

Association of Diet-Induced Hyperinsulinemia With Accelerated Growth of Prostate Cancer (LNCaP) Xenografts

Vasundara Venkateswaran, Ahmed Q. Haddad, Neil E. Fleshner, Rong Fan, Linda M. Sugar, Rob Nam, Laurence H. Klotz, Michael Pollak

- Background** Prior research suggested that energy balance and fat intake influence prostate cancer progression, but the influence of dietary carbohydrate on prostate cancer progression has not been well characterized. We hypothesized that hyperinsulinemia resulting from high intake of refined carbohydrates would lead to more rapid growth of tumors in the murine LNCaP xenograft model of prostate cancer.
- Methods** Athymic mice were injected subcutaneously with LNCaP human prostate cancer cells and, when tumors were palpable, were randomly assigned (n = 20 per group) to high carbohydrate–high fat or low carbohydrate–high fat diets. Body weight and tumor volume were measured weekly. After 9 weeks, serum levels of insulin and insulin-like growth factor 1 (IGF-1) were measured by enzyme immunoassay. AKT activation and the levels of the insulin receptor in tumor cells were determined by immunoblotting. The in vitro growth response of LNCaP cells to serum from mice in the two treatment groups was measured based on tetrazolium compound reduction. All statistical tests were two-sided.
- Results** After 9 weeks on the experimental diets, mice on the high carbohydrate–high fat diet were heavier (mean body weight of mice on the high carbohydrate–high fat diet = 34 g versus 29.1 g on the low carbohydrate–high fat diet, difference = 4.9 g, 95% CI = 3.8 to 6.0 g; $P = .003$), experienced increased tumor growth (mean tumor volume in mice on high carbohydrate–high fat diet = 1695 versus 980 mm³ on low carbohydrate–high fat diet, difference = 715 mm³, 95% CI = 608 to 822 mm³; $P < .001$), and experienced a statistically significant increase in serum insulin and IGF-1 levels. Tumors from mice on the high carbohydrate–high fat diet had higher levels of activated AKT and modestly higher insulin receptor levels than tumors from mice on the low carbohydrate–high fat diet. Serum from mice on the high carbohydrate–high fat diet was more mitogenic for LNCaP cells in vitro than serum from mice fed the low carbohydrate–high fat diet.
- Conclusion** A diet high in refined carbohydrates is associated with increased tumor growth and with activation of signaling pathways distal to the insulin receptor in a murine model of prostate cancer.

Obesity is associated with increased cancer mortality (1), and its increasing prevalence (2) may hinder progress in cancer control. Recent studies focusing on prostate cancer have shown that obesity is associated with only a small increase in the risk of this disease but that it is an important adverse prognostic factor (3–11). The mechanisms that link obesity to prostate cancer prognosis are incompletely characterized. It is possible that the association of obesity with prostate cancer prognosis is mediated by adipokines (signaling molecules produced by adipocytes) and that the amount of adipose tissue is, therefore, of key importance (12). However, it has also been suggested that it is the hormonal profile associated with excess energy intake over expenditure that mediates the effects of obesity on prostate cancer prognosis (13–16) and that obesity is associated with prostate cancer prognosis because it is surrogate for excess energy intake.

Recent evidence from population studies indicates that hyperinsulinemia is also related to adverse outcome in prostate cancer

(17–21). This relationship is plausible because insulin and insulin-like growth factor 1 (IGF-1) receptors activate downstream signaling pathways that involve AKT, mTOR, and other molecules that positively regulate protein translation and proliferation and inhibit apoptosis (22). Insulin levels, like obesity, increase with greater

CONTEXT AND CAVEATS

Prior knowledge

The influence of diet on prostate cancer progression was not well characterized.

Study design

Mice carrying human prostate cancer xenografts were randomly assigned to high carbohydrate–high fat and low carbohydrate–high fat diets, and differences in tumor growth and hormone status were recorded.

Contribution

This study found that a diet high in refined carbohydrates was associated with increased tumor growth and higher levels of insulin and insulin-like growth factor 1.

Implications

Additional research is needed to determine whether diets associated with reductions in insulin levels may benefit prostate cancer patients.

Limitations

Mice on the high carbohydrate–high fat diet also consumed less protein, and this may have confounded the associations between carbohydrate intake and cancer growth.

energy intake, particularly in the form of rapidly absorbed carbohydrates (23). However, the influence of diet-induced hyperinsulinism on experimental prostate cancer models has not been examined. In this report, we examine the relationship between dietary carbohydrate intake and insulin-mediated signaling using a xenograft murine model of human prostate cancer progression.

Materials and Methods

Establishment of the LNCaP Xenograft Model of Prostate Cancer

Mice were maintained in a sterile and pathogen-free facility, with cages, bedding, and water autoclaved before use. Animal care conformed to institutional guidelines including the Care and Use of Experimental Animals guidelines of the Canadian Council on Animal Care. Human prostate cancer cells (LNCaP) (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium with 10% fetal bovine serum. Using a 27-gauge needle, we injected 1×10^6 LNCaP cells in 0.2 mL Matrigel into the flanks of 6- to 8-week old athymic male Swiss nu/nu mice (Taconic, New York) that had been anesthetized with methoxyfluorane. Two weeks after injection, all mice, all with palpable tumors, were randomly assigned to one of the two diets, low carbohydrate–high fat or high carbohydrate–high fat ($n = 20$ per group).

Diet Formulation and Treatment

The high carbohydrate–high fat diet included 40% carbohydrate, 45% fat, and 15% protein. The low carbohydrate–high fat diet consisted of 10% carbohydrate, 45% fat, and 45% protein (Table 1). Protein levels were adjusted in the diets to ensure an equivalent amount of calories (*see* Table 2), such that the mice were fed 4.76

and 4.58 Kcal/g in the high carbohydrate–high fat and low carbohydrate–high fat diets, respectively, for a period of 9 weeks. The high-carbohydrate diet was created by the addition of dextrin (30.5%) and sucrose (17%). Diets were stable for 6 months, as determined by the manufacturer (Purina Mills Test Diets, Richmond, IN, who reported that they were free of phytoestrogens), stored at 4 °C, and sterilized by irradiation before administration. Mice were allowed ad libitum access to food and water throughout the treatment period and were killed at the end of the treatment or when a tumor reached maximum permissible tumor diameter (17 mm), in accordance with the Canadian Council on Animal Care and Cancer Endpoint Guidelines.

Assessment of Body Weight and Tumor Volume

Body weight and tumor measurements were recorded weekly. Tumor size was assessed by measuring the (longest and shortest) two tumor diameters with a caliper. Tumor volume was calculated by the formula ($\text{short length}^2 \times \text{long length}/2$).

Preparation and Analysis of Blood and Tissue

At necropsy, blood was drawn from all mice by direct heart puncture, serum was separated, and aliquots of serum were stored at –80 °C. Tumors were excised, weighed, and processed for histopathologic studies or immunoblotting as described below. Tissue for histopathology was fixed in 10% v/v buffered formalin. Sections (5- μm thick) were cut from the paraffin-embedded tissue, mounted on slides, and stained with hematoxylin and eosin.

Immunohistochemical Analysis of Proliferative Marker (Ki-67) in Tumor Tissue

Immunostaining was performed on primary tumor sections using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, paraffin-embedded tissues were deparaffinized with xylene and dehydrated using washes of decreasing ethanol concentration (100%, 95%, and 80%). Antigen retrieval was performed by boiling the sections in 10 mM citrate buffer for 10 minutes. Sections were then blocked with diluted normal goat serum (blocking serum in Vectastain Elite ABC kit) and incubated overnight with primary antibody, Ki-67 (rabbit polyclonal antibody; 1:25 dilution; Abcam, Cambridge, MA). Endogenous peroxidase was blocked and sections incubated with secondary antibody (biotin-labeled anti-rabbit IgG, Vector Laboratories),

Table 1. Composition of low-carbohydrate and high-carbohydrate diets*

Dietary parameters	High carbohydrate	Low carbohydrate
Composition of diet, % weight		
Carbohydrate (dextrin + sucrose)	47.5	11.4
Fat	23.8	22.8
Protein	17.9	51.5
Others (minerals)	10.8	14.3
Energy contribution, %		
Carbohydrate (dextrin + sucrose)	40	10
Fat	45	45
Protein	15	45

* These data were provided in the specification sheets of the manufacturer.

according to the manufacturer's instructions, followed by incubation with streptavidin–peroxidase and 3, 3'-diaminobenzidine. Sections were counterstained with hematoxylin. The Ki-67 labeling index was determined by counting 1000 tumor cells at $\times 40$ magnification in 10 microscopic fields. Brown, granular nuclear staining was considered to be positive for Ki-67. Labeling indices were calculated as the percentages of tumor cells with positive nuclear staining. Immunostaining was performed blindly and scored on prostate tumor tissue sections from each mouse ($n = 10$ per group; two sections from each tumor) by an independent pathologist.

Plasma Insulin Measurements

Serum insulin was measured in duplicate ($n = 20$ per treatment group) using a rat insulin ELISA kit (Crystal Chem Inc., IL), according to the manufacturer's instructions. Insulin in the sample was bound to guinea pig anti-rat insulin antibody coated on microplate wells, and unbound materials were removed by washing with the washing buffer provided by the manufacturer. Horseradish peroxidase-conjugated anti-guinea pig antibody was then added to the insulin complex immobilized on the microplate well, and excess peroxidase was removed with washing buffer. The peroxidase conjugate on the microplate well was detected using 3,3',5,5'-tetramethylbenzidine substrate solution. The enzymatic reaction was stopped by the addition of 100 μ L of enzyme reaction stopping solution (provided in the kit) and absorbance measured by a plate reader within 30 minutes (measuring wavelength: 450 nm, background wavelength: 530 nm). Appropriate insulin standards ranging in concentration from 0 to 6.4 ng/mL and internal controls (provided by the manufacturer) were used.

Plasma Insulin-Like Growth Factor 1 Measurements

IGF-1 was measured in duplicate by Enzyme Immunoassay (Diagnostic Systems Laboratories, Inc., TX) in serum samples from all mice in the two treatment groups. Samples were incubated for 1 hour with biotin-labeled mouse IGF-1 and goat anti-mouse IGF-1 antiserum in microtitration wells coated with rabbit anti-goat gamma globulin (according to the manufacturer's instructions). After incubation and washing with washing solution (saline solution with a nonionic detergent; provided in the kit), the wells were incubated with streptavidin-horseradish peroxidase, which binds to biotinylated mouse IGF-1. Unbound peroxidase was washed with washing buffer and the wells incubated with tetramethylbenzidine. An acidic stopping solution containing 0.2 M sulfuric acid was then added and the degree of enzymatic turnover of the substrate determined. Absorbance was measured by a plate reader within 30 minutes (measuring wavelength: 450 nm, background wavelength: 530 nm). Appropriate standards ranging in concentration from 0 to 4000 ng/mL and internal controls (provided by the manufacturer) were used.

Immunoblotting

Tumor tissues from five mice in each group were cut into 1-mm pieces and homogenized separately in ice-cold RadioImmuno Precipitation Assay lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA) supplemented with a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride and

0.02 mg/mL each of aprotinin, leupeptin, and pepstatin; Sigma Chemical Company, St. Louis, MO). Protein amounts were determined by Bradford analysis. Protein (40 μ g suspended in lysis buffer) was loaded in lanes of sodium dodecyl sulfate-containing polyacrylamide gels, electrophoresed, and transferred to membranes (Immobilon transfer membrane, Millipore, Bedford, MA). Blots were blocked for 1 hour with blocking buffer (5% nonfat dry milk in phosphate-buffered saline containing 0.2% Tween 20), followed by sequential incubation with primary and secondary antibodies. The following primary antibodies were used: β -actin mouse monoclonal antibody (Sigma) at 1:20000 dilution, AKT antibody (recognizing phosphorylated and unphosphorylated forms) at 1:1000 dilution (Cell Signaling, Danvers, MA), phospho-AKT (Ser⁴⁷³) polyclonal antibody (Cell Signaling) at 1:1000 dilution, and mouse anti-insulin receptor (β -subunit) monoclonal antibody (Chemicon, Temecula, CA) at 1:200 dilution. Secondary antibodies were horseradish peroxidase-labeled anti-mouse IgG, 1:5000 dilution or horseradish peroxidase-labeled anti-rabbit IgG, 1:1000 dilution. Antibody–protein complexes were visualized by electrochemiluminescence.

In Vitro Mitogenicity Assay

LNCaP cells (5×10^3 per well) were plated in 96-well plates. After 24 hours, cells were washed twice with phosphate-buffered saline (to remove serum) and treated (6 wells per treatment) with serum-free media for an additional 24 hours. Then cells were treated for 72 hours with serum (1% in media) obtained at necropsy from individual mice on the different diets ($n = 20$ per group). The 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method was used to measure cell proliferation as previously described (24).

Statistical Analysis

The statistical significance of differences between dietary groups with respect to body weight, tumor volume, tumor wet weight, serum insulin levels, serum IGF-1 levels, and serum in vitro mitogenicity data were calculated using the two-tailed Student's *t* test. Results were considered to be statistically significant at *P* less than .05. All statistical tests were two-sided.

Results

Influence of Diet on Body Weight

To investigate the influence of diet on body weight, mice bearing palpable tumors were randomly assigned to a high- or low-carbohydrate diet for 9 weeks and food consumption was recorded. Diets were well tolerated. There was no statistically significant difference in grams of food consumed per day between the groups (4.77 g in the high-carbohydrate group versus 4.83 g in the low-carbohydrate group, difference = -0.06 g, 95% CI = -1.02 to 0.90 g; *P* = .893). The total calories consumed were also similar (Table 2), but mice on the high-carbohydrate diet obtained fewer calories from protein, as expected. Mice on the high carbohydrate–high fat diet began to gain weight relative to the mice on the low carbohydrate–high fat diet after approximately 5 weeks of treatment (Fig. 1), although differences in body weight between the treatment groups did not become statistically significant until the conclusion of the experiment (after 9 weeks of treatment), at

Table 2. Calculated daily intakes and the caloric values for the treatment groups

Variable	High carbohydrate	Low carbohydrate
Food consumed (g)/day per mouse	4.77	4.83
Total energy value of diets, Kcal/g*	4.76	4.58
Total energy consumed, Kcal/day per mouse†	22.7	22.1
Grams consumed/day per mouse of‡		
Carbohydrate	2.27	0.55
Fat	1.14	1.1
Protein	0.85	2.49
Energy (Kcal) from dietary constituent/day per mouse§		
Carbohydrate	9.08	2.2
Fat	10.2	9.9
Protein	3.4	9.96

* Data provided in the specification sheets of the manufacturer.

† Total energy value × g of diet consumed.

‡ Composition × g consumed/100.

§ Calculated as grams of diet consumed/day per mouse × physiologic fuel value (carbohydrate, fat, and protein are approximately 4, 9, and 4 Kcal/g, respectively).

which time mice on the high-carbohydrate diet were 15% heavier than mice on the low-carbohydrate diet (mean body weight of mice on a high carbohydrate–high fat diet = 34 versus 29.1 g on the low carbohydrate–high fat diet; difference = 4.9 g, 95% CI = 3.8 to 6.0 g; $P = .003$; Fig. 1).

Influence of Diet on Tumor Volume

Tumors were measured weekly. Statistically significant differences in tumor volume were observed from week 5 (mean tumor volume of mice on the high-carbohydrate diet = 901 versus 588 mm³ for mice on the low-carbohydrate diet, difference = 313 mm³, 95% CI = 219 to 407 mm³; $P = .035$; Fig. 2, A) By the end of the 9-week treatment period, there was a statistically significant increase (45%) in tumor volume in the mice on a high-carbohydrate diet relative to animals

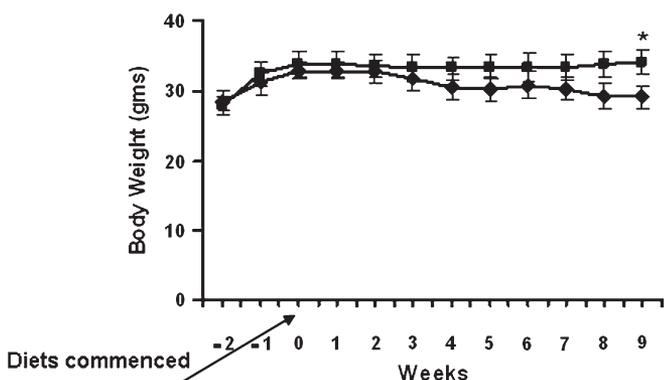


Fig. 1. Effect of high carbohydrate–high fat and low carbohydrate–high fat diets on body weight. Mice ($n = 20$ per group) injected with LNCaP human prostate cancer cells were confirmed to have tumors after 2 weeks and assigned to either the high carbohydrate–high fat diet (black square) (40% carbohydrate, 45% fat, and 15% protein) or the low carbohydrate–high fat diet (black diamond) (10% carbohydrate, 45% fat, and 45% protein). Body weight was measured weekly over a period of 9 weeks. Error bars correspond to upper 95% confidence intervals of the means. Means were statistically significantly different based on the two-sided Student's t test ($*P < .05$).

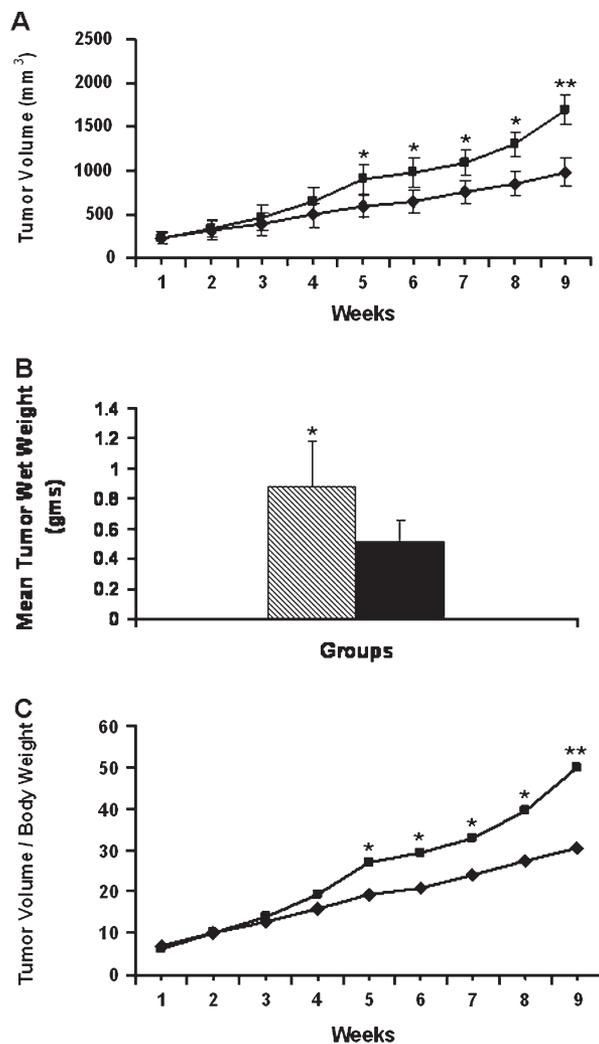


Fig. 2. Effect of diet on tumor growth. **A)** Tumor volume of mice injected with LNCaP human prostate cancer cells and assigned ($n = 20$ per group) 2 weeks later to the high carbohydrate–high fat (black square) or low carbohydrate–high fat (black diamond) diet was measured using calipers and recorded weekly over the 9-week treatment period. Error bars correspond to the 95% confidence intervals (CIs) of the means. **B)** At the end of the treatment period, mice on high carbohydrate–high fat (hatched bar) and low carbohydrate–high fat (black bar) diets were killed and tumors excised and weighed. Error bars correspond to the upper 95% CIs of the means. **C)** Ratio of mean prostate tumor volume to body weight. The weekly change over the treatment period in the ratio of the mean tumor volume to body weight for mice on the high carbohydrate–high fat (black square) and low carbohydrate–high fat (black diamond) diets is shown. Means were statistically significantly different based on the two-sided Student's t test ($*P < .05$, $**P < .001$).

on a low-carbohydrate diet. Mean tumor volume in mice on a high-carbohydrate diet was 1695 versus 980 mm³ in the low-carbohydrate diet (difference = 715 mm³, 95% CI = 608 to 822 mm³; $P > .001$).

There was also an increase in the ratio of the mean tumor volume to body weight in the mice on a high-carbohydrate compared with the low-carbohydrate group (Fig. 2, C). The mean tumor volume to body weight ratio at week 9 was 49.9 and 30.6 in the high-carbohydrate and low-carbohydrate group, respectively (difference = 19.3, 95% CI = 15.9 to 22.7; $P = .001$). At the end of the 9-week treatment period, mice were killed, and tumors were excised and weighed. Tumor weights ranged from 0.6 to 1.4 g in mice on the

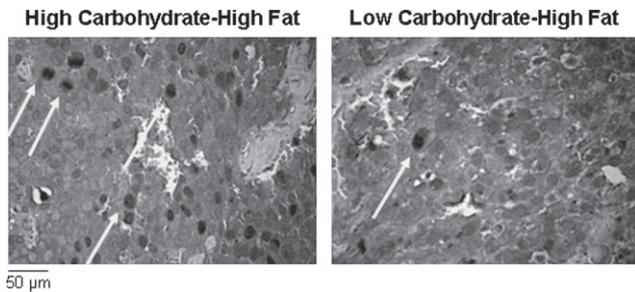


Fig. 3. Immunohistochemistry of proliferation marker Ki-67 in tumor tissue from mice on high carbohydrate-high fat and low carbohydrate-high fat diets. Immunostaining was performed on paraffin-embedded tumor sections ($n = 10$ per group; two sections from each tumor) using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Cells with **brown**, granular nuclear staining (indicated by **arrows**) were considered to be positive for Ki-67. A section of a tumor from each treatment group is shown.

high-carbohydrate diet and from 0.2 to 0.8 g in mice on the low-carbohydrate diet. Mean tumor weight in mice on the high-carbohydrate diet was 0.88 versus 0.51 g in the low-carbohydrate diet (difference = 0.37 g, 95% CI = 0.13 to 0.61 g; $P = .04$; Fig. 2, B).

Expression of Ki-67 Proliferation Marker on LNCaP Xenografts

To better understand the basis for the effect of diet on tumor volume, we studied markers of proliferation in the xenograft tumors, as well as circulating insulin and IGF-1 levels in tumor-bearing mice. Immunohistochemical analysis of a proliferation marker (Ki-67) was performed on paraffin-embedded prostate tumor tissues from 10 mice per treatment group; two sections were examined from each tumor. There was a substantial increase in the percentage of positively stained nuclei in tumors from the high-carbohydrate group (27%) compared with that in tumors from the low-carbohydrate group (5.7%) (difference = 21.3%, 95% CI = 13.6 to 28.8%; $P < .001$; Fig. 3).

Influence of Diet on Serum Insulin and IGF-1 Levels

To explore dietary effects on insulin and IGF-1 levels, blood samples were collected from mice in both groups by direct heart puncture, serum was separated from plasma, and aliquots of serum were stored at -80°C . The mean insulin level in serum samples of mice on the high-carbohydrate diet was statistically significantly higher than that of mice on the low-carbohydrate diet (1.45 versus 0.45 ng/mL, difference = 1 ng/mL, 95% CI = 0.95 to 1.06 ng/mL; $P = .039$; Fig. 4, A). Serum from mice on the high-carbohydrate diet had a 21.4% higher level of IGF-1 compared with that in mice on the low-carbohydrate diet (1868.5 versus 1468.5 ng/mL; difference = 400 ng/mL, 95% CI = 358.3 to 441.7 ng/mL; $P = .022$; Fig. 4, B).

Influence of Diet on Phosphorylation of AKT in Prostate Cancer Tissue

In view of the diet-induced changes in insulin and IGF-1 levels, we next investigated if the diets influenced signaling pathways in the experimental tumors. First, we measured phosphorylation of AKT, a signaling molecule that is downstream of the insulin and IGF-1 receptors (22), in lysates from tumor tissues ($n = 5$ per group). The amount of phosphorylated AKT was below the detection limit in

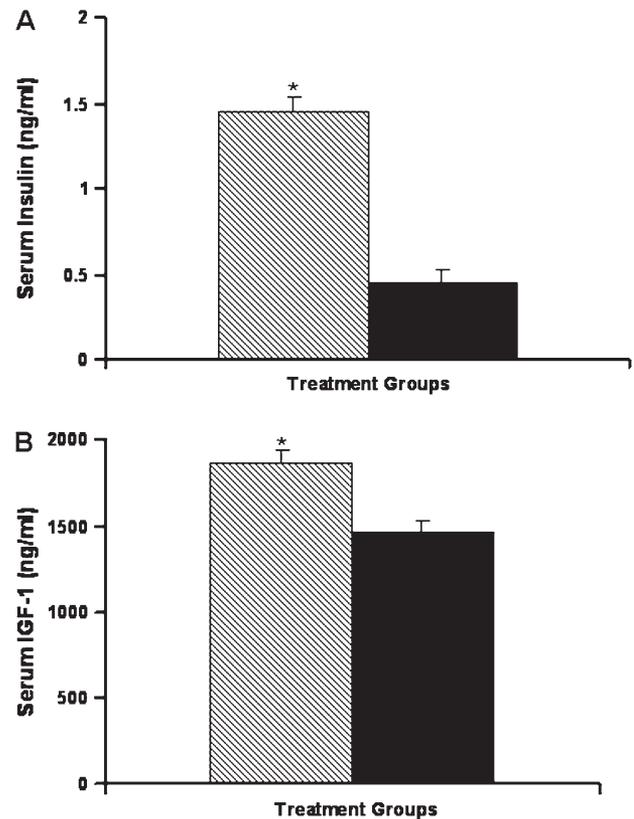


Fig. 4. Serum insulin and insulin-like growth factor levels in mice on high- and low-carbohydrate diets. **A)** Serum insulin in mice ($n = 20$ per group) that had been assigned to high carbohydrate-high fat (**shaded bar**) and low carbohydrate-high fat (**black bar**) diets was measured after 9 weeks. Means were statistically significantly different based on the two-sided Student's t test. **Error bars** correspond to the upper 95% confidence intervals (CIs) of the means. **B)** Serum IGF-1 in mice ($n = 20$ per group) that had been assigned to high carbohydrate-high fat (**shaded bar**) and low carbohydrate-high fat (**black bar**) diets was measured after 9 weeks using an immunoassay. The degree of enzymatic turnover of the substrate was determined by dual absorbance at 450 and 620 nm. Difference was statistically significantly different based on the two-sided Student's t test. **Error bars** correspond to the upper 95% CIs of the means ($*P < .05$).

tumor tissue lysate from the mice on the low-carbohydrate diet, but a band corresponding to the phosphorylated kinase was clearly visible in the immunoblots obtained from tumor tissue lysates from the mice on high-carbohydrate diet (Fig. 5). Thus, the high-carbohydrate diet increased AKT activation in the LNCaP xenograft tissue. Insulin receptor levels were also higher in the neoplastic tissue of the mice on the high-carbohydrate diet ($n = 5$ per group) (Fig. 5).

Mitogenicity of Mouse Serum In Vitro

In view of the pronounced changes in hormone-mediating signaling we wished to investigate if serum from mice on the carbohydrate diets altered the growth of human prostate cancer cells in vitro. LNCaP cells were treated with serum obtained from mice on the high- and low-carbohydrate diet ($n = 20$ per group). The MTS method was used to measure cell proliferation as previously described (24). LNCaP cells treated with 1% serum from mice on the high-carbohydrate diet showed greater proliferation than cells treated with serum from mice on the low-carbohydrate diet (optical

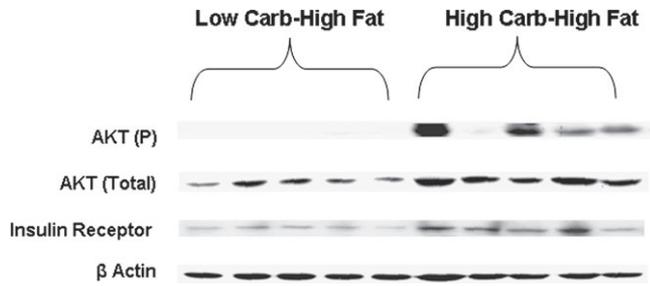


Fig. 5. Comparison of activated AKT and insulin receptor levels in tumors from mice on low carbohydrate-high fat and high carbohydrate-high fat diets. Total AKT, phosphorylated AKT, and insulin receptor in individual lysates (5 per group) prepared from tumors excised at the end of the dietary treatment were detected on immunoblots by electrochemiluminescence using antibodies specific for the insulin receptor and the phosphorylated form of AKT. β actin was used as an internal control for protein loading and transfer.

density at 490 nm from MTS cell proliferation assay = 0.64 versus 0.29, difference = 0.35, 95% CI = 0.3 to 0.4; $P = .005$; Fig. 6).

Discussion

We examined the relationship between dietary carbohydrate intake and prostate cancer behavior using the LNCaP model and observed that increasing carbohydrate intake (without altering fat intake or total calories) augmented the growth rate of prostate cancer xenografts. The high-carbohydrate diet was also associated with a major increase in host insulin levels, a modest increase in host IGF-1 levels, and, in neoplastic tissue, an increase in insulin receptor levels, AKT activation, and the rate of cell proliferation. Most prior studies of modulation of signaling at critical nodes such as AKT (which is downstream from receptors of the tyrosine kinase class and upstream of mTOR) involved pharmacologic interventions (25); our data provide evidence for an effect of macronutrient composition on AKT activation in LNCaP xenografts.

There was a small difference in the weight gain of mice between the dietary groups (Fig. 1), although the magnitude of the difference in tumor weight was greater than the difference in body weight. It is possible that mice on the high-carbohydrate diet were slightly heavier due to fluid retention because insulin has been reported to have antinatriuretic properties (26).

Rapidly absorbed sugars are important sources of carbohydrate in modern American diets and evoke high surges in insulin levels (27–29). However, our results do not establish that the effect of host diet on tumor behavior was attributable to effects of diet on insulin. Insulin is, nevertheless, a candidate mediator of the effect of the high-carbohydrate diet on prostate cancer behavior, in view of 1) the well-known association of diets high in sugars with higher insulin levels, 2) the detection of insulin receptors in the LNCaP xenografts, 3) enhanced activation of AKT (a signaling protein that is downstream of the insulin receptor) in tumors of mice on the high-carbohydrate diet, and 4) our observation of increased proliferation in vitro of human prostate cancer cells that were exposed to serum from mice on the high-carbohydrate diet compared with that of prostate cancer cells that were exposed to serum from mice on the low-carbohydrate diet. These results of our study are consistent with an effect of hyperinsulinemia related

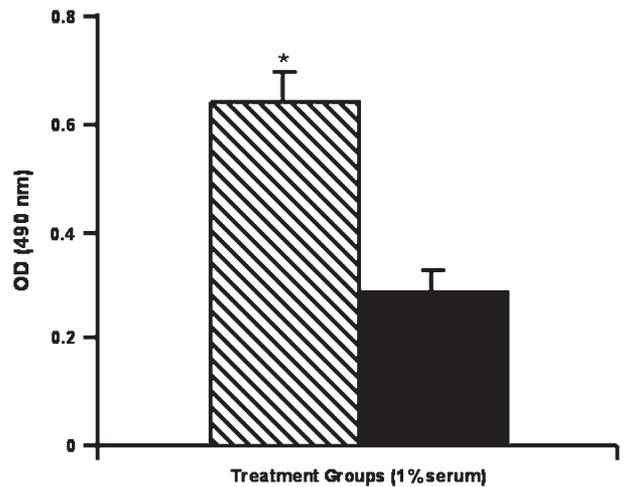


Fig. 6. In vitro mitogenicity of serum from mice on high carbohydrate-high fat and low carbohydrate-high fat diets. LNCaP cells were plated at 5×10^3 per well and treated for a period of 72 hours with serum (1% in media) obtained from individual mice on the different diets ($n = 20$ per group). High carbohydrate-high fat (hatched bar) and low carbohydrate-high fat (black bar). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) method was used to measure cell proliferation. Difference was statistically significantly different based on the two-sided Student's t test. Error bars correspond to the upper 95% confidence intervals of the means ($*P < .05$).

to the ingestion of sugars, and they might not have been observed if an isocaloric starch-based carbohydrate diet had been used.

One limitation of our study is that we cannot exclude the possibility that some of our observations are related to decreased protein intake. To maintain caloric equivalence, the diet containing higher sugar levels had lower protein levels. Although it would also be of interest to compare the effects of isocaloric isoprotein diets that vary in the nature of the carbohydrate (sugar versus starch), this is technically challenging because of differences in palatability that could lead to differences in consumption. Another limitation of this study is that our results derive from a single model of prostate cancer. The relationships between diet and prostate cancer progression we have observed need to be tested in other models.

Our findings are consistent with population studies that showed an association between higher levels of insulin or c-peptide (a surrogate marker of insulin levels that can be conveniently measured in serum, especially when fasting samples are not available) with adverse outcome in prostate cancer (18,21). Further studies will be required to determine if insulin directly mediates the effect of the diet, indirectly mediates the effect through an insulin-regulated host factor that acts on neoplastic cells, or if insulin is simply a surrogate for another effector molecule. However, prior circumstantial evidence consistent with a role for insulin in neoplasia includes 1) the association of hyperglycemia with cancer risk and prognosis (30); 2) the finding, over 20 years ago, that insulin deficiency is associated with reduced tumor growth (31); and 3) the observation that transplantation of pancreatic islets to an ectopic site can lead to carcinogenesis in that site (32). Regardless of the exact role of insulin signaling in regulating tumor growth, it will be important to describe more comprehensively the dietary-induced changes in signaling pathways in neoplastic tissue and to determine if the activation state of insulin receptor substrate family

members, mammalian target of rapamycin, and S6-kinase are altered by the dietary manipulation.

We also observed that the higher IGF-1 levels in mice on the high-carbohydrate diet were associated with more rapid tumor growth. These experimental results are consistent with epidemiologic data (33,34), showing that increased risk of prostate cancer, particularly advanced prostate cancer (35), is associated with higher serum IGF-1 concentration. IGF-1-mediated signaling has received more attention in the context of neoplasia than insulin signaling, but our data are compatible with the hypothesis that insulin itself may play a role in mediating the effect of macronutrient composition of diets on tumor growth (22,36).

The association of the high-carbohydrate diet with elevated circulating insulin levels was expected based on previous results (23,37,38), but the increased levels of insulin receptors in the neoplastic tissue was an unexpected finding for which the mechanism is unknown. Although IGF-1 receptors on prostate cancer cells have been studied extensively (39–41), there is only limited evidence that prostate cancer cells display insulin receptors (42), and our results justify further research to better characterize insulin and insulin/IGF hybrid receptors in prostate cancer using both laboratory models and tissue microarrays of human tumors.

Androgen deprivation therapy for prostate cancer, which is effective and in widespread use, induces hyperinsulinemia in a substantial proportion of men (43,44). This adverse effect has been discussed in terms of its effect on quality of life and cardiovascular health in long-term prostate cancer survivors (45,46). However, it is possible that the hyperinsulinemic state also influences risk of progression to aggressive, androgen-independent disease. This possibility is being addressed by ongoing studies that seek to determine if interindividual differences in degree of castration-induced hyperinsulinism are related to time to emergence of androgen independence.

Our results provide support for the concept that diets associated with reduction in insulin level may have benefits for prostate cancer patients (47), particularly for the subset of patients who are hyperinsulinemic. It should be noted that although obese men tend to be hyperinsulinemic, some individuals described as “metabolically obese, normal weight” (48) are also hyperinsulinemic, and cancer patients in this group (as well as obese men) may benefit from strategies to reduce insulin levels. Our experimental data justify clinical research to determine if optimization of macronutrient intake to meet, but not exceed, nutritional requirements and to minimize insulin levels, may lower prostate cancer risk and/or improve prostate cancer prognosis. Pharmacologic agents such as metformin (49), which reduce hyperinsulinemia and associated metabolic abnormalities, may also have a role to play in the treatment of metabolically defined subsets of prostate cancer patients.

References

- (1) Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003;348:1625–38.
- (2) Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999–2004. *JAMA* 2006;295:1549–55.
- (3) Amling CL, Riffenburgh RH, Sun L, Moul JW, Lance RS, Kusuda L, et al. Pathologic variables and recurrence rates as related to obesity and race in men with prostate cancer undergoing radical prostatectomy. *J Clin Oncol* 2004;22:439–45.
- (4) Amling CL. Relationship between obesity and prostate cancer. *Curr Opin Urol* 2005;15:167–71.
- (5) Freedland SJ, Terris MK, Presti JC Jr, Amling CL, Kane CJ, Trock B, et al. Obesity and biochemical outcome following radical prostatectomy for organ confined disease with negative surgical margins. *J Urol* 2004;172:520–4.
- (6) Freedland SJ, Aronson WJ, Kane CJ, Presti JC Jr, Amling CL, Elashoff D, et al. Impact of obesity on biochemical control after radical prostatectomy for clinically localized prostate cancer: a report by the Shared Equal Access Regional Cancer Hospital database study group. *J Clin Oncol* 2004;22:446–53.
- (7) Freedland SJ. Obesity and prostate cancer: a growing problem. *Clin Cancer Res* 2005;11(Pt 1):6763–6.
- (8) Freedland SJ, Giovannucci E, Platz EA. Are findings from studies of obesity and prostate cancer really in conflict? *Cancer Causes Control* 2006;17:5–9.
- (9) Rodriguez C, Freedland SJ, Deka A, Jacobs EJ, McCullough ML, Patel AV, et al. Body mass index, weight change risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort. *Cancer Epidemiol Biomarkers Prev* 2007;16:63–9.
- (10) Rohrmann S, Roberts WW, Walsh PC, Platz EA. Family history of prostate cancer and obesity in relation to high-grade disease and extra-prostatic extension in young men with prostate cancer. *Prostate* 2003;55:140–6.
- (11) Strom SS, Wang X, Pettaway CA, Logothetis CJ, Yamamura Y, Do KA, et al. Obesity, weight gain risk of biochemical failure among prostate cancer patients following prostatectomy. *Clin Cancer Res* 2005;11(Pt 1):6889–94.
- (12) Trayhurn P, Wood IS. Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochem Soc Trans* 2005;33(Pt 5):1078–81.
- (13) Chan JM, Gann PH, Giovannucci EL. Role of diet in prostate cancer development and progression. *J Clin Oncol* 2005;23:8152–60.
- (14) Hsieh LJ, Carter HB, Landis PK, Tucker KL, Metter EJ, Newschaffer CJ, et al. Association of energy intake with prostate cancer in a long-term aging study: Baltimore Longitudinal Study of Aging (United States). *Urology* 2003;61:297–301.
- (15) Huffman DM, Johnson MS, Watts A, Elgavish A, Eltoum IA, Nagy TR. Cancer progression in the transgenic adenocarcinoma of mouse prostate mouse is related to energy balance, body mass, and body composition, but not food intake. *Cancer Res* 2007;67:417–24.
- (16) Platz EA, Leitzmann MF, Michaud DS, Willett WC, Giovannucci E. Interrelation of energy intake, body size, and physical activity with prostate cancer in a large prospective cohort study. *Cancer Res* 2003;63:8542–8.
- (17) Hammarsten J, Hogstedt B. Clinical, haemodynamic, anthropometric, metabolic and insulin profile of men with high-stage and high-grade clinical prostate cancer. *Blood Press* 2004;13:47–55.
- (18) Hammarsten J, Hogstedt B. Hyperinsulinaemia: a prospective risk factor for lethal clinical prostate cancer. *Eur J Cancer* 2005;41:2887–95.
- (19) Hsing AW, Chua S Jr, Gao YT, Gentschein E, Chang L, Deng J, et al. Prostate cancer risk and serum levels of insulin and leptin: a population-based study. *J Natl Cancer Inst* 2001;93:783–9.
- (20) Lehrer S, Diamond EJ, Stagger S, Stone NN, Stock RG. Serum insulin level, disease stage, prostate specific antigen (PSA) and Gleason score in prostate cancer. *Br J Cancer* 2002;87:726–8.
- (21) Ma J, Li H, Pollak M, Kurth T, Giovannucci E, Stampfer MJ. Prediagnostic plasma c-peptide levels and prostate cancer incidence and survival. *Proceedings of AACR Frontiers in Cancer Prevention Research Meeting*; 2006 Nov 12; Philadelphia (PA). Boston (MA): AACR; 2006.
- (22) Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 2004;4:505–18.
- (23) Fernandez ML. The metabolic syndrome. *Nutr Rev* 2007;65(Pt 2):S30–4.
- (24) Venkateswaran V, Klotz LH, Fleshner NE. Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. *Cancer Res* 2002;62:2540–5.
- (25) Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005;4:988–1004.

- (26) Friedberg CE, van Buren M, Bijlsma JA, Koomans HA. Insulin increases sodium reabsorption in diluting segment in humans: evidence for indirect mediation through hypokalemia. *Kidney Int* 1991;40:251–6.
- (27) Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. *Horm Metab Res* 2003;35:694–704.
- (28) Wu T, Giovannucci E, Pischon T, Hankinson SE, Ma J, Rifai N, et al. Fructose, glycemic load, and quantity and quality of carbohydrate in relation to plasma C-peptide concentrations in US women. *Am J Clin Nutr* 2004;80:1043–9.
- (29) McCarty MF. Insulin and IGF-I as determinants of low. “Western” cancer rates in the rural third world. *Int J Epidemiol* 2004;33:908–10.
- (30) Jee SH, Ohrr H, Sull JW, Yun JE, Ji M, Samet JM. Fasting serum glucose level and cancer risk in Korean men and women. *JAMA* 2005;293:194–202.
- (31) Pavelic K, Slijepcevic M, Pavelic J, Ivic J, Audy-Jurkovic S, Pavelic ZP, et al. Growth and treatment of Ehrlich tumor in mice with alloxan-induced diabetes. *Cancer Res* 1979;39:1807–13.
- (32) Dombrowski F, Mathieu C, Evert M. Hepatocellular neoplasms induced by low-number pancreatic islet transplants in autoimmune diabetic BB/Pfd rats. *Cancer Res* 2006;66:1833–43.
- (33) Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998;279:563–6.
- (34) Wolk A, Mantzoros CS, Andersson SO, Bergstrom R, Signorello LB, Lagiou P, et al. Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study. *J Natl Cancer Inst* 1998;90:911–5.
- (35) Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, et al. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. *J Natl Cancer Inst* 2002;94:1099–106.
- (36) Kurmasheva RT, Houghton PJ. IGF-I mediated survival pathways in normal and malignant cells. *Biochim Biophys Acta* 2006;1766:1–22.
- (37) McKeown NM, Meigs JB, Liu S, Saltzman E, Wilson PW, Jacques PF. Carbohydrate nutrition, insulin resistance, and the prevalence of the metabolic syndrome in the Framingham Offspring Cohort. *Diabetes Care* 2004;27:538–46.
- (38) Yang EJ, Kerver JM, Park YK, Kayitsinga J, Allison DB, Song WO. Carbohydrate intake and biomarkers of glycemic control among US adults: the third National Health and Nutrition Examination Survey (NHANES III). *Am J Clin Nutr* 2003;77:1426–33.
- (39) Krueckl SL, Sikes RA, Edlund NM, Bell RH, Hurtado-Coll A, Fazli L, et al. Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. *Cancer Res* 2004;64:8620–9.
- (40) Hellawell GO, Turner GD, Davies DR, Poulson R, Brewster SF, Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Res* 2002;62:2942–50.
- (41) Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *J Cell Biochem* 2006;99:392–401.
- (42) Pandini G, Mineo R, Frasca F, Roberts CT Jr, Marcelli M, Vigneri R, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res* 2005;65:1849–57.
- (43) Basaria S, Muller DC, Carducci MA, Egan J, Dobs AS. Hyperglycemia and insulin resistance in men with prostate carcinoma who receive androgen-deprivation therapy. *Cancer* 2006;106:581–8.
- (44) Smith MR, Lee H, Nathan DM. Insulin sensitivity during combined androgen blockade for prostate cancer. *J Clin Endocrinol Metab* 2006;91:1305–8.
- (45) Braga-Basaria M, Dobs AS, Muller DC, Carducci MA, John M, Egan J, et al. Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. *J Clin Oncol* 2006;24:3979–83.
- (46) Keating NL, O’Malley AJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. *J Clin Oncol* 2006;24:4448–56.
- (47) Mavropoulos JC, Isaacs WB, Pizzo SV, Freedland SJ. Is there a role for a low-carbohydrate ketogenic diet in the management of prostate cancer? *Urology* 2006;68:15–8.
- (48) St Onge MP, Janssen I, Heymsfield SB. Metabolic syndrome in normal-weight Americans: new definition of the metabolically obese, normal-weight individual. *Diabetes Care* 2004;27:2222–8.
- (49) Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 2006;66:10269–73.

Funding

Prostate Cancer Research Foundation (Toronto, ON, Canada to V. V.); Prostate Cancer Foundation (Los Angeles, CA, to M. P.).

Notes

We thank Mrs Crocetta Accardi and Ms Heather Goodman for excellent technical assistance.

The authors take full responsibility for the study design, data collection, analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

Manuscript received March 12, 2007; revised September 15, 2007; accepted October 22, 2007.