Fructose diet and VEGF-induced plasma extravasation in hamster cheek pouch[1]

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KEY WORDS diabetes mellitus; capillary permeability; endothelial growth factors; fructose

ABSTRACT

AIM: To determine in the hamster cheek pouch whether or not the changes in plasma extravasation induced by vascular endothelial growth factor (VEGF) could be affected by fructose diet. **METHODS:** Hamsters were subjected to control drinking water or to water containing fructose (10 %) for 18 weeks. **RESULTS:** The fructose diet induced a small but significant increase in glycemia (0.80±0.11 and 1.09±0.15, n= 8 and 9 for control and fructose-treated animals, respectively, P<0.05). Bradykinin-induced plasma extravasation was not affected by the fructose diet while the effects of VEGF were markedly increased (maximal number of leakage sites: 76±20 and 126±55, n = 8 and 9 for control and fructose-treated animals, respectively, P<0.01). **CONCLUSION:** Even moderate changes in glycemic levels can produce profound alteration in the VEGF response.

INTRODUCTION

Vascular endothelial growth factor (VEGF) has been independently isolated as both an endothelial growth factor and as a vasopermeability factor. The designation VEGF currently includes a family of six known members that can interact with at least six different membrane "receptors[1,2]." VEGF itself consists of five polypeptides of different sizes that derive from the same gene by alternative splicing but $VEGF_{165}$ is likely to be the predominant molecular species[1,2]. VEGF induces an increase in vascular permeability *in vitro*[3] and in various vascular beds from different species *in vivo*[4] including the hamster cheek pouch[5].

In both type-I and type-II diabetes, micro- and macro-angiopathies are a major cause of morbidity and mortality. The pleiotropic nature of these events is far from understood. However, an earlier sign of vascular dysfunction appears to be an increase in vascular leakage linked to hyperglycemia and increase in VEGF production[6,7]. Type-II diabetes (NIDDM) is characterized by hyperglycemia, hyperinsulinemia, insulin resistance, and subsequent glucose intolerance. Most of these features are reproduced in various animal species by a diet rich in fructose[8-10]. The purpose of this study was to determine, in the hamster, the metabolic changes produced by a diet moderately enriched in fructose and whether or not VEGF-induced plasma extravasation could be affected.

MATERIALS AND METHODS

Male Harlan golden hamsters (60-80 g; Charles River, France) were housed four by four in a temperature and light cycle-controlled colony room. They were fed *ad libitum* with standard diet (protein: 23 %, glucid: 51 %, and lipid: 3.5 %) and had free access to tap drink-

[1]Project integrally financed by the Institut de Recherches Servier.
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Received 2002-07-25 Accepted 2002-12-30

ing water or to *D*-fructose dissolved in drinking water (10 %) during 18 weeks (n=35 in each group).

Metabolic parameter Global water consumption was measured from week 1 to week 18. After 18 weeks of treatment, animals were weighed, and after anaesthesia with isoflurane, blood was taken from the retro-orbital sinus in order to measure non-fasting plasma glucose (GOD-PAP, Boehringer), insulin (Phasadeph Insulin RIA, Pharamacia and Upjohn), and glycosylated hemoglobin (HbA_{1c}, Unimate 3 HBA1C, Roche). Thereafter, the treatment was maintained and ten animals from each group were taken at random to study plasma extravasation during the following days. Finally, in half of the remaining animals, plasma triglycerides (Unimate 7 TRIG, Roche) and total cholesterol was further assessed (Unimate 7 CHOL, Roche). One animal died in the control group for an unknown reason.

Hamster cheek pouch As described previously [11], hamsters were anaesthetized with sodium pentobarbital (80 mg/kg, ip) and the body temperature controlled at 37 °C. The cheek pouch was exteriorised, dissected, and fixed upon a perfusion chamber. The pouch was superfused (6 mL/min) with a modified Ringer's solution of the following composition (mmol/L): NaCl 124, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 2, $NaHCO_3$ 25, and HEPES 30 (pH 7.4; 36°C, bubbled with 5 % CO_2-95 % N_2 gas mixture) and observed under the microscope either under visible light or under UV illumination (magnification×50). After a 30-min stabilization period, fluorescein isothiocyanate dextran (FITC dextran, M_r 150 000) was injected through the femoral vein (250 mg/kg, 5 mL/kg). Anaesthesia was maintained with chloralose (25 mg/kg, 0.1mL, iv) when needed as previously described[12,13].

VEGF-induced increase in vascular leakage The following protocol was subsequently applied[5]. At the beginning of the experiment, the pouch was superfused with non-recirculating solution. Preparations with more than 10 spontaneous leakage sites (an objective sign of damage during the surgical procedure) and preparations developing petechia during the course of the experiments (a gross sign of circulatory and vascular dysfunction) were excluded (two in the control group, one in the fructose diet group). Forty minutes after the FITC-dextran administration, bradykinin (300 nmol/L), a concentration that represents the EC_{50} under those experimental conditions[13], was superfused for 5 min and the number of leakage sites counted (surface area of the microscope field: 0.2 cm^2). The preparation was then washed for 40 min by which time the number of leakage sites had returned to control value. The pouch was then superfused at the same rate (6 mL/min) with recirculating control solution (total volume: 20 mL) for 20 min. Then $VEGF_{165}$ was directly added to the recirculating solution (maximum volume added <500 μL) in order to produce a final concentration of 0.1 mg/L or 2.4 nmol/L. Each animal was exposed only once to a single concentration of $VEGF_{165}$ and only one pouch was studied for a given hamster. At the end of the experiments the pouch was re-exposed to bradykinin (300 nmol/L). Topical application of bradykinin can be repeated up to five times and evokes reproducible changes in permeability[13]. The changes in permeability provoked by bradykinin are independent of pre-exposure to VEGF or even to the presence of VEGF[5].

Substances Bradykinin, chloralose (Sigma, La Verpillère, France), human recombinant vascular endothelial growth factor ($VEGF_{165}$, R&D systems, Abington, UK), and *D*-fructose (Prolabo, France) were used in this study.

Statistical evaluation Data were shown as mean±SD; n represents the number of animals studied. Statistical evaluation was performed using ANOVA1 or 2 followed by the appropriate post hoc test. The differences were considered as statistically significant when $P<0.05$.

RESULTS

Metabolic parameters The diet in fructose induced an immediate and statistically significant increase in fluid intake [week #1: (7.5±2.3) and (10±5) mL/d per hamster, in control and fructose treated animals, respectively, ANOVA2, $P<0.01$] that lasted the duration of the treatment [week #18: (7.2±1.7) and (12±3) mL/d per hamster, n=34 and 35, respectively, ANOVA2, $P<0.01$] but the body weight was not affected. The fructose diet induced a significant increase in glycemia but without any significant changes in glycosylated hemoglobin, insulinemia, and triglyceridemia. Total cholesterol was significantly decreased in the fructose group (Tab 1).

Plasma extravasation Basal FITC-dextran extravasation was similar in control and fructose-treated animals [(2.4±2.0) and (2.0±0.9) μg/L, n=8 and 9, in hamsters submitted to control and fructose diet, respectively].

Bradykinin (300 nmol/L) was topically applied

Tab 1. Metabolic changes induced by fructose (10 %) in drinking water. Mean±SD. ANOVA1, $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

	Control	Fructose
Weight/g	115±10, *n*=34	121±14, *n*=35
Glycemia/g·L^{-1}	0.90±0.17, *n*=34	1.01±0.24^{b}, *n*=35
Insulinemia/mU·L^{-1}	11±6, *n*=34	14 ±11, *n*=35
HbA_1c/%	5.53±0.23, *n*=34	5.7±0.6, *n*=35
Triglyceridemia/g·L^{-1}	3.1±0.9, *n*=12	2.8±0.9, *n*=14
Cholesterol/g·L^{-1}	2.02±0.24, *n*=12	1.52±0.22^{c}, *n*=14

before and after $VEGF_{165}$ (2.4 nmol/L). Both bradykinin and VEGF produced an increase in vascular leakage in both group of animals. The increase in vascular leakage produced by bradykinin was similar when applied at the beginning or the end of the experiments. Furthermore, the number of leakage sites produced by the application of bradykinin was not significantly affected by the chronic fructose-diet (Fig 1). The increase in vascular permeability produced by bradykinin was not significantly influenced either by the order of the application or by the chronic treatment with fructose (Fig 1). In contrast, the increase in plasma extravasation produced by $VEGF_{165}$ was significantly increased by the fructose diet, the maximal number of leakage sites being increased by 64 % (Fig 2). The increase in plasma extravasation produced by VEGF was significantly larger in the fructose-treated group (ANOVA2 followed by a Bonferroni post hoc test, $P<0.05$, $P<0.01$ *vs* control). The blood glucose levels values for these two groups of animals were 0.80±0.11 and 1.09±0.15, *n*=8 and 9 for control and fructose treated animals, respectively (ANOVA1, $P<0.05$, Fig 2).

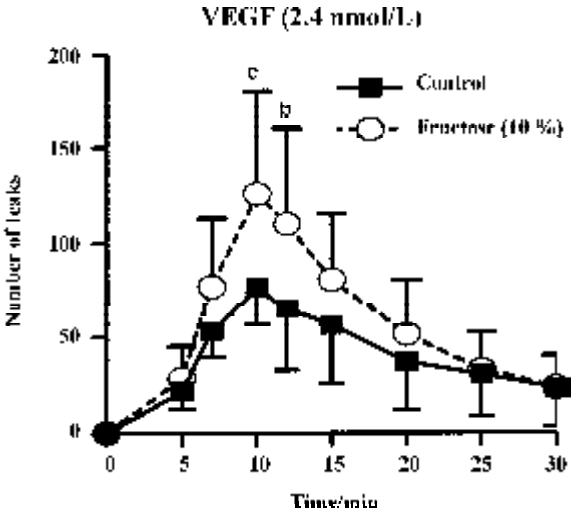

Fig 2. VEGF and vascular leakage in the hamster cheek pouch. Time-dependent changes in the number of microvascular leakage sites induced by $VEGF_{165}$ (2.4 nmol/L) in control hamsters or hamsters subjected to *D*-fructose (10 %) in their drinking water. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

DISCUSSION

This study showed that even moderate changes in glycemic levels could produce profound alteration in the VEGF response.

In the present study, the extent of the metabolic changes induced by fructose was markedly less severe that the one reported in similar studies. Indeed, in various animal models of fructose diet including in the

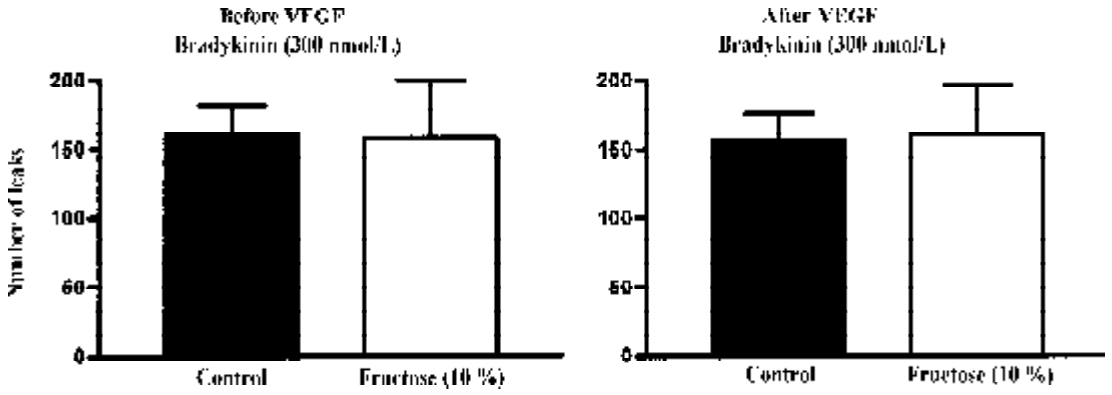

Fig 1. Bradykinin and vascular leakage in the hamster cheek pouch. The figure showed the maximum number of leakage sites produced by the application of bradykinin (300 nmol/L) either at the beginning of the experiment (before VEGF application, left panel) or at the end of the experiment (after VEGF application, right panel) in control hamsters or hamsters subjected to *D*-fructose (10 %) in their drinking water. Mean±SD.

hamster, hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia, and insulin resistance were reported[10,14]. In contrast, fasted glycemic levels were not affected in these previous studies. These discrepancies with the present study could be explained firstly by the major difference in the severity of the diet. Most studies are performed with food containing 60 % fructose, leading to a daily intake of approximately 16 g of fructose/hamster[10] instead of the 1 to 1.2 g ingested in the present study. Secondly, the small, but highly significant increase in plasma glucose, reported in the present study is most probably linked to the difference in experimental conditions as the present study measured non-fasted plasma glucose while other works reported fasted plasma glucose. Similarly, a trend toward an increase in the insulin level did not reach statistical significance probably because of the wide dispersion of the individual insulin values collected in these non-fasted animals. Interestingly, in this model of moderate fructose diet, in contrast to the other models, hamsters do not develop hypertension[15]. So the only measurable detrimental impact of this moderate fructose diet was a small but significant increase in glucose exposure. The basal vascular permeability was not significantly different in the control and the fructose treated group suggesting that, *per se*, the modest increase in glycemic level did not alter the integrity of the capillary network. Similarly, the increase in plasma extravasation produced by bradykinin was not affected by fructose. Under our experimental conditions, the concentration of bradykinin used (300 nmol/L) represented the EC_{50} for this peptide under those experimental conditions[13]. So the effect of bradykinin at this concentration was sub-maximal and an increase in the number of leakage sites could have still been possible. In contrast, the increase in plasma extravasation produced by VEGF was markedly increased by the fructose diet. This specific alteration of the VEGF response may be linked to the mechanism of action of VEGF. In the hamster cheek pouch, the plasma extravasation produced by VEGF is dependent on the protein kinase C pathway while the effect of bradykinin is not[5]. Interestingly, during diabetes and hyper-glycemia, the activation of protein kinase C and the increase in diacylglycerol levels have been linked to vascular dysfunction, including vascular leakage[16]. Furthermore, vascular hyperpermeability and increased blood flow caused by elevated tissue glucose can be blocked by VEGF antibodies[6].

This study suggests that, during diabetes, alteration in VEGF-induced plasma leakage can be an early marker of vascular dysfunction and can be observed even with moderate increase in blood glucose levels.

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