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The duration of feeding on a sucrose-rich diet determines variable in vitro effects of insulin and fructose on rat liver triglyceride metabolism

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The aim of the present study was to investigate under controlled conditions the in vitro metabolic effects of fructose and insulin on the triglyceride formation by the isolated perfused livers obtained from hypertriglyceridemic rats that had been fed a sucrose-rich diet for a long-term (15 week) period as compared with those fed sucrose for a short-term (3 week) period. Our findings indicate a significantly higher increase in triglyceride formation by perfused livers of rats fed the sucrose-rich diet for a long-term period in the presence of oleate as a triglyceride-forming substrate (15 weeks, 6-fold increase; 3 weeks, 2-fold increase). Though the contribution of net triglyceride secretion to this increase in triglyceride formation was about twice as high at both durations of feeding on a sucrose-rich diet, a strikingly elevated liver triglyceride accumulation was recorded for a long-term period (15 week, 10-fold increase; 3 week, 4-fold increase). The addition of fructose in the perfusate further increased the output of triglycerides from livers of animals fed the sucrose diet at both durations of feeding. Despite this finding, long-term sucrose led to even higher hepatic triglyceride storage under the present experimental conditions (μmol/liver at 15 week; 318.2 ± 17.2 vs. 63.0 ± 10.0 at 3 week; $P < 0.001$). Insulin in the presence of fructose promoted synthesis of liver triglycerides at both durations of feeding on the sucrose-rich diet. However, different insulin responses have been observed, showing an increase (3-fold) in triglyceride storage only in rats fed the sucrose diet for 3 weeks and an inhibition (32%) on the net triglyceride output only in animals fed the sucrose diet for 15 weeks. Our findings indicate important differences in the in vitro effects of fructose and insulin on hepatic triglyceride formation characterized by an imbalance between secretion and storage depending on the duration of feeding on the sucrose-rich diet. Thus care should be taken when generalizing conclusions on the effects of nutrients or hormones in this nutritionally induced hyperlipemic experimental animal model, since variable metabolic milieu may emerge at different durations of feeding on the diet. (J. Nutr. Biochem. 6:422–430, 1995.)

Keywords: sucrose-rich diet; triglyceride metabolism; liver perfusion; triglyceride secretion; hypertriglyceridemia

Introduction

The intake of a diet high in fructose or sucrose has long been known to result in elevated levels of plasma triglycerides in both humans and experimental animals.[1–3] Until the present time, most of the available animal studies on the mechanisms underlying carbohydrate-induced hypertriglyceridemia were conducted in short- or medium-term (a few weeks) trials.[3–7] Studies on the long-term metabolic consequences of high carbohydrate feeding are limited,[8–11] and, particularly, those concerning very low density lipoprotein-triglycerides (VLDL-TG) secretion and removal are contradictory.[8,9] Thus Schonfeld et al.[9] showed a rise of in vitro VLDL-TG output by livers obtained from rats fed fructose

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(10%) for 103 to 110 days when perfused with oleate at different concentrations as the only triglyceride-forming precursor. However, we were unable[8] to find a significant increase of the in vivo triglyceride output in normal Wistar rats fed for 15 weeks on an isocaloric 63% sucrose-rich diet (SRD).

Hepatic triglyceride synthesis and the rate of VLDL-TG output are determined by plasma- or perfusate-free fatty acid concentrations as well as by the precedent nutritional and hormonal status of the animal.[12,13] Insulin is believed to play a key role in the development of hypertriglyceridemia. Its lipogenic role is supported not only by the relationship between hyperinsulinemia and VLDL-TG production in vivo,[14] but also by findings that insulin, under specific conditions, promoted lipogenesis and enhanced VLDL-TG secretion from in vitro perfused livers.[15,16] The latter observation however, is not a consistent finding, since the precise effects of insulin seem to be critically dependent upon the conditions of perfusion.[16]

Our own laboratory reported over the past few years[17,18] that feeding a high sucrose diet to rats for long time periods resulted in a multiphasic metabolic syndrome, characterized by three sequential periods. In the induction period (2 to 5 weeks on the SRD), hypertriglyceridemia appeared to be the combined result of increased liver VLDL-TG secretion and decreased plasma triglyceride clearance. Both hyperinsulinemia and glucose intolerance were present in this period.[8,17] Similar to others,[9] we also documented spontaneous normalization of those parameters in the medium term (5 to 8 weeks) on the SRD (adaptation period). However, hypertriglyceridemia as well as glucose intolerance reappeared from the 8th week on the SRD onward (recurrence period), despite normal insulin responses to i.v. glucose and only a slight increment of insulin secretion in vitro recorded at this time period.[17,18] Thus, liver triglyceride metabolism in SRD-fed rats may differ depending on (1) the amount of carbohydrate and the duration of the diet, and (2) the metabolic and hormonal milieu present when variable time periods on the diet are considered.

The present work was designed to study, under controlled conditions, the regulatory effects of fructose and insulin on the triglyceride accumulation and secretion in the isolated perfusion of livers from rats fed a sucrose-rich diet for a long-term period (15 weeks) when compared with those fed the SRD for a short-term period (3 weeks).

Methods and materials

Male Wistar rats, weighing 190 to 220 g, were randomly divided in two groups according to diets: the STD group, standard laboratory rodent chow (Ralston Purina, St. Louis, MO USA) containing per weight 62.5% starch (corn, sorghum, wheat, oats, barley), 22.5% protein, 3.5% fat, 6% fiber, 1% vitamin mixture, and 4% salt mixture as stated by the manufacturer; and SRD group, sucrose-rich diet containing per weight 63% sucrose, 17% casein, 5% corn oil, 10% cellulose, 1% vitamin mixture (AIN-76A), 3.5% salt mixture (AIN-76A), and 0.5% choline chlorhydrate. Both diets provided approximately 15.28 kJ/g of chow. Preparation, contents, and handling of both diets have previously been reported in detail.[17] We have recently shown[19] that rats fed the standard laboratory rodent chow compared with rats maintained on a semisynthetic starch diet for an experimental period of up to 15 weeks did not show significant differences in daily food intake and weight gain over the experimental period; basal plasma glucose, insulin, triacylglycerol, and free fatty acid levels; or liver triacylglycerol content.

These results show that rats fed a chow diet, despite the minor differences in the components, behave similarly to rats fed a semisynthetic starch diet for the time period studied. The composition of the semisynthetic starch diet was identical in all the components to the SRD with the only exception being that sucrose was replaced by starch. Food was available ad libitum, and the rats were maintained on their respective diets for 3 or 15 weeks. The weight of each animal was recorded twice a week. In a separate experiment, the individual caloric intake and weight gain of at least 10 animals in each group were assessed twice a week. On the day of the experiment, food was removed at 7:00 a.m., unless otherwise indicated, and experiments were performed between 9:00 a.m. and noon.

Isolated liver perfusion

Fed rats were anesthetized intraperitoneally with pentobarbital (60 mg/kg of body weight), and in situ perfusion of isolated liver was carried out by the recirculation technique described by Hems et al.[20] Briefly, the perfusate (initial volume 120 mL) consisted of 90 mL of Krebs–Henseleit bicarbonate buffer (pH 7.4), 25 mmol/L of glucose, 3% (wt/vol) fatty acid-free[21] bovine serum albumin (BSA) (fraction V), and 30 mL of washed bovine erythrocytes. The medium was gassed continuously with 95% O_2 + 5% CO_2. Livers were perfused at constant average flow rates of 1.2 mL/(min · g of liver) and pO_2 of 12 to 13 kPa for a total of 140 min. Livers were perfused in the absence or presence of exogenous fatty acids as substrate. In the last case, a fatty acid complex containing 68 g/L of purified BSA and 12 mmol/L of oleic acid was prepared.[22] After a 20 min equilibration period, a rapid priming infusion of the complex (72 μmol oleic acid) was followed by a constant rate infusion of 165 μmol oleic acid/hr during 2 hr, to maintain a constant concentration of nonesterified fatty acids within the range of 0.5 to 0.7 mmol/L.

When studying the in vitro effects of fructose on triglyceride secretion, fructose was dissolved in 0.9% NaCl and infused after the equilibration period as a rapid priming dose (4 mmol/L), followed by a constant infusion with the oleate-albumin complex, in order to achieve a final concentration between 1.4 and 2.5 mmol/L. The upper limit of this range has previously been recorded in the portal vein of rats absorbing oral fructose.[23]

In the experiments designed to study the effects of fructose plus insulin on the triglyceride secretion rate, glucagon-free insulin (Sigma Chemical CO, St. Louis, MO USA) was dissolved in HCl (pH 3), diluted with 0.9% NaCl to reach pH 6, and infused at a rate of approximately 55 pmol(h · g of liver). Reproducible perfusate insulin concentrations (1.45 to 2.15 nmol/L) were attained in all experiments. All the other components of the perfusion medium, including the oleate–albumin complex and fructose, were kept as mentioned above.

Aliquots of the perfusate (2 mL) were obtained for chemical analysis at the end of the equilibration period and at 30 min intervals thereafter in all experiments. Viability of the perfused liver was assessed by liver color, the ratio of liver wet/dry weight, and the release of glutamate-pyruvate transaminase and glutamate-oxalacetate transaminase into the perfusion medium. The liver wet/dry weight ratios obtained was 3.51 ± 0.02 (±SEM of 80 perfusions). Values of glutamate-pyruvate transaminase were as follows (±SEM, U/[h · g of liver]): 3 weeks on diet: SRD, 0.04 ± 0.02 vs. 0.04 ± 0.01 in STD; 15 weeks on diet: SRD, 0.04 ± 0.01 vs. 0.03 ± 0.01 in STD and glutamate-oxalacetate transaminase (±SEM, U/(h · g of liver): 3 weeks on diet: SRD, 0.09 ± 0.01 vs. 0.07 ± 0.01 in STD; 15 weeks on diet: SRD, 0.08 ± 0.01 vs. 0.07

± 0.01 in STD. These findings are in agreement with those formerly reported by us as well as other investigators.[24,25]

Analytical

Lipids were extracted from liver homogenates and cell-free perfusate, and the triglyceride concentration was analyzed as described previously.[26] Hepatic content and output of triglyceride recorded after the perfusion was compared with the content and output obtained from livers perfused with the medium without the triglyceride-forming substrates over the normal duration of perfusion experiments.

Acetoacetate, 3-hydroxybutyrate and fructose in protein-free perfusate were determined by enzymatic methods.[27] The net production of ketone bodies (acetoacetate plus 3-hydroxybutyrate) and triglycerides were corrected for their concentrations in the perfusate at time 0 and also for sampling losses and infusate additions at every time point between 30 and 120 min.

Since the stimulation of triglyceride formation probably mirrors the portal rather than the peripheral concentration of insulin, we measured the insulin as well as the glucagon and glucose concentrations in portal circulation. Two hours after the beginning of the light period, animals were anesthetized and kept in a box at 37°C throughout the experiment. Immediately after laparotomy, blood samples were withdrawn from the portal vein, keeping the time-dependent operational stress at a minimum. The samples were collected in ice-cold centrifuge cups containing 1000 IU of Trasylol and 2 mg of EDTA/ml of blood and centrifuged at 3,500 *g* for 15 min. The supernatant was frozen in liquid nitrogen and kept at −30°C for no longer than 3 weeks before being assayed. Immunoreactive insulin (IRI) was measured by the method of Herbert et al.[28] The IRI assay was calibrated against rat insulin Novo Nordisk, Bagsvaerd, Denmark). The immunoreactive glucagon (IRG) assay was performed with the Diagnostic Products Corporation kit. Glucose was determined by a spectrophotometric method.[27]

In a separate group of animals handled in the same way as mentioned above, blood samples were obtained from the jugular vein, placed immediately in chilled tubes, centrifuged at 4°C, and plasma or serum was rapidly aliquoted. Plasma was immediately deproteinized for the determination of ketone bodies.[27] Serum was stored for no longer than 3 days at −20°C and assayed by spectrophotometric methods for triglycerides[26] and free fatty acids.[29]

Results are expressed as mean ± SEM. Statistical significance were determined by Student's *t*-test or by analysis of variance (ANOVA) followed by the Scheffe's test.[30] Differences having *P* values less than 0.05 were considered to be statistically significant.

Reagents

Enzymes, substrates, and coenzymes were purchased from Sigma Chemical Company or from Boehringer Mannheim Biochemicals (Indianapolis, IN USA). Highly purified rat insulin was kindly provided by the Novo Company. All other chemicals were of reagent grade.

Results

Animals fed on the SRD presented an average food consumption (kJ/day) similar to animals fed the standard chow (STD) throughout the 15 week observation period (±SEM; 303.6 ± 10.8 vs. 305.2 ± 6.2, respectively). Average weight gain (g/day) was also similar (2.20 ± 0.10 vs. 2.10 ± 0.15, respectively) indicating that the SRD was readily consumed and utilized by the experimental animals.

Effects of the SRD on plasma levels of triglycerides, free fatty acids, total ketone bodies, glucose, insulin, and glucagon

Plasma triglycerides (TG) and free fatty acids (FFA) are significantly elevated after 3 weeks on the SRD (±SEM; TG, SRD: 1.73 ± 0.05 vs. 0.58 ± 0.13 mmol/L in STD, $P < 0.01$; FFA, SRD: 521.0 ± 38.9 vs. 284.0 ± 16.3 μmol/L in STD, $P < 0.01$). This hyperlipemic effect was further enhanced when the SRD was given for 15 weeks (TG, SRD: 2.66 ± 0.19 vs. 0.60 ± 0.05 mmol/L in STD, $P < 0.01$; FFA, SRD: 734.0 ± 55.8 vs. 286.0 ± 30.0 μmol/L in STD, $P < 0.01$). In contrast, plasma total ketone bodies were not significantly different from those observed in animals fed the STD at either feeding period. Values recorded were as follows: (±SEM, μmol/L) 3 weeks on diet SRD, 168.0 ± 15.0 vs. 170.0 ± 13.0 in STD; 15 weeks on diet SRD, 192.0 ± 17.6 vs. 196.0 ± 20.8 in STD.

Portal vein levels of glucose, insulin, and glucagon were assayed 2 hr after the beginning of the light period. Plasma glucose concentrations were significantly higher in rats on the SRD for either 3 or 15 weeks. Values recorded were as follows: (±SEM, mmol/L) 3 weeks on SRD, 7.30 ± 0.15 vs. 6.48 ± 0.13 in STD, $P < 0.01$; 15 weeks on SRD, 7.90 ± 0.09 vs. 6.63 ± 0.12 in STD, $P < 0.01$). A 3-fold increase in insulin levels was recorded in the rats fed the SRD for 3 weeks when compared with controls (±SEM; nmol/L, SRD: 4.13 ± 0.10 vs. 1.31 ± 0.08 in STD, $P <$ 0.01). However, similar portal insulin levels were observed at 15 weeks on the SRD (SRD, 1.93 ± 0.47 vs. 1.38 ± 0.10 in STD, *P* NS). Since portal glucagon levels remained unchanged by the SRD at either time period (±SEM; pmol/L, 3 weeks on SRD: 45.0 ± 3.2 vs. 44.0 ± 2.8 in STD; 15 weeks on SRD: 54.0 ± 2.3 vs. 46.0 ± 3.2 in STD), the insulin/glucagon (I/G) molar ratio was three times higher in the SRD group after 3 weeks on the experimental diet (±SEM, SRD: 92.0 ± 3.9 vs. 29.0 ± 1.8 in STD, $P <$ 0.01). A rather different hormonal picture emerged after 15 weeks on the SRD. At this time period, the I/G molar ratio recorded in SRD fed rats was similar to that observed in age-matched controls fed the STD. Values recorded were as follows: (±SEM) SRD: 36.0 ± 2.5 vs. 30.0 ± 2.4 in STD.

Triglycerides secretion from the isolated perfused rat liver

Figure 1 (*A* and *B*) shows that the accumulative secretion of TG was linear throughout the entire perfusion period in both dietary groups. In the absence of TG-forming substrates, the triglyceride secretion by the SRD livers was significantly augmented without respect to the duration of feeding. Values obtained were as follows: (±SEM; μmol/[liver · 2 hr]) 3 weeks on diet SRD: 13.84 ± 0.17 vs. 8.12 ± 0.61 in STD, $P < 0.001$; 15 weeks on diet SRD: 16.26 ± 1.17 vs. 9.69 ± 0.50 in STD, $P < 0.001$.

When only oleate was present in the perfusion medium, livers from animals fed the SRD for either 3 or 15 weeks secreted about twice the amount of triglycerides than their respective age-matched control (STD) livers.

The presence of fructose in addition to oleate in the

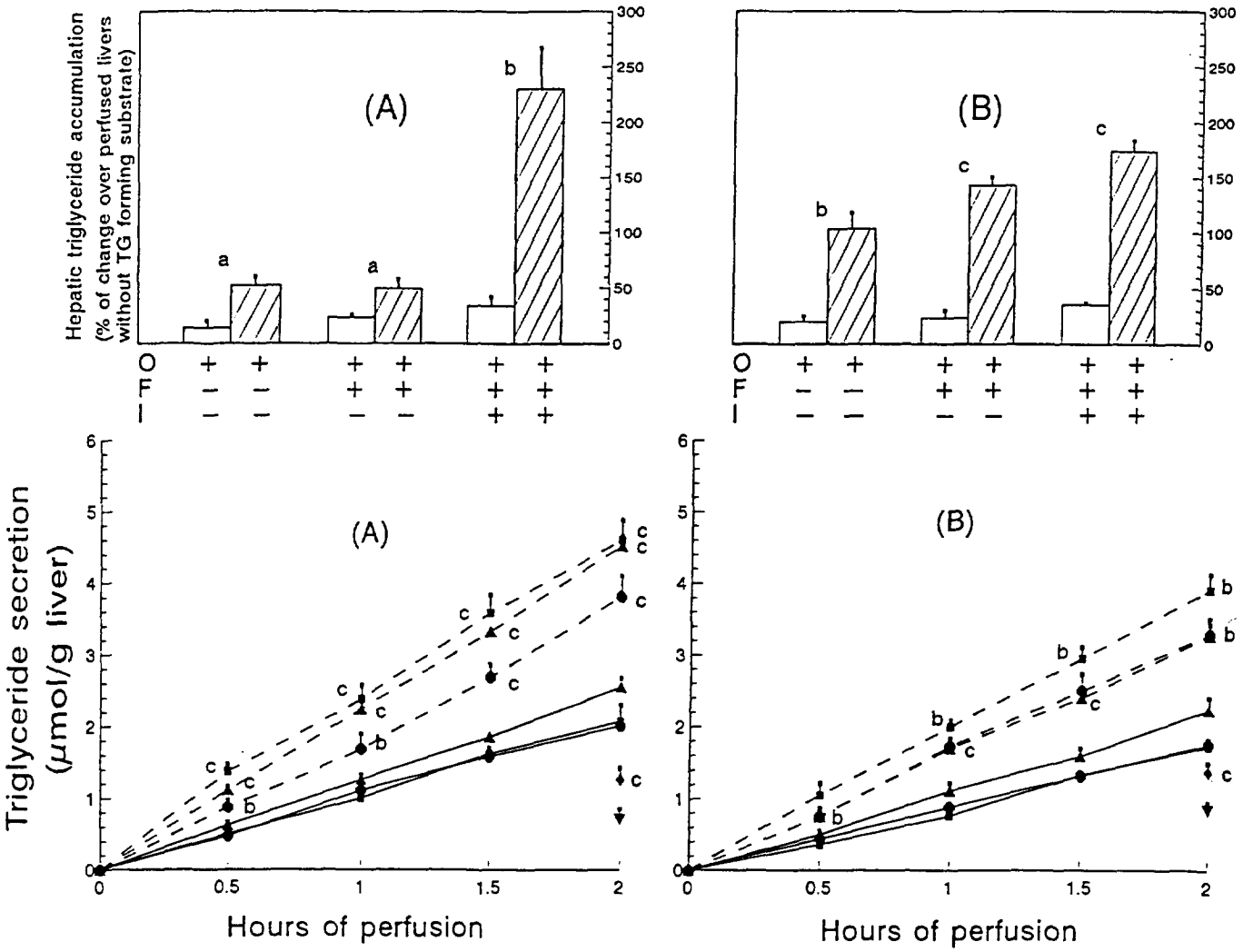

Figure 1 Triglyceride secretion from isolated perfused rat livers. Livers from STD- and SRD-fed rats were perfused as described in text. Data presented are mean ± SEM of the cumulative secretion of triglycerides at 30 min intervals. The following additions were made to the perfusate as described in Methods and materials Non triglyceride forming substrate: ▼ STD; ♦ SRD. Oleate (O): —●— STD; – –●– – SRD. Oleate + fructose (F): —■— STD; – –■– – SRD. Oleate + fructose + insulin (I): —▲— STD; – –▲– – SRD. Time zero was the time at which infusion of the above substrates and insulin was started. Animals were fed with STD □ and SRD ▨ for either 3 weeks (*Figure 1A*) or 15 weeks (*Figure 1B*). At least seven perfused livers were processed in each experiment. (Inset) Hepatic triglyceride accumulation as % of change/perfused livers without triglyceride-forming substrates. SRD values designated as a, b, and c are significantly different from the STD values at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

perfusate further increased the secretion of triglycerides from livers of rats for either 3 or 15 weeks on the SRD. In contrast, fructose by itself had no significant effects on the triglyceride secretion from livers of rats fed the STD.

Livers from animals fed the SRD for 15 weeks showed a small but significant decrease in the triglyceride secretion when perfused with the additional presence of insulin. However, under those experimental conditions, no changes in the triglyceride secretion was seen in livers of rats fed the SRD for 3 weeks, while an approximately 30% increase was recorded from livers of control animals. Since the perfused liver continually removes some lipids from the perfusate during recirculation, this observed accumulation of triglycerides in the perfusate should be taken as net accumulation.

In the absence of TG-forming substrate, only livers from rats fed a SRD for 15 weeks show a significantly higher triglyceride content. When perfused with oleate, fructose, and insulin, an important increase in liver triglyceride content at the end of the perfusion period was observed in animals fed the SRD for either 3 or 15 weeks, but only livers donated by rats fed the SRD during 15 weeks showed an increase in their triglyceride content after being perfused with either oleate alone or oleate plus fructose. It should be pointed out that the triglyceride content of livers obtained from rats fed the STD was not significantly increased after being perfused with oleate, oleate plus fructose, or oleate plus fructose plus insulin (inset *Figures 1A* and *1B*).

Table 1 shows the balance of triglyceride formation by livers of rats fed the SRD or STD for either 3 or 15 weeks in the presence of oleate as the single substrate. Triglyceride balance was estimated from the mass of triglyceride accu-

Table 1 Effects of diet and time on diet on triglyceride formation in perfused livers

Diet	Time on diet (weeks)	Hepatic triglyceride content: Without substrate (A) (μmol/liver)	Hepatic triglyceride content: With oleate (B) (μmol/liver)	Net hepatic change (B − A) (μmol/liver)	Net triglyceride output (C) (μmol/[liver · 2 hr])	Triglyceride balance (B − A + C) (μmol/liver)
STD	3	96.7 ± 1.3[a]	110.2 ± 5.7[a]	13.5 ± 6.1[a]	12.5 ± 2.3[a]	26.0 ± 6.8[a]
SRD	3	122.0 ± 3.5[c]	185.9 ± 9.1[c]	63.8 ± 9.8[c]	28.9 ± 3.6	92.8 ± 10.5[c]
STD	15	107.6 ± 5.8[b]	128.9 ± 5.3[b]	21.3 ± 5.3[b]	15.5 ± 0.7[b]	36.4 ± 4.8[b]
SRD	15	222.9 ± 25.1	453.4 ± 32.0	230.6 ± 31.9	26.5 ± 1.0	257.7 ± 32.3
				Two-way ANOVA (2 × 2)*		
Diet		S	S	S	S	S
Time		S	S	S	NS	S
Diet × time		S	S	S	S	S
Residual mean square		430.4	2131.0	2063.3	35.3	2139.0

Livers were perfused with Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 25 mmol/L of glucose, 3% (wt/vol) bovine serum albumin fraction V and 25% (vol/vol) washed bovine erythrocytes. After a 20 min equilibration period, the oleate–albumin complex was added as substrate. Liver and perfusate triglyceride levels were analyzed as previously described. Net triglyceride output (C) is the difference between the TG output from livers perfused with and without the triglyceride-forming substrates (for details see Methods and materials). Values are given as mean ± SEM. Seven animals were perfused in each experiment. *S: significant ($P < 0.05$); NS: not significant. $P < 0.05$ by Scheffe's test when compared: [a]STD vs SRD at 3 weeks; [b]STD vs SRD at 15 weeks; and [c]SRD at 3 weeks vs. SRD at 15 weeks.

mulation in both the liver (B-A) and in the perfusate (C) at the end of the perfusion period, respectively, to livers perfused without TG-forming substrate. As can be seen, although the balance of triglyceride formation was significantly increased in livers donated from animals fed the SRD, this effect was more pronounced after 15 weeks on the experimental diet. Whereas the contribution of net triglyceride output to this net TG balance was considerably important in livers of SRD-fed rats for 3 weeks, the accumulation of TG in livers of rats fed the SRD for 15 weeks was the major influence on the net TG balance.

Table 2 depicts the influence of insulin on triglyceride balance when added in the presence of oleate and fructose as compared with the effects of oleate plus fructose alone. Effects of the diet itself, time on the diet, and the addition of fructose and insulin as well as their interaction are analyzed by three-way ANOVA (middle panel). Insulin increased approximately three times the balance of triglyceride formation in livers from rats fed SRD for 3 weeks. Since no significant changes were recorded in the net triglyceride output, this resulted in an accumulation of triglycerides in the liver. The rats fed the SRD for 15 weeks showed a small increase in their balance of triglyceride formation when exposed to insulin, probably as a result of the observed decrease in the net triglyceride output.

The lack of an insulin effect on net triglyceride output from livers of SRD-fed rats when in the presence of fructose indicated the need for analyzing the isolated effects of insulin and time on SRD livers through two-way ANOVA (Table 2, bottom panel). As can be seen, the hormone decreased the net triglyceride output from livers of SRD-fed rats at 15 weeks, whereas it actually increased the triglyceride output from livers of rats fed STD for either 3 or 15 weeks.

The cumulative production of total ketone bodies of perfused livers obtained from STD- or SRD-fed rats is shown in *Figures 2A* and *2B*. When oleate was infused alone, ketogenesis, as indicated by total ketone bodies production, was significantly lower in livers obtained from rats fed the SRD for either 3 or 15 weeks when compared with that seen in age-matched STD controls. The perfusion with fructose in addition to oleate did not change the net output of ketone bodies from either SRD or STD livers. However, a significant antiketogenic effect was observed in perfused liver donated by either SRD or STD rats when insulin, in addition to fructose and oleate, was present in the perfusate. The length of time on the sucrose-rich diet did not influence these reported changes in ketogenesis induced by either oleate alone, oleate plus fructose, or oleate plus fructose plus insulin.

Discussion

It is generally agreed that the hypertriglyceridemia induced by feeding rats diets rich in sucrose or fructose is a result of either or both an overproduction of VLDL-TG or a reduction of TG lipolysis by the endothelial lipases. Although in a short-term feeding (days to a few weeks) this conclusion is supported by several studies in vivo and in vitro[3,4,7,10] including ours,[8] the situation is rather controversial when the period of feeding a sucrose-rich diet is extending for up to 15 weeks (long-term).[8,9]

We have investigated using isolated liver of rats fed an SRD under defined composition of perfusion medium, the effect of duration of feeding (short, 3 weeks, vs. long, 15 weeks) on triglyceride metabolism regulated by fructose and insulin.

In the absence of TG-forming substrates, the triglyceride output depends on the novo synthetized fatty acids and on the hepatic stores of triglycerides. Under these conditions, the sucrose-fed livers secreted about 2-fold more triglyceride than the control (*Figure 1*) during both dietary periods. However, in contrast to our own former findings in vivo,[8] in vitro perfusion of livers obtained from rats fed a sucrose-rich diet for 15 weeks with oleate showed a significant increase in triglyceride secretion when compared with ap-

Table 2 Effects of insulin and fructose on triglyceride formation in perfused livers of rats fed short- (3 weeks) and long- (15 weeks) term with a sucrose-rich diet

Diet	Time on diet (weeks)	Additions to the perfusion medium	Hepatic triglyceride content (B) (μmol/liver)	Net hepatic change (B − A) (μmol/liver)	Net triglyceride output (C) (μmol/[liver · 2 hr])	Triglyceride balance (B − A + C) (μmol/liver)
STD (7)	3	F	119.5 ± 2.1[a]	22.8 ± 2.3	13.1 ± 0.8[ah]	35.9 ± 2.6[a]
SRD (7)	3	F	184.9 ± 9.2[fi]	63.0 ± 10.0[fi]	43.1 ± 2.1	106.1 ± 9.6[fi]
STD (7)	15	F	132.8 ± 6.6[b]	25.2 ± 7.1[b]	17.6 ± 0.5[b]	42.8 ± 7.3[b]
SRD (7)	15	F	541.1 ± 15.9[k]	318.2 ± 17.2	40.2 ± 3.8[k]	358.4 ± 18.0
STD (7)	3	F + I	129.7 ± 7.0[c]	32.9 ± 7.7[c]	22.4 ± 1.2[c]	55.4 ± 7.4[c]
SRD (7)	3	F + I	401.7 ± 41.3[g]	280.8 ± 45.3	44.2 ± 1.1[g]	324.9 ± 37.7
STD (7)	15	F + I	145.6 ± 1.8[d]	38.0 ± 2.0[d]	23.6 ± 0.6	61.6 ± 2.0[d]
SRD (7)	15	F + I	610.4 ± 28.0	387.5 ± 22.2	27.3 ± 1.7	414.8 ± 17.9
				Three-way ANOVA (2 × 2 × 2)*		
Diet			S	S	S	S
Time			S	S	S	S
Additions			S	S	NS	S
Diet × time			S	S	S	S
Diet × additions			S	S	S	S
Time × additions			S	S	S	S
Diet × time × additions			S	S	S	S
Residual mean square			2023.4	2389.7	24.8	2407.3
				Two-way ANOVA (2 × 2)* of triglyceride output from livers of SRD fed rats		
Time					S	
Additions					S	
Time × additions					S	
Residual mean square					40.0	

Livers were perfused with Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 25 mmol/L of glucose, 3% (wt/vol) bovine serum albumin fraction V and 25% (vol/vol) washed bovine erythrocytes. After a 20 min equilibration period, the oleate-albumin complex and fructose (F) or fructose + insulin (F + I) were infused. Liver and perfusate triglyceride levels were analyzed as described previously. Net triglyceride output (C) is the difference between the TG output from livers perfused with and without triglyceride-forming substrates (for details see Methods and materials). Values are given as mean ± SEM. Number of rats per group is shown in parentheses. (A) Values have been given in *Table 1*. *S: significant ($P < 0.05$); NS: not significant. $P < 0.05$ by Scheffe's test when compared: [a]STD vs. SRD at 3 weeks with the addition of F; [b]STD vs. SRD at 15 weeks with the addition of F; [c]STD vs. SRD at 3 weeks with the addition of F + I; [d]STD vs. SRD at 15 weeks with the addition of F + I; [e]3 weeks vs. 15 weeks within the STD with the addition of F; [f]3 weeks vs. 15 weeks within the SRD with the addition of F; [g]3 weeks vs. 15 weeks within the SRD with the addition of F + I; [h]F vs. F + I in the STD group at 3 weeks; [i]F vs. F + I in the SRD group at 3 weeks; [j]F vs. F + I in the STD group at 15 weeks; and [k]F vs. F + I in the SRD group at 15 weeks.

propriate age-matched control livers donated by rats fed the STD. Moreover, these results indicate that the TG secretion is dependent upon the presence of oleate in the perfusate. It is to be pointed out that this increased triglyceride secretion was recorded at perfusate-free fatty acid levels similar to those attained in vivo in the plasma of rats in the fed state kept on an SRD for the same period of time.

The addition of fructose to the perfusate in concentrations comparable to that seen in vivo in the portal vein of SRD animals in the fed state resulted in a further increase in triglyceride output. These results are similar to those observed during short-term feeding (3 weeks) on the SRD by Mayes et al.[31] Fructose had no stimulatory effects on triglyceride secretion from livers donated by control animals fed the standard chow.

A significant increase in triglyceride content was observed at the end of the perfusion period in livers of animals fed a SRD for either 3 or 15 weeks. Considering short-term feeding, these findings are in agreement with previous reports of Boogaerts et al.[32] in cultured hepatocytes from rats fed sucrose-rich diet (30%) for 3 days and Yamamoto et al.[4] in perfused livers from rats fed the SRD for 10 days and indicate that under the present experimental conditions the triglyceride export system became saturated and that any further uptake or synthesis of triglycerides resulted in an increased cellular storage. However, a striking difference has been noted in the relative contribution of the net TG accumulation over the net TG balance in the livers from animals fed the SRD for 15 weeks as compared with those obtained from rats fed for 3 weeks either without substrate, with oleate, or with oleate plus fructose. Thus, these findings clearly show the relationship between the worsening hepatic triglyceride accumulation and the duration of the diet. Moreover, Schonfeld et al.,[9] working with perfused livers of rats fed for 103 to 110 days a diet containing 10% fructose, were unable to show any significant increase in liver triglyceride content. Since their animals were fed for a similar period of time, the lower amount of the fructose used may explain the differences with our present results.

It may also be assumed that the metabolic and hormonal conditions prevailing in livers of animals fed the SRD may be significantly different when variable time periods (3 or 15 weeks) on the diet are considered. Thus, we have previously reported[8] that the VLDL-TG secretion in vivo after

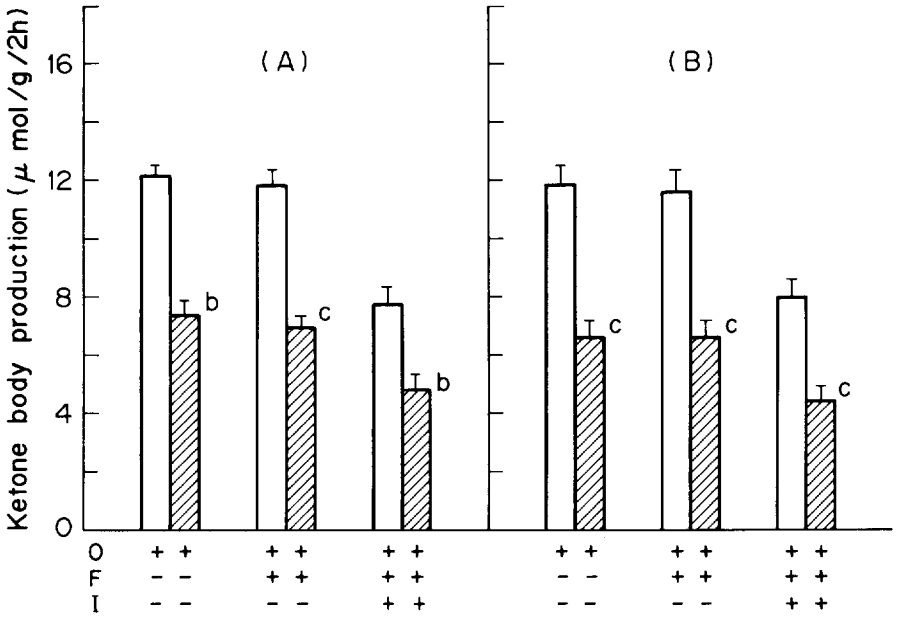

Figure 2 Effects of oleate (O), fructose (F), and insulin (I) on hepatic ketone bodies production. Livers from rats fed STD □ and SRD ▨ were isolated and perfused in vitro as described in Methods and materials. Results are expressed as mean ± SEM of at least seven experiments in each group. Animals were fed the SRD for either 3 weeks (*Figure 2A*) or 15 weeks (*Figure 2B*). SRD values designated b and c are significantly different from the STD values at $P < 0.01$ and $P < 0.001$, respectively.

Triton WR 1339 treatment behaved differently in rats fed the SRD for 3 versus 15 weeks. While short-term feeding resulted in a large increase in VLDL-TG output when compared with age-matched controls fed the STD, no significant increases in triglyceride secretion could be obtained after long-term SRD feeding. However, our finding of a sustained and progressive hypertriglyceridemia in the presence of elevated liver triglyceride content, suggested that a combination of an impaired triglyceride export system and defective removal mechanisms are operating in vivo in animals fed the SRD for 15 weeks.

Finally, since we are unaware of published data on the actual composition of VLDL lipoprotein at this precise stage of high sugar intake, we cannot rule out the possibility that an impaired Apo B availability and/or assembling with the lipid fraction could be underlying the increased lipid deposition.[33–35]

Our data show that the addition of insulin to the perfusate diminished the net triglyceride output from livers of rats fed the SRD for 15 weeks. However, insulin did not change the triglyceride output from those fed the SRD for 3 weeks but rather significantly increased their liver triglyceride contents. These findings are showing additional evidence of different response to effectors or insulin when the duration of feeding is considered.

It is generally agreed that in a broad sense insulin stimulates hepatic triglyceride synthesis as evidenced by animal experiments using cultured hepatocytes,[36] liver slices,[37] or perfused liver.[23] In contrast, there is conflicting information on whether insulin enhances[15,16] or inhibits[38] VLDL-TG secretion from the liver. Working in the in vitro isolated perfused liver of rats fed for 9 days with a sucrose-rich diet, Reaven et al.[13] reported that the degree to which triglyceride output is stimulated by a given perfusate-free fatty acid concentration varies considerably depending on the preexisting in vivo levels of plasma insulin. Thus, the effects of insulin on VLDL-TG secretion may be highly dependent on the hormonal and metabolic conditions prevailing within the hepatocyte at the time of obtaining the liver for the experiment. Since the metabolic conditions in livers obtained from animals fed the SRD for either time period facilitated accelerated lipogenesis, it may be assumed that the effects of insulin would preferentially increase cellular triglyceride storage rather than promote triglyceride secretion.

A different metabolic response to insulin was recorded in livers donated by control rats fed the standard chow. Since insulin actually increased the net triglyceride output into the perfusate, net triglyceride accumulation within the liver was thus not observed. These results in STD-fed animals are in agreement with previous data shown by Topping et al.[23]

The graded effects on the balance of triglyceride formation in livers donated by SRD rats was: oleate plus fructose plus insulin > oleate plus fructose > oleate. Livers from rats fed the STD showed the same qualitative graded pattern.

A comparable ketogenic response was observed in livers obtained from rats fed the SRD for 3 or 15 weeks when perfused with oleate or oleate plus fructose, in agreement with previous observations by Yamamoto et al.,[4] in perfused livers of rats fed a sucrose-rich diet for just 10 days. Moreover, insulin promoted a similar antiketogenic effect on livers from animals fed the SRD for 3 or 15 weeks.

Present data indicate that the length of time on the diet is a critical factor in determining the development and worsening of the dyslipidemia induced by a high sucrose intake because of evolving changes in the hormonal and metabolic milieu. Under these experimental conditions, liver triglyceride metabolism is characterized by an imbalance between triglyceride synthesis and secretion, which finally leads to increased liver triglyceride storage.

In synthesis, as a result of SRD, hormonal and substrate effectors lead to several changes involving the hepatic lipid metabolism depending on the duration of feeding. Com-

pared with 3 weeks, triglycerides produced from livers of rats fed the SRD for 15 weeks are preferentially stored. Moreover, the in vitro addition of insulin produces a genuine inhibitory effect on triglyceride output only in animals fed the SRD for 15 weeks. Thus, care should be exerted on generalizing conclusions on the effects of nutrients or hormones in this nutritionally induced hyperlipemic experimental animal model, since variable metabolic milieu may emerge at different time length periods on the diet.

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