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Insulin Protects against Hepatic Bioenergetic Deterioration Induced by Cancer Cachexia: An *in Vivo* ^{31}P Magnetic Resonance Spectroscopy Study

Manfred Brauer, Richard I. Inculet,[1] Gopal Bhatnagar, Gregory D. Marsh, Albert A. Driedger, and R. Terry Thompson

Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1 [M. B.], and Department of Surgery and Nuclear Medicine, Victoria Hospital [R. I. I., G. B., A. A. D.], and Department of Nuclear Medicine and Magnetic Resonance, Lawson Research Institute, St. Joseph's Health Care Centre, The University of Western Ontario [G. D. M., R. T. T.], London, Ontario, Canada N6G 3V4

ABSTRACT

The bioenergetic effects of cancer cachexia on the livers of male Fischer rats inoculated with a methylcholanthrene-induced sarcoma were assessed using serial *in vivo* ^{31}P magnetic resonance spectroscopy. Rats were randomized into three groups: tumor-bearing controls (n = 7); an insulin-treated group receiving 2 units/100 g body weight/day starting 21 days after implantation (n = 8); and a chronic insulin-treated group receiving insulin every day after implantation (n = 3). During the 32-day study, serial measurements of food intake, body weight, and tumor volume were taken, and ^{31}P magnetic resonance spectroscopy analyses of the livers were conducted every 7 days after tumor implantation. Neither the short-term nor the chronic insulin treatment regimens stimulated the progress of tumor growth. However, both treatments prevented body weight loss, and the short-term insulin treatment prevented tumor-induced decrease in food intake relative to the control group. Liver bioenergetic deterioration was evaluated from the increase in the ratio of P_i to ATP obtained from the hepatic ^{31}P magnetic resonance spectra. At day 28 postimplantation, control rats exhibited appreciable hepatic bioenergetic deterioration, *i.e.*, a P_i/ATP ratio of 1.41 ± 0.35 (SE), significantly higher ($P < 0.05$) than the P_i/ATP ratio for short-term or chronic insulin treatment groups (P_i/ATP 0.92 ± 0.22 and 0.84 ± 0.22, respectively) or rats before tumor implantation (P_i/ATP 0.76 ± 0.14). This insulin-induced bioenergetic protection occurred at any given tumor burden up to at least 10%. Thus, both short-term insulin given just prior to the frank manifestations of cancer cachexia and chronic insulin treatment given throughout tumor growth ameliorated host hepatic bioenergetic deterioration without significantly stimulating tumor growth. Insulin may act by altering the host metabolism (stimulation of liver glucose uptake and utilization, decreased energy-requiring gluconeogenesis, and general protein-sparing action) at the expense of the tumor.

INTRODUCTION

The clinical manifestations of cancer cachexia including anorexia, relentless weight loss, and dwindling body cell mass have been well documented and contribute significantly to patient morbidity and mortality (1–3). It has been estimated that two-thirds of cancer patients die from the effects of cachexia (*e.g.*, increased susceptibility to infection) rather than from the tumor itself (1). The cachectic wasting of host tissue also makes cancer patients less able to withstand the rigors of chemotherapeutic or surgical intervention.

The tumor-host interactions responsible for cancer cachexia are mediated by cytokines such as tumor necrosis factor and various interleukins, as well as by hormones such as glucagon and catecholamines (3). Cachexia increases the levels of tumor necrosis factor, glucagon, and catecholamines and decreases the levels of important anabolic hormones such as insulin and growth hormone. Cachexia-induced changes in these modulators cause appreciable metabolic alterations to host tissue metabolism including lactic acidemia, increased hepatic gluconeogenesis, decreased glucose utilization, increased glucose recycling in the liver and the periphery, excessive lipolysis in the adipose tissues, and increased muscle proteolysis (2, 4). A driving force for the dramatic increase in host tissue catabolism is to provide gluconeogenic precursors to the liver for the dramatically increased glucose consumption by the growing tumor (2). Even small tumors consume large amounts of glucose to form lactate via "aerobic glycolysis," an effect well known since the days of Warburg (5). The demand of the tumor for glucose from the gluconeogenic precursors, lactate (from the tumor), glycerol (from the adipose tissues), and amino acids (from muscle) places significant bioenergetic stress on the liver since gluconeogenesis is an energy-expensive process. While the mechanisms responsible for cancer cachexia remain obscure, a recent hypothesis has emphasized the central role of the liver (1, 3). It has been proposed that the presentation of increasing levels of gluconeogenic precursors to the liver requires progressively greater amounts of energy for hepatic biosynthesis of glucose for the rapidly growing tumor. This is supported by evidence demonstrating increased oxygen consumption of isolated hepatocytes from a tumor-bearing host (1). Ultimately, significant deterioration of host liver bioenergetic status occurs, contributing to the progressive metabolic decline of the host.

While defects in host intermediary metabolism of carbohydrates, proteins, and fats resemble the effects of starvation, these defects persists despite the provision of additional nutrients. The administration of preoperative parenteral hyperalimentation alone may minimize postoperative complications in grossly cachectic patients, but the ability to halt or reverse the underlying metabolic manifestation of cancer cachexia is limited. Recently, manipulation of the substrate metabolism of the host through hormone therapy has been attempted. The anabolic actions of exogenously administered insulin (protein sparing, inhibited lipolysis, and enhanced host carbohydrate utilization) would be advantageous to the patient suffering from cancer cachexia. Studies using cachectic tumor-bearing rats have demonstrated that insulin is effective in promoting weight gain and increasing food intake (6). Compositional analysis shows that the insulin-induced weight gain is a proportional gain of protein, water, and fat similar to observations in normal rat tissue. While insulin is apparently effective in improving host weight, potential effects on intracellular bioenergetic status, particularly in the liver, have not been investigated. Determination of insulin-induced changes in intracellular energy levels would be an important step both in clarifying the mechanism of insulin action in cancer cachexia and in elucidating the biochemical etiology of the cancer cachexia syndrome.

The purpose of this paper is to describe the effects of insulin in sparing the bioenergetic status of the liver. We were able to serially measure *in vivo* concentrations of phosphorus-containing metabolites using ^{31}P MR[2] spectroscopy.

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[1] To whom requests for reprints should be addressed, at Department of Surgery, Victoria Hospital, 375 South Street, London, Ontario, Canada N6G 3V4.

[2] The abbreviations used are: MR, magnetic resonance; RM-ANOVA, univariate repeated-measures analysis of variance; MRS, magnetic resonance spectroscopy.

MATERIALS AND METHODS

Animal Preparation and Monitoring. Eighteen male Fischer 344 rats (200–225 g) were used. A laparotomy placing the left hepatic lobe s.c. was performed as described previously (7). During recovery all animals were individually housed under controlled conditions. Rats were adapted to a nonscatterable C-21 casein base paste diet during the 7-day recovery period. Animal weight and food intake were recorded every 48 h.

Tumor. A 1-mm^3 fragment of nonmetastasizing methylcholanthrene-induced fibrosarcoma was implanted s.c. in the left flank of each animal following initial MRS analysis. The methylcholanthrene-induced sarcoma is not hormone dependent and grows in a reliable fashion causing observable cachexia by postimplant day 24 and death by day 35–40 (4, 6, 7). When tumors became palpable, linear measurements in 3 orthogonal dimensions were obtained 3 times weekly. At sacrifice, tumors were excised, measured, and weighed and tumor burden was calculated (4, 7).

Insulin Administration. Animals were randomly assigned to one of three groups. Group 1 (no insulin) rats (n = 7) received saline equivalent to the volume of insulin solution given to groups 2 and 3. Group 2 (insulin-treated) rats (n = 8) began receiving insulin (2 units/100 g body weight/day) on post-tumor implant day 21. Group 3 (chronic insulin-treated) rats (n = 3) began receiving insulin by s.c. injection on the tumor implant day.

^{31}P MRS. ^{31}P spectra were obtained using a two-turn, 0.9-cm doubly tuned surface coil and a ORS TMR-32 spectrometer with a 1.89-T superconducting magnet. Animals were anesthetized (pentobarbital, 35 mg/kg i.p.) prior to scanning. A pulse repetition rate of 756 ms was used to obtain two 512-scan hepatic spectra. Skeletal muscle scans were performed on the right thigh muscle using the same pulse parameters. Scans were collected at 7-day intervals for 28 days.

The free induction decays were collected using 2-K data points and a 4000-Hz bandwidth. Prior to Fourier transformation, the data were zero-filled to 4 K and multiplied by a 10-Hz exponential to improve the signal/noise ratio. The broad hump was removed in a reproducible manner by standard deconvolution techniques.

The relative areas under individual peaks were determined using a nonlinear least square algorithm to fit the spectrum to a sum of 7 Lorentzian curves. The spectra were normalized to the area under the phosphocreatine peak and the skeletal muscle contamination of the hepatic spectra was eliminated by subtracting the peak areas of skeletal muscle spectra from that of the hepatic spectra (8). The hepatic P_i/ATP ratio was then calculated from the areas under the P_i and ATP resonances (β-phosphate).

Statistical analysis to assess treatment effects of groups over time was performed using SAS to calculate RM-ANOVA after ensuring that the orthogonal components were uncorrelated and had equal variances. Differences between treatment groups were assessed by the least significant difference method. A paired Student t test was used to assess effects on individual animals before and after acute insulin treatment, at days 21 and 28, respectively. Statistical significance was set at $P < 0.05$.

RESULTS

Animal Monitoring. Daily food intake was measured and plotted as a function of time after tumor implantation (Fig. 1*A*). Food intake was not significantly different among the groups until day 22 (post-tumor implant) when intake for the control tumor-bearing rats started to decrease substantially. This is in agreement with previous studies using this same animal model (6, 7). After day 22, food intake for the short-term insulin group (group 2) became significantly higher ($P < 0.05$, RM-ANOVA) than that of the control group (group 1), indicating the beneficial effects of insulin on cachexia-induced anorexia. The chronic insulin-treated group (group 3) initially consumed more food than groups 1 and 2 did over the first 10–15 days of the study. However, chronic insulin treatment did not appear to prevent the later cachexia-induced decrease in food intake after day 20; food intake after day 22 was not significantly different from controls ($P > 0.05$, RM-ANOVA).

Total body weight (Fig. 1*B*) rose in all groups until day 22 when the control group body weights reached a plateau and began to decrease. After day 22, the short-term insulin group (group 2) and the chronic insulin-treated rats (group 3) had significantly higher body weight ($P < 0.05$, RM-ANOVA) than the control group. Differences between body weights of groups 2 and 3 were not significant over this period ($P > 0.05$, RM-ANOVA). The body weights of the chronic insulin-treated groups had reached a plateau by day 22 and were starting to decrease. Although the short-term insulin and chronic insulin-treated rats had a decline in carcass weight (total body weight minus tumor weight) the loss was significantly less ($P < 0.05$) than that observed in the control group.

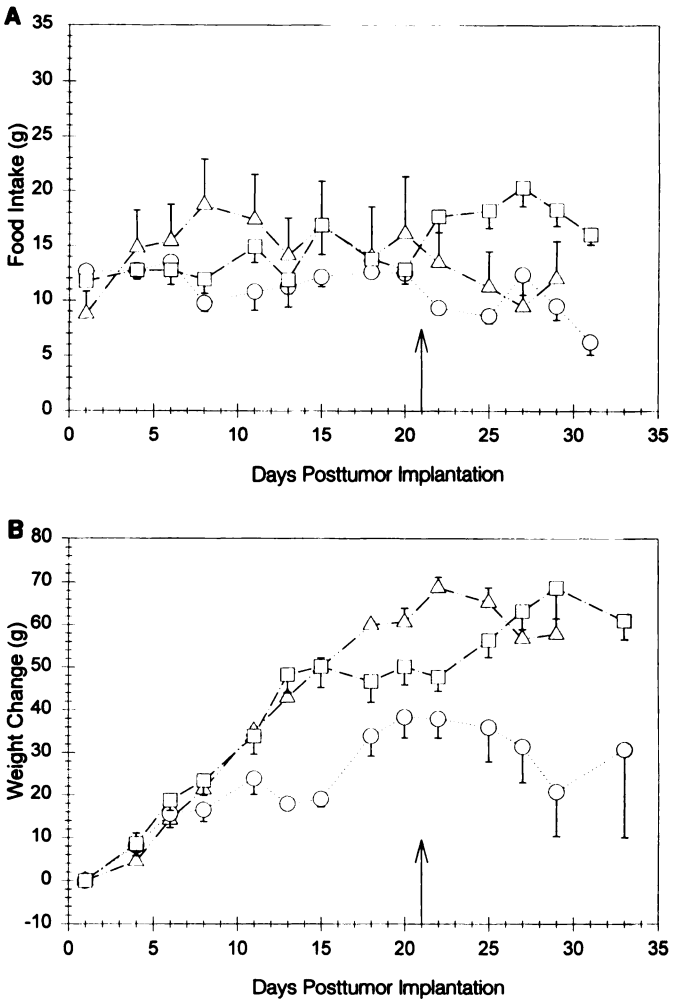

Fig. 1. *A*, plot of food intake *versus* time after tumor implantation. Daily food intake was measured for: group 1, control tumor-bearing rats, n = 7 (○); group 2, short-term insulin-treated tumor-bearing rats, n = 8 (□); group 3, chronic insulin-treated tumor-bearing rats, n = 3 (△). *Arrow*, time when insulin was started for group 2. *Points*, mean; *bars*, SE. *B*, plot of body weight gain *versus* time after tumor implantation. Body weight gain was measured for the same three groups as in *A*.

Moley *et al.* (6) used the same animal model for cachexia and found very similar effects of acute insulin (given 20 days after tumor implantation, for a 5-day period) and chronic insulin (started 10 days after implantation). These workers reported that acute insulin increased both body weight gain and daily food intake by amounts similar to those in our present study. Their longer-term insulin treatment initially increased food intake but by days 20–25 the intakes were decreasing just as in our study. Total body weight had also reached a plateau by days 25–30 in the study of Moley *et al.* (6), which was similar to the results we found. This suggests a longer-term (>7 days) adaptation to the beneficial anabolic effects of insulin as tumor burden increases.

Tumor Burden. The implanted tumor had an exponential growth rate (Fig. 2). From day 21 to day 31, there were no significant differences between the groups except on day 29, when tumor burden for groups 2 and 3 was significantly lower than in group 1. These data show that the insulin treatments did not stimulate tumor growth and in fact tended to inhibit the progression of tumor burden.

Schneeberger *et al.* (7) and Moley *et al.* (6) showed similar rates of tumor progression in control rats, although Moley *et al.* (6) did not find significant differences in tumor growth among control, acute insulin, and longer-term insulin-treated groups. Thus, the results of Figs. 1 and 2 are consistent with other studies using this cancer cachexia model and hence validate the ^{31}P MRS results that we will now describe.

^{31}P MRS. ^{31}P MR spectra for liver and skeletal muscle were acquired serially over the course of tumor development as described previously (7, 8). Initially (day 0), the P_i/ATP (β-phosphate) ratio for the control group was 0.76 ± 0.14 (SE) (Fig. 3). This ratio did not differ significantly from the acute or chronic insulin groups ($P > 0.05$, RM-ANOVA). Prior to insulin administration, groups 1 and 2 had a rise in the P_i/ATP ratio from day 0 to day 21. There was no significant difference ($P > 0.05$, RM-ANOVA) between the control and the acute insulin groups from day 0 to day 21 (before insulin treatment). After insulin administration on day 21, the short-term (7-day) insulin group (group 2) animals showed a decline in the P_i/ATP ratio from 1.06 ± 0.05 to 0.92 ± 0.08. This indicates that the 7-day insulin treatment tended to reverse the increase in hepatic P_i/ATP ratio in these animals, although the effect was not statistically significant ($P = 0.29$, paired Student's t test). From day 21 to day 28, the P_i/ATP ratio continued to rise in the tumor-bearing controls (group 1). By day 28 the control group had a P_i/ATP ratio of 1.41 ± 0.35, which was significantly higher ($P < 0.05$, RM-ANOVA) than both the short-term insulin-treated (0.92 ± 0.22) and the chronic insulin-treated group (0.84 ± 0.22). On day 28 groups 2 and 3 did not differ statistically ($P > 0.05$, RM-ANOVA). Thus, both short-term insulin treatment given just prior to frank manifestations of cancer cachexia and chronic insulin treatment given throughout the full course of tumor growth significantly decrease the bioenergetic deterioration of the liver.

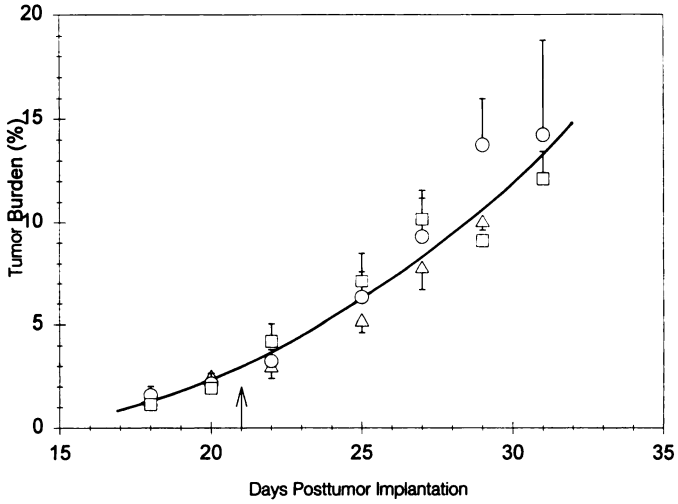

Fig. 2. Plot of tumor burden (weight of tumor/total body weight) *versus* time after tumor implantation. Tumor burdens were determined for: group 1, control tumor-bearing rats, $n = 7$ (○); group 2, short-term insulin-treated tumor-bearing rats, $n = 8$ (□); group 3, chronic insulin-treated tumor-bearing rats, $n = 3$ (△). *Arrow*, time when insulin was started for group 2. *Points*, mean; *bars*, SE.

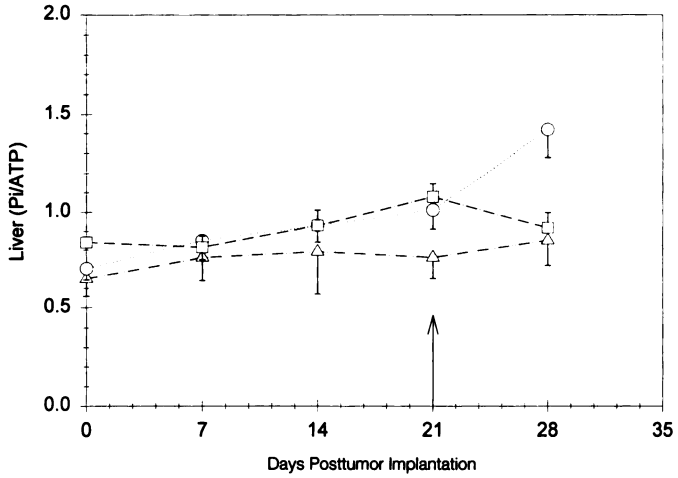

Fig. 3. Plot of hepatic P_i/ATP ratios *versus* time after tumor implantation. Serial hepatic P_i/ATP ratios were measured for: group 1, control tumor-bearing rats, $n = 7$ (○); group 2, short-term insulin-treated tumor-bearing rats, $n = 8$ (□); group 3, chronic insulin-treated tumor-bearing rats, $n = 3$ (△). *Arrow*, time when insulin was started for group 2. *Points*, mean; *bars*, SE. At day 28, the P_i/ATP ratios for groups 2 and 3 were significantly lower than for group 1.

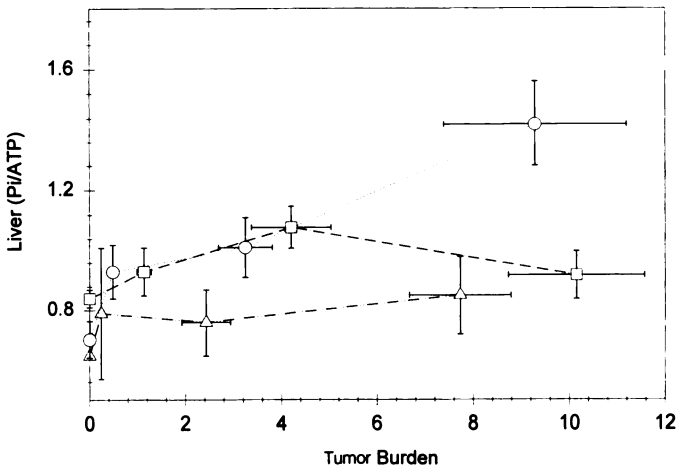

Fig. 4. Plot of hepatic P_i/ATP ratios *versus* tumor burden. Serial hepatic P_i/ATP ratios were measured for: group 1, control tumor-bearing rats, $n = 7$ (○); group 2, short-term insulin-treated tumor-bearing rats, $n = 8$ (□); group 3, chronic insulin-treated tumor-bearing rats, $n = 3$ (△). *Points*, mean; *bars*, SE, with variability in both P_i/ATP and in tumor burden indicated. Group 2 began to receive insulin when tumor burden was less than 4%. At tumor burdens greater than 6%, the P_i/ATP ratios for groups 2 and 3 were significantly lower than for group 1.

The P_i/ATP ratios for the various groups were plotted as a function of tumor burden (Fig. 4). The control group showed a substantial increase in P_i/ATP as the tumor burden increased. The P_i/ATP ratios for the chronic insulin-treated rats were largely unaffected by the degree of tumor burden, up to at least 10%. The group 2 rats experienced the same increase in P_i/ATP, with increasing tumor burden, as did the control group until insulin was given at day 21. The P_i/ATP ratio for the group 2 rats then decreased to that of the chronic insulin-treated group, although the tumor load was quite high (>10%). For tumor burdens >6%, group 3 and group 2 after insulin exhibited significantly lower P_i/ATP ($P < 0.05$, RM-ANOVA) than controls. Thus, the two insulin treatments were able to prevent (group 3) and even reverse (group 2) the hepatic bioenergetic decline induced by cancer cachexia.

DISCUSSION

In vivo ^{31}P MRS is a noninvasive technique which allows for repeated analysis of the major phosphorus-containing metabolites in a particular organ or tissue within the intact animal or human. This method thus has distinct advantages over invasive methods, such as needle biopsies, or noninvasive methods, which

nonspecifically monitor bioenergetic parameters of the whole organism. For example, Moley *et al.* (6) found that insulin had no effect on total body energy expenditure but did not have a means of assessing bioenergetic status for the liver specifically. This study focuses on the liver since this is the key organ in coordinating the metabolism of the host in response to the tumor and plays a central role in cachexia etiology.

Analysis of the P_i/ATP ratio is a well accepted indicator of liver bioenergetics (7, 9, 10). ATP is the main high energy compound within the hepatocyte (there is no phosphocreatine in the liver), and P_i and ADP are the main breakdown products of ATP hydrolysis. Thus, the P_i/ATP ratio gives an estimate of the phosphorylation potential for the cell and an indication of the steady-state balance of ATP-generating processes such as glycolysis and oxidative phosphorylation *versus* ATP (or energy)-requiring processes such as fatty acid biosynthesis or gluconeogenesis. Cancer cachexia has been shown to increase P_i/ATP ratios in the liver (7); hepatic P_i/ATP ratios increase as the tumor burden increases. However, there is no indication that the ability of the liver to function normally and generate ATP is compromised at tumor burdens of 2–10% (1, 2, 4). At 6% tumor burden, for example, the liver can still metabolize a fructose load as rapidly as non-tumor-bearing animals while maintaining the same ATP levels as non-tumor-bearing rats.[3] Thus, the increase in P_i/ATP detected by *in vivo* ^{31}P MRS must be due to the increased demand for energy-requiring processes in the liver. Given the presence of a rapidly growing tumor and the fact that tumors preferentially utilize glucose as their energy source (2, 5), increased hepatic gluconeogenesis is the most likely candidate for the increase in ATP-requiring processes involved in cancer cachexia. Six ATP equivalents are required to convert two molecules of lactate to one molecule of glucose. Gluconeogenic precursors include lactate, the end product of tumor metabolism and, to a lesser degree, α-ketoacids from the proteolysis of muscle to amino acids and glycerol from lipolysis. (It should also be noted that the ammonia released from the breakdown of amino acids must be detoxified in the liver via the urea cycle, another major energy-requiring process.) Such a "hypermetabolic state" of increased energy expenditure in the liver with an increased P_i/ATP ratio, has been observed in other conditions, such as during chronic exposure to ethanol (10) or after a 48-h fast (9). This increase in energy expenditure would not be detectable using whole-body calorimetric methods (6) since the effect on the liver would be obscured by the rest of the body.

In this study, we have demonstrated not only that cancer cachexia causes a bioenergetic imbalance in the liver but also that insulin ameliorates and even reverses this effect. This is likely because insulin inhibits the energy-requiring processes which are stimulated in cancer cachexia. Insulin is protein sparing and inhibits the degradation of proteins to amino acids to α-ketoacids. Insulin has particularly been implicated in increasing the utilization of glucose by the host liver, at the expense of the tumor, *i.e.*, more hepatic glucose absorption and utilization, less gluconeogenesis, less glucose made available to the tumor and hence less lactic acid produced by the tumor (1, 2, 4, 6). This shift in nutrients from the tumor to the host decreases the bioenergetic demands placed on the liver, reestablishing the steady-state balance between ATP synthesis and utilization and bringing P_i/ATP ratios back toward normal levels.

[3] K. E. Gehman, R. I. Inculet, G. D. Marsh, M. Brauer, A. A Driedger, and R. T. Thompson. Early detection of cancer cachexia using hepatic ^{31}P magnetic resonance spectroscopy and a fructose stress test, submitted for publication.

The only other study which has examined at the adverse metabolic effects of a remote tumor on the liver and the reversal of these effects by hormones and growth factors was that of Dong *et al.* (11). They found that insulin-like growth factor 1 and insulin improved hepatic mitochondrial redox potential, as reflected in acetoacetate/β-hydroxybutyrate ratios in tissue extracts after removal of the liver from tumor-bearing rats. However, these studies were suspect due to the invasive nature of their techniques. Very rapid metabolic changes occur in the liver in <5 s of interruption of blood flow to the liver (12) so that quick freeze-clamping of the tissue (which was not done in this study) must be done. This underscores the value of a noninvasive technique such as *in vivo* ^{31}P MRS. A very recent report by Dagnelie *et al.* (13) found, via ^{31}P MRS, that remote prostate tumors induced an increase in the hepatic P_i/ATP ratio, which is in agreement with our studies. However, the effects of insulin were not assessed. Insulin was recently shown to improve the bioenergetic status, *i.e.*, increase the phosphorylation potential ([ATP]/[ADP] [P_i]) of a normal glucose-perfused rat heart (14).

Thus, the development of cachexia is associated with an increase in hepatic P_i/ATP which can be detected by ^{31}P MRS. The changing ratio is most likely due to a reduction in the phosphorylation potential caused by the high energy demands of the liver, reflecting an abscopal effect on the liver of a remote tumor. Insulin counteracts the effects of the tumor on P_i/ATP, presumably mediated through one or more of the known anabolic effects of the hormone, likely alleviating the high-energy-requiring processes involved in gluconeogenesis.

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