An adipogenic serum factor in genetically obese rodents

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The adipose conversion of 3T3-Li cells depends on a serum factor present in high amounts in fetal calf serum, which is heat stable and can be extracted from serum by ethanol precipitation. Sera of two genetically obese rodent species, fa/fa Zucker rats and C57Bl/KsJ–*db/db* mice, contain a high adipogenic activity which is very similar to that found in fetal calf serum. In contrast, sera of their lean siblings (Fa/Fa-Zucker rats and C57Bl/KsJ–+/+ mice) are devoid of adipogenic activity.

3T3-Li Preadipocyte *Adipogenic serum factors* *Genetically obese rodent*

1. INTRODUCTION

3T3-Li fibroblasts originally isolated in [1] are able to differentiate into adipocytes. During differentiation the activity of lipogenic and lipolytic enzymes markedly increases, simultaneously the cells develop insulin receptors and a hormone-sensitive adenylate cyclase system [2]. When subcutaneously injected into intact mice 3T3 preadipocytes differentiate into fat pads, which do not differ morphologically from normal adipose tissue [3]. The process of differentiation, which is accelerated by insulin, isobutylmethylxanthine and dexamethasone [2,4–6], depends on a serum factor present in high activity in fetal calf serum [7], and to a lesser degree, also in adult calf serum [7] and human serum [8,9]. However, nothing is known about the role of such a factor for adipogenesis and obesity. This report describes a study on the adipogenic activity in serum and serum extracts of genetically obese rodents in comparison to sera from normal animals.

2. METHODS

2.1. *Cell culture and assays*

3T3-Li cells were obtained from Flow Labs (Irvine). They were inoculated in 35 mm plastic dishes at 1000 cells/cm^2 and grown to confluence in Dulbeccos modified Eagle (DME) medium, containing 10% newborn calf serum and glucose (1 g/l), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After reaching confluence differentiation was started by replacing the medium with DME-medium supplemented with fetal calf serum, rodent serum or 10% newborn calf serum together with serum extracts (section 2.2). Insulin was added to 5 μg/ml medium and, for the first 2 days after confluence, isobutylmethylxanthine (0.5 mmol/l). During the exponential growth phase and during differentiation cells were refed every other day with fresh medium. Usually 8 days after having reached confluence the cells showed considerable lipid accumulation and were harvested in 1 ml phosphate-buffered saline. Glycerophosphate dehydrogenase activity was determined as a measure of adipose differentiation in sonicated cell extracts as originally described in [10]. The determination of cellular DNA was done as in [11].

2.2. *Preparation of serum extracts*

The preparation of serum extracts was performed as follows: 5–100 ml serum were dialyzed for 24 h against phosphate buffer 5 mmol/l (pH 7.5). Thereafter the serum was kept in a waterbath for 15 min at 100°C and then stored for 12 h at −80°C. After thawing the preparation was centrifuged at 35000 × *g* for 20 min (heat extract). The clear supernatant was brought to pH 4.5 by

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addition of acetic acid (2 mol/l), thereafter ethanol was added to 40% final conc. After centrifugation at 2000 × *g* the clear supernatant, which contained the serum factor, was lyophilized and stored at −20°C. For appropriate experiments, it was solubilized with NaCl 0.15 mol/l (ethanol serum extract). Protein concentrations in sera and serum extracts were determined as in [12].

3. RESULTS

The effects of native sera on the adipose conversion of 3T3-Li cells are summarized in table 1. Glycerophosphate dehydrogenase activity as a measure of lipogenic activity, increases markedly upon addition of fetal calf serum as compared to newborn calf serum. A similar rise of glycerophosphate dehydrogenase was also seen, when sera of genetically obese C57Bl/KsJ–*db/db* mice or obese fa/fa Zucker rats were used instead of fetal calf serum. Interestingly the sera of phenotypically normal siblings (C57Bl/KsJ− + / + mice and Fa/Fa Zucker rats) as well as sera from normal Swiss mice did not promote adipose conversion of 3T3-Li cells as morphologically visualized by the lack of lipid accumulation, and as measured by the low activities of glycerophosphate dehydrogenase.

Sera of all rodents under study exhibited a marked mitogenic activity as can be seen from the elevated DNA-levels. It was difficult to obtain a clear relationship between adipose conversion and the concentration of native serum, possibly due to the presence of inhibitory mitogenic factors [8]. Therefore a separation of the adipogenic activity from most of the serum proteins had to be established (table 2). After dialysis 75% of serum proteins were removed by heat precipitation.

Table 1

Influence of native sera on adipose conversion of 3T3-Li cells

Donor of serum	GPDH (mU/dish)	DNA (μg/dish)
Newborn calf	11	17
Fetal calf	241	21
Swiss mice (non-obese mice)	7	39
C57Bl/KsJ–*db/db* (diabetes mice)	222	41
C57Bl/KsJ− + / + (control mice)	2	33
fa/fa Zucker rats (obese rats)	182	38
Fa/Fa Zucker rats (control rats)	53	44

Cells were incubated with sera at 5% for 12 days. Glycerophosphate dehydrogenase (GPDH) and DNA were measured as in section 2. Each incubation was performed in duplicate. A representative experiment from at least 3 expts is shown

Table 2

Partial purification of an adipogenic factor from fetal calf serum

	Adipogenic activity (mU GPDH/dish)	Serum or serum fraction (mg protein/ml medium)	Specific activity (mU GPDH/mg protein)
Native serum	173.4	4.1	42.2
Native serum, dialyzed	186.1	3.4	54.7
Heat extract	233.3	1.0	233.3
Ethanol extract	218.4	0.13	1676

Fetal calf serum or serum extracts were added to confluent 3T3-Li cells in the concentrations as indicated. After 8 days, cells were harvested and GPDH activity was measured (section 2). Incubations with heat extract and ethanol extract were carried out in the presence of newborn calf serum (10%), whose adipogenic activity (22 mU GPDH/dish) was subtracted

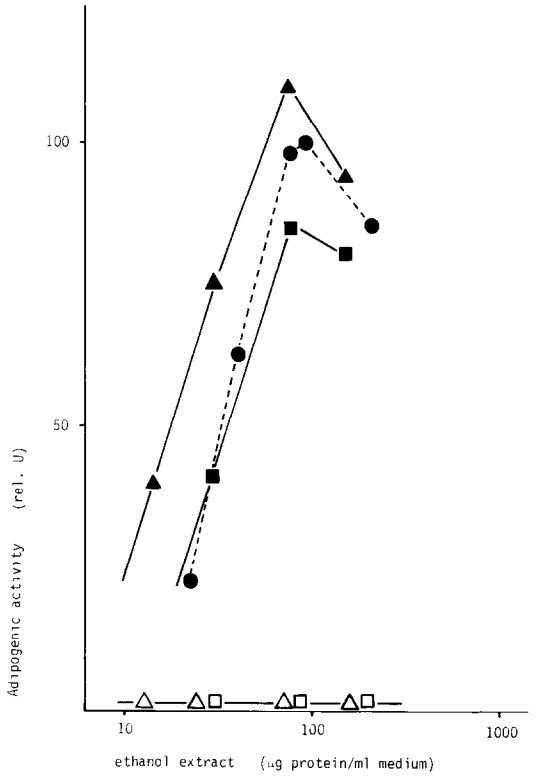

Fig.1. Adipogenic activity in ethanol extracts from serum. Confluent 3T3-Li cells were incubated in the presence of ethanol extracts from fetal calf serum (●---●), fa/fa Zucker rat serum (▲—▲), Fa/Fa Zucker rat serum (△—△), C57Bl/KsJ *db/db* mouse serum (■—■) and C57Bl/KsJ +/+ mouse serum (□—□) for 8 days; see section 2. 100 rel.U of adipogenic activity correspond to GPDH activity found in confluent 3T3-Li cells after incubation for 8 days with ethanol extract from fetal calf serum at 100 µg protein/ml medium. Each point represents the mean of 3 identical experiments.

Treatment of this fraction with ethanol (40%) at pH 4.5, did remove additional 80% of proteins, apparently without loss of adipogenic activity. When expressed as activity/mg serum protein, the adipogenic factor is concentrated ~40-fold.

A clear dose relationship of adipose conversion was obtained with serum extracts (fig.1). Ethanol extracts from fetal calf serum as well as from sera of obese *db/db* mice and from obese fa/fa Zucker rats gave nearly identical parallel dose–response relationships. In contrast ethanolic serum extracts of the lean siblings (+/+ mice and Fa/Fa Zucker rats) were obviously devoid of adipogenic activity in the concentration range studied.

4. DISCUSSION

The importance of a serum factor for the adipose conversion of 3T3-Li cells has been clearly demonstrated [7]. Rather limited information is available as to the exact nature of the differentiation factor. In fetal calf serum the factor is heat stable and not dialyzable [7], implying that it is a protein or that it is bound to serum proteins. Here, a separation of the factor from most of the serum proteins was performed. After heating to 100°C the factor remained fully active in the supernatant, whereas most of the serum proteins were denatured. A further purification was achieved by an ethanol precipitation at pH 4.5. In this preparation the factor has app. M_r ~4000, as shown by chromatography on Sephacryl AcA 202 and is easily destroyed by treatment with subtilisin (not shown).

The demonstration of a clear dose–response relationship between adipose conversion and the amount of native fetal calf serum turned out to be difficult possibly due to the presence of inhibitors [8]. In contrast a dose–response curve could be obtained very easily using ethanolic serum extracts (fig.1). An adipogenic activity could also be demonstrated in native sera or serum extracts from the genetically obese rodents studied. As compared to normal controls, obese animals exhibited elevated blood glucose and plasma triglyceride concentrations as well as a marked hyperinsulinemia (table 3). Due to the fact that adipose conversion also occurred when dialysed serum extracts were added, it seems rather unlikely that glucose is responsible for the adipogenic activity. Insulin-stimulated adipogenesis can also be excluded since even high insulin concentrations added to serum or serum extracts from lean control animals were without any effect. The same is true for serum triglycerides, the concentration of which is highly elevated in fa/fa Zucker rats and which get lost during the preparation of ethanol serum extracts.

The marked parallelism of dose–response relationships obtained with ethanol serum extracts from both *db/db* mice and fa/fa Zucker rats and from ethanol extracts from fetal calf serum, in-

Table 3

Blood concentrations of glucose, triglycerides and insulin in the rodents studied

	Zucker rat		C57Bl/KsJ mouse	
	Fa/Fa	fa/fa	+/+	*db/db*
Blood glucose (mg/100 ml)	100 ± 2.7	210 ± 38	144 ± 4.1	399 ± 22
Serum insulin (μU/ml)	29 ± 3.3	96.4 ± 8.2	15 ± 1.2	134.8 ± 18.3
Serum triglycerides (mmol/l)	1.55 ± 0.05	21.05 ± 1.19	1.25 ± 0.06	2.79 ± 0.32

Figures represent means ± SEM from at least 10 individual determinations

dicates that the adipogenic factors from bovine serum and from rodent serum are very similar.

These data suggest that the adipogenic factor present in fetal calf serum and in the serum of obese rodents is a peptide with hormonal activity. A number of peptide hormones proved to be ineffective or even inhibitory in stimulating the adipose conversion of 3T3 preadipocytes [8]; they include FGF, PDGF, EGF, TRH-LH, LRH, TRH, ACTH, FSH, vasopressin and oxytocin as well as endorphin and enkephalin. However, growth hormone initiated adipose conversion from 3T3 preadipocytes [9,13]. Given that the serum concentrations of growth hormone and prolactin are reduced in obese *db/db* mice and fa/fa Zucker rats [14–17], it is rather unlikely that growth hormone is responsible for the adipogenic activity in the sera of obese rodents. In addition, growth hormone gets lost during the preparation of ethanolic serum extracts.

The fact, that a factor leading to adipose conversion of 3T3-Li cells could be demonstrated in native sera and serum extracts of two species of genetically obese rodents, points to its importance for the development of this kind of obesity, which at least in its early stages, is rather hyperplastic. It seems not unlikely that in these animals the development of obesity is initiated by an abnormally high level of adipogenic serum factor, which leads to an increased adipose differentiation of pre-determined preadipocytes, originating possibly from undifferentiated stem cells, as proposed in [18].

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