed on a torsion balance. Paraffin sections, 4 μ thick, were then prepared and differentially stained according to Moewis' modification [15] of Maxwell's method [13]. The contribution of acidophilic [alpha] cells to the total volume of the anterior pituitary was estimated at a magnification of $500 \times$ by application of the point sampling method of Chalkley [4]. A square ruled grid with 121 intersection points was placed in the focal plane of the microscope eye-piece; the number of intersections ("hits") superimposed on acidophilic cells and other hypophyseal tissue being counted. In each animal a minimum of 1800 hits from at least 6 different sections were used to compute the relative volume of

alpha cells. The nuclear volume of the alpha cells relative to that of the cytoplasm was similarly estimated by point sampling at a magnification of $1250\times$; the number of hits sampled per animal in this case was at least 2500.

For electron microscopy of the pituitaries, 7 lean and 2 obese mice, 5 months old, were anaesthetized with Nembutal® and perfused with an aldehyde mixture composed of 1% formaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer adjusted to pH 7.4. The pituitary gland was then dissected out and postfixed in 1% osmium tetroxide dissolved in 0.06 M phosphate buffer, pH 7.4. After embedding in Epon ultrathin sections were prepared with an LKB Ultratome III. The sections were stained with uranyl acetate [22] followed by lead citrate [16] and analysed in a RCA-EMU-3B electron microscope with a primary magnification of up to $10000\times$.

Results

Bioassays. The effect on the sulfate incorporation of GH-administration to obese-hyperglycemic mice and their lean littermates is shown in Fig. 1. At the

Fig. 1. The effect of GH administration ($500\text{ }\mu\text{g}$ daily for 3 days) on the sulfate incorporation (ng of sulfate incorporated/5 pairs of ribs/30 min) into the costal cartilage of lean (hatched) and obese-hyperglycemic (cross-hatched) mice. Means $\pm$ S.E.M. Figures within parentheses denote the number of animals

beginning of the experiment the animals were about 8 weeks old; the mean body weight of the lean animals being $17.1\pm0.6\text{ g}$ and of the obese ones $27.8\pm1.4\text{ g}$. Ovine GH was injected on 3 consecutive days in a daily dose of $500\text{ }\mu\text{g}$ per animal. As can be seen in the Figure both groups of mice responded to the treatment with increased sulfation activities. In the lean group the mean increase was about 120% of the control value and in the obese group about 100%. There were no significant differences ($P>0.05$) in weight gain between the GH-treated and the control mice.

The influence of GH on the serum glucose and insulin levels were analysed in a separate experiment. Young obese mice (4–6 weeks old; mean body weight at the start of the experiment $15.9\pm1.4\text{ g}$) received $125\text{ }\mu\text{g}$ of bovine GH in daily injections for 5 days and the results were compared with those obtained in a control group of obese mice injected only with the medium used for dissolving the hormone. As can be seen in Table 1 this treatment did not cause any significant differences ($P>0.05$) in the body weight, blood sugar concentration or sulfation activity between the two groups of mice. By contrast, at the end of the experiment the mean serum insulin level was more than twice as high in the GH-treated animals as in the controls ($P<0.001$).

Table 1. Effects of bovine GH given in a dose of $125\text{ }\mu\text{g}$ daily for 5 days on the body weights, serum glucose, serum insulin and sulfate incorporation of obese-hyperglycemic mice. Numbers of animals within parentheses. Means $\pm$ S.E.M.

Animals	Body weight g	Serum glucose mg%	Serum insulin ng/ml	Sulfate incorporation ng/5 pairs of ribs/30 min
Controls (8)	20.8 ± 1.3	142 ± 5	64 ± 10	179 ± 10
GH-treated (8)	22.2 ± 1.3	150 ± 9	137 ± 18	234 ± 33

Fig. 2. Sulfate incorporation (Means $\pm$ S.E.M.) into the costal cartilage of obese-hyperglycemic (●—●) and lean (○—○) mice of different ages

The rate of sulfate incorporation into cartilage tissue of obese mice and their lean littermates at various ages has been reported in detail elsewhere [8], and a summary of the results is given in Fig. 2. It is evident that the pattern of sulfation activity was quite different in the two groups of animals. In the lean mice the highest rate of sulfate incorporation was found among the 1 month old animals and thereafter it decreased smoothly until a steady state level was reached at about 5 months of age. In the 1 month old obese mice the rate of sulfate incorporation was similar

to that of the lean controls. However, in the older age groups the obese animals showed a markedly higher activity, which increased up to a peak value at 5 months of age and then gradually decreased. Levels of the same order as in the control group were not recorded until the age of about 17 months.

Fig. 3. The epiphyseal widths (Means $\pm$ S.E.M.) of obese-hyperglycemic (●—●) and lean (0—0) mice of different ages. The number of animals in each group is given within parentheses

in the lean littermates by a rapid rate of decrease, which appeared to fall off after the age of about 5 months. The epiphyseal width of the obese animals decreased almost linearly throughout the period of observation; in each age group the mean value being above that of the corresponding controls.

Morphological studies. The light microscopic evaluation of the pituitary morphology has been confined to the alpha cells, which are generally held to be the cellular source of GH. With the present staining

Table 2. A comparison between obese-hyperglycemic mice and their lean littermates with regard to pituitary weights, percentage contribution of alpha cells to the total anterior pituitary volume and of the cell nuclei to the total alpha cell volume. Numbers of observations within parentheses. Means $\pm$ S.E.M.

Animals	Pituitary weight mg	Alpha cells in % of the anterior pituitary volume	Nuclei in % of the alpha cell volume
Lean	1.33 $\pm$ 0.05 (8)	45.7 $\pm$ 1.0 (6)	34.3 $\pm$ 0.3 (6)
Obese	1.37 $\pm$ 0.04 (8)	43.0 $\pm$ 0.2 (5)	33.0 $\pm$ 0.7 (5)

Fig. 4 a. Paraffin section of the anterior pituitary from an obese-hyperglycemic mouse. Dark (red) alpha cells are dispersed all over the section surface; the highest cell density being seen in the lower left part of the section. Green filter. $\times$ 100

Fig. 4 b. High power view of a pituitary section from an obese mouse. Numerous round alpha cells with dark staining cytoplasm. Green filter. $\times$ 1000

As can be seen in Fig. 3 there were marked differences also between the mean epiphyseal widths of the obese and lean mice. An initially high value at the age of about 2 months in both types of animals was followed

method the alpha cells were brought out in an intensely red against the remaining pituitary cells, which could not be further differentiated (Fig. 4). At a low magnification alpha cells could be identified in

all regions of the anterior pituitary, although the cellular density seemed most pronounced in the sub-capsular part of the organ (Fig. 4a). At a higher magnification the cells appeared rounded with prominent nuclei (Fig. 4b). Distinct granules were not recognized in the strongly staining cytoplasm. There were no obvious differences in distribution, staining intensity or frequency between the alpha cells of obese and lean mice.

have been used. In the lean mice the alpha cells were frequent; they were round and of medium size with a rather large nucleus. As can be seen in Fig. 5a an abundance of large and electron dense granules were present in the cytoplasm. These granules were spherical with a mean diameter of 300–400 m μ . In most alpha cells the secretion granules were absent from a perinuclear area, in which the rough surfaced endoplasmic reticulum was particularly prominent. Rounded or rod

Fig. 5 a. Electron microscopic view of cells from the anterior pituitary of a lean mouse. Two alpha cells with prominent secretory granules are situated on each side of a cytoplasmic process from a beta cell, which extends towards a capillary in the upper part of the Figure. $\times 7000$

Quantitative analyses showed that the mean pituitary weight was similar ($P > 0.05$) in the two kinds of animals (Table 2). The percentage contribution of alpha cells to the total volume of the anterior pituitary appeared somewhat smaller in the obese than in the lean animals ($P < 0.05$). No differences were found as regards the relative nuclear volume of the alpha cells ($P > 0.05$).

In the electron microscopic investigation of the pituitary gland attention was also focussed on the alpha cells. In identifying this as well as the other pituitary cell types the criteria described by Barnes [2]

shaped mitochondrial profiles were scattered throughout the cytoplasm.

In addition to the alpha cells also beta, gamma and delta cells were identified. A low number of remaining cells lacked secretory granules and were therefore not classified according to type.

A comparison between the ultrastructure of the alpha cells in the lean and obese mice revealed no differences either with regard to their frequency or to their general appearance (Fig. 5 b). The alpha cells of the obese mice also contained an abundance of secretory granules, whose distribution, size and electron

density appeared similar to those observed in the lean mice.

Comparisons between the beta and gamma cells in the pituitaries of the two types of mice revealed no differences. Because of the paucity of delta cells comparative studies of this cell type were omitted.

Liver enzymes. The K_m -value for liver glucokinase in lean mice was calculated as 17 mM and in obese animals as 18 mM. The activities of both hexokinase

Discussion

The *in vivo* incorporation rate of sulfate into the costal cartilage of hypophysectomized mice has previously been shown to be a sensitive index of the circulating growth hormone activity [7]. The present results indicate that also in intact mice of this particular strain administration of GH in high doses stimulates the *in vivo* sulfation activity of cartilage. Moreover, GH given in lower doses, which did not stimulate

b. The same types of cells as seen in Fig. 5 a but from an obese-hyperglycemic mouse. Intercalated between two alpha cells there is a cytoplasmic process from a beta cell in broad contact with a capillary in the upper left part of the Figure. $\times 7000$

and glucokinase in the livers of lean and obese female mice are given in Fig. 6. The hexokinase activity was similar in the two groups of mice ($P > 0.05$), whereas the glucokinase activity was considerably higher in the obese group ($P < 0.001$). This was the case irrespective of whether the values were calculated in relation to the protein concentration or the wet weights of the livers. Starvation of the animals for 3 days did not cause any marked changes of the hexokinase activities; neither were the mean values of the glucokinase activities in the starved animals significantly lower than those in the controls ($P > 0.05$).

the sulfate incorporation, seemed to increase further the insulin resistance of the obese mice as evidenced by a raised concentration of serum insulin without any concomitant decrease of the blood sugar level. This finding supports previous reports in which an increased serum glucose level in GH-treated obese-hyperglycemic mice was interpreted to indicate a decreased peripheral glucose utilization [14, 20]. The possibility of an additional, direct action of GH on the pancreatic B-cells should, however, also be kept in mind [12].

The present observations of both an enhanced sulfate incorporation and increased epiphyseal widths

of the obese mice at various ages would appear to support the hypothesis that the endogenous GH level of these animals is raised. This view must, however, be critically evaluated in the light of previous observations indicating that in some respects the actions of GH and insulin on the cartilage metabolism are similar. It is of particular interest in this context that the effect of insulin has been shown to mimic that of the GH dependent sulfation factor [18]. Thus, extremely high insulin levels enhanced the *in vitro* sulfate incorporation of cartilage; the minimum effective concentration being of the order of 1–10 mU/ml. The obese mice have high levels of immunoreactive serum insulin during approximately the same period of life as their sulfation activity is raised. In fact, insulin levels of about 20 mU/ml, have been reported to be present in 6 months old obese-hyperglycemic mice [23]. The enhanced sulfate incorporation of these animals may

Fig. 6. Activities of hepatic hexokinase (HK) and glucokinase (GK) of obese mice and their lean littermates with free access to food (□) or after 3 days starvation (▨). Numbers of animals within parentheses. Means $\pm$ S.E.M.

thus be accounted for by the hyperinsulinemia rather than by (or in addition to) an increased circulating GH activity. Whether the enlargement of the epiphyseal lines in the obese mice reflects the same basic mechanism as does the enhanced rate of sulfate incorporation is not clear. Administration of insulin in a high dose, 10 U/kg body weight, for 5 days has previously been claimed to bring about a moderate increase of the epiphyseal width of the tibia in intact rats [1], but mediation by a stimulated release of GH was not ruled out in this study. In the present investigation the epiphyseal width of the obese animals decreased between the age of 2 and 6 months, whereas during the same age period there was a marked increase of the sulfate uptake. It is to be noted, however, that both the rate of sulfate incorporation and the epiphyseal widths differed significantly between the lean and obese animals.

In an attempt to clarify the ambiguous results referred to above a comparison was made between the

pituitary structure in the lean and obese mice. The lack of more pronounced differences between the pituitary alpha cells of the two kinds of animals does not necessarily lend support to the view that the results obtained with the bioassays reflect the extremely high levels of serum insulin rather than an increased circulating GH activity in the obese-hyperglycemic syndrome. An apparently normal store of secretion granules in a cell does not exclude a raised turn-over rate of the secretion product. It may therefore be concluded that further studies are required to evaluate the level of circulating GH activity in the obese-hyperglycemic mice. Attempts to devise a radioimmunoassay of this hormone in mice are now in progress.

The extreme insulin resistance of the obese-hyperglycemic mouse raises the question, whether this property is a characteristic of some or all cells, which are regarded as sensitive to the hormone in normal animals. A pronounced insulin resistance of muscle and to a somewhat less marked extent of fat cells has been demonstrated previously in the obese mice [21]. As discussed above, the present results, however, do not exclude that the cartilage cells of these animals may respond to the raised serum insulin levels. The central role of the liver in the over-all glucose metabolism makes it particularly important to evaluate to what extent this organ is sensitive to insulin in obese-hyperglycemic mice. Previous studies have shown that in normal liver cells the activity of glucokinase is regulated by insulin [17], whereas hexokinase is controlled by other factors. In the present study the occurrence of glucokinase activity, characterized by a high K_m -value, was demonstrated in the livers of both lean and obese mice. The pronounced increase of this enzyme activity in the livers of the obese animals strongly suggests that the liver cells are, in fact, sensitive to the raised levels of plasma insulin. This observation confirms a previous report by Seidman *et al.* [19], indicating elevated levels not only of glucokinase but also glucose-6-phosphatase and fructose-1, 6-diphosphatase in the livers of these animals. The present failure to reduce the glucokinase activity in both lean and obese mice by fasting for 3 days agrees with similar observations in normal mice reported by Lauris and Cahill [10]. The sensitivity of glucokinase to starvation may, however, be sex-dependent, since in another strain of obese mice, New Zealand Obese, a significant reduction of the liver glucokinase activity was observed in a group of male mice starved for 3 days, whereas no effect was recorded in a group of similarly treated female animals (Borglund, to be published).

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