A mechanism to explain how regular exercise might reduce the risk for clinical prostate cancer

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Epidemiological studies report that regular physical activity can reduce the risk for prostate cancer. This study was conducted to investigate possible mechanisms to explain the epidemiological data. Serum from sedentary controls or men with regular (5 days/week) aerobic exercise was used to stimulate lymph node cancer of the prostate (LNCaP) tumor cells *in vitro*. Growth and apoptosis were assessed and cell lysate p53, p21 and Bcl-2 proteins measured. Tryphostin was used to block the insulin-like growth factor-I receptor. Exercise serum-stimulated growth was reduced at 2 and 4 days while apoptosis was increased. Tryphostin reduced growth in the control but not in the exercise serum-stimulated samples. Total cell lysate p53 protein was higher in the exercise serum-stimulated cells at both 2 and 4 days. The levels of p21 protein, a downstream effector of p53, were elevated at 2 days but were normal at 4 days. Bcl-2, an antiapoptotic protein, was significantly reduced at 2 days in the exercise serum-stimulated lysates. These results indicate that exercise training alters serum insulin-like growth factor axis factors *in vivo* that increase LNCaP cellular p53 protein content *in vitro* leading to reduced growth via p21 and induced apoptosis via the mitochondrial pathway.
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Introduction

The pathologic incidence of small, latent or subclinical prostatic carcinoma is similar across many different populations (Breslow *et al.*, 1977). Marked variations, however, exist in the clinical incidence of prostate cancer (PCa), ranging from a low of 1/100 000 in China to 45–65/100 000 in whites in the United States to 102/100 000 in African-Americans in the United States (Hsing *et al.*, 2000). The low rates in East Asian men change dramatically when men relocate to the United States and adopt a western lifestyle (Muir *et al.*, 1991; Shimizu *et al.*, 1991). These data all point to the fact that a Western lifestyle significantly increases the risk for clinical PCa. One aspect that has received much attention is the lack of physical activity. In a recent review of the literature on physical activity and the risk for PCa, Thune and Furberg (2001) found that 14 of 28 epidemiological studies reported that increased occupational or leisure time activity reduced the risk for PCa by 10–70%. In a more recent study (Giovannucci *et al.*, 2005), it was reported that regular vigorous activity among men in the Health Professionals Follow-up Study slowed the progression of PCa and reduced PCa mortality.

In previous studies (Tymchuk *et al.*, 2001; Ngo *et al.*, 2002, 2003a, b; Barnard *et al.*, 2003), we have demonstrated that a low-fat diet and/or regular physical activity results in changes in serum *in vivo* that reduced the proliferation and increased apoptosis of serum-stimulated lymph node cancer of the prostate (LNCaP) and Los Angeles prostate cancer-4 (LAPC-4), androgen-dependent, prostate tumor cell lines *in vitro*. It is well recognized that the induction of apoptosis is important for determining the progression of clinical cancer (Gurumurthy *et al.*, 2001). The changes in serum with a low-fat diet and/or regular exercise included reductions in insulin, insulin-like growth factor-I (IGF-I) and free testosterone along with increases in sex hormone-binding globulin and insulin-like growth factor binding protein-1 (IGFBP-1) (Tymchuk *et al.*, 1998; Barnard *et al.*, 2003). Changes in the IGF axis appeared to be the most important. When IGF-I was added back to the post-diet and exercise serum or the exercise alone serum, the reduced growth in LNCaP cells was eliminated (Barnard *et al.*, 2003). When IGFBP-1 was added to the pre-diet and exercise serum, LNCaP growth was reduced and apoptosis increased (Ngo *et al.*, 2003b).

The current study focuses on the ability of exercise training to alter serum factors that increase the function of the p53 gene in PCa tumor cells. The specific biological roles attributed to p53 are very complex, but p53 essentially protects the genome from mutations and/or genetic alternations. When defects are found in DNA, the p53 protein is phosphorylated and stabilized,

which activates other downstream genes or factors to cause cell cycle arrest, DNA repair, or to induce apoptosis (Gurumurthy *et al.*, 2001; Wahl and Carr, 2001). IGF-I has been documented to suppress these actions of p53 (Gurumurthy *et al.*, 2001; LeRoith and Roberts, 2003). As we have documented that regular exercise decreases serum levels of IGF-I while increasing serum IGFBP-1 to further reduce free IGF-1, we hypothesized that these serum changes may increase tumor p53 protein content, reduce serum-stimulated tumor cell growth and initiate apoptosis. In an initial study, we documented an increase in p53 protein in exercise serum-stimulated LNCaP cell lysates (Leung *et al.*, 2004). To further test this hypothesis, we have measured the effect of exercise on serum-stimulated p53 protein content as well as related factors including p21 and Bcl-2 in the LNCaP prostate cell line. We also used tyrphostin, a specific IGF-I/insulin receptor tyrosine kinase inhibitor, to evaluate the effects of these growth factors on LNCaP cell growth.

Materials and methods

Study participants

Similar aged (62 ± 2, 60 ± 3 years) groups of control ($n = 10$) and exercise ($n = 12$) volunteer men were studied. The control group consisted of obese men with a sedentary lifestyle, that is no regular exercise program, and all had prostate specific-antigen values in the normal range (< 4.0 ng/dl). The body mass index of the control group was 38 ± 2 vs. 26 ± 1 for the exercise group. The exercise group attended the Adult Fitness Program at the University of Nevada, Las Vegas. Volunteers were requested from among those who had participated in the program for at least 10 years; the average was 14.7 years. The program was held 5 days/week for 1 h and consisted of warm-up and flexibility activities followed by 45–50 min of continuous, strenuous exercise including calisthenics and swimming laps in the pool. Attendance was checked daily and the 12 participants averaged 4.7 days/week over the 14.7 years. Years of participation in the program ranged from 3 to 25. No dietary intervention occurred in the Adult Fitness Program of the University of Nevada, Las Vegas. Although one might expect individuals who exercise on a regular basis to also eat a healthy diet, this did not appear to be the case with these participants as their fasting total and low-density lipoprotein cholesterol levels were not significantly different from that of the control group (Barnard *et al.*, 2003). Fasting blood samples were obtained from the participants in the morning, allowed to clot, and then were centrifuged to obtain serum. The serum was frozen and stored at −80°C until used for analyses. Serum samples were analyzed for insulin, IGF-I and IGFBP-1 using the enzyme-linked immunosorbent assay (ELISA) kits from Diagnostic Systems Inc. (Webster, Texas, USA) and were reported in earlier papers (Barnard *et al.*, 2003; Leung *et al.*, 2004). The University of California Los Angeles' Institutional Review Board approved the study and informed consent was obtained from the participants.

Cell culture

Androgen-dependent LNCaP prostate tumor cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were grown in 75-cm^2 flasks (Falcon Primaria, Bedford, Massachusetts, USA) in RPMI-1640 medium without phenol red, supplemented with 10% fetal bovine serum (FBS), 200 IU penicillin, 200 mg/ml streptomycin and 4 nmol/l L-glutamine (Omega Scientific Inc., Tarzana, California, USA). The cultures were maintained at 37°C and supplemented with 5% CO_2 in a humidified incubator. Cells were passaged routinely at 80% confluence and fresh medium was replaced every third day. Cells used in experiments were not passaged more than 10 times.

Growth assay

Cells in the Falcon flasks were detached with 0.25% trypsin-ethylene diamine tetraacetic acid solution (Sigma Chemical Co., St Louis, Missouri, USA), centrifuged at 3000*g* for 5 min at 10°C and resuspended in fresh medium. Cell viability was assessed via trypan blue exclusion. Cells were plated (5×10^3 cells/well) in 96-well plates and allowed to attach and stabilize for 24 h. After the 24-h stabilization period, fresh medium (RPMI-1640 medium, 200 IU penicillin, 200 mg/ml streptomycin and 4 nmol/l L-glutamine) with 10% FBS or 10% human serum was added to the wells in triplicates. The plates were then incubated (37°C, 5% CO_2) for 2 or 4 days. Cell growth was determined by CellTiter 96AQ Assay (Promega Corporation, Madison, Wisconsin, USA). This method has been shown to correlate with manual counting of cells in our laboratory ($r = 0.98$).

In order to further investigate the involvement of IGF in LNCaP cell growth an IGF-I receptor/insulin receptor tyrosine kinase inhibitor, tryphostin, (AG 1024 from A.G. Scientific, San Diego, California, USA) was added to the cultures (10 nmol/ml) for both control and exercise serum stimulation and the cells were grown for 2 or 4 days.

Apoptosis assay

Experiments were carried out in 96-well tissue culture plates at a density of 10×10^3 cells/well. Cells were prepared and plated for 24 h as mentioned above. Apoptosis was measured at the end of 2 or 4 days of cell growth with FBS, control or exercise sera by Cell Death Detection ELISA Plus (Roche Applied Science, Indianapolis, Indiana, USA). This apoptosis assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, and allows the specific determination of mononucleosomes and oligonucleosomes in the cytoplasm fraction of lysates. Background using incubation

buffer and ABTS solution was subtracted from the absorbance measurements (405–490 nm). The results are expressed in microunits of mononucleosomes and oligonucleosomes. In previous studies with the same serum samples, we have used terminal deoxytransferase uridine triphosphate nick-end labeling and annexin-V assays to determine apoptosis and found results similar to those reported for the Cell Death Detection ELISA (Barnard *et al.*, 2003).

Cell lysate assays

LNCaP cells were initially plated at a density of 150 000 cells/10-cm dish, and allowed to attach and stabilize for 24 h. After the 24-h stabilization period, fresh medium (RPMI-1640 medium, 200 IU penicillin, 200 mg/ml streptomycin and 4 nmol/l L-glutamine) with 10% human serum was added to the dishes. Cells were then incubated for 2 or 4 days. After the incubation period, cells were washed once in phosphate-buffered saline and were lysed with 5 × passive lysis buffer containing protease inhibitors (Promega). After 30 min of incubation at room temperature, lysates were centrifuged for 10 min at 3000*g* and the supernatants collected. A Bio-Rad protein assay (Bio-Rad Laboratory, Richmond, California, USA) was used to determine total protein concentration in lysates and cell suspension buffer was used to adjust the protein concentrations to 10 μg/ml.

Following protein determination, a p53 ELISA kit (Oncogene Research Products, San Diego, California, USA) was used to measure p53 protein concentration in cell lysate supernatants according to the manufacturer's protocol. A $p21^{WAF1}$ ELISA kit (Oncogene Research Products) was used to quantify p21 protein. For Bcl-2 determination, 20 μg of lysate protein was loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. Immunoblotting was performed with anti-Bcl-2 and anti-heat shock protein-60 (HSP-60) antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse antibodies as secondary antibodies and detected by chemiluminescence (ECL, Amersham, Piscataway, New Jersey, USA).

Statistical analysis

All growth and apoptosis assays for both the control and exercise samples were carried out on the same 96-well plates and the results were expressed as a percentage of the control results. Statistical analysis (InStat Statistical Software, Graphpad Prism, San Diego, California, USA) was performed by a Student's *t*-test for two-variable data sets. For more than two groups, data were analyzed by analysis of variance followed by Newman–Keuls post-hoc analyses. $P < 0.05$ was considered significant. Data are expressed as means ± standard error.

Results

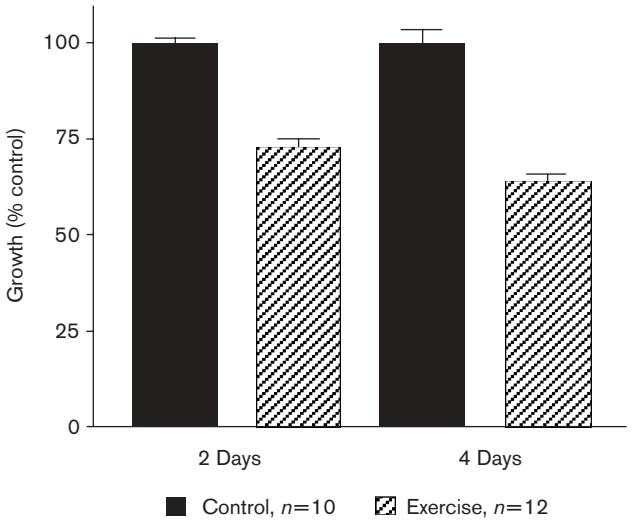
Lymph node cancer of the prostate growth and apoptosis

Figure 1 shows the data for LNCaP cell growth following stimulation with exercise or control participants' serum. Cell proliferation was significantly lower ($P < 0.05$) after both 2 (27%) and 4 (36%) days of exercise serum-stimulation relative to control with no significant difference between the 2 and 4 days. When the tyrosine kinase inhibitor tyrphostin was added to the control serum-stimulated cultures, growth at 2 days was reduced by 40 ± 4.4% and was not significantly different from the growth seen with the exercise samples. When the inhibitor was added to the exercise serum-stimulated cultures no further reduction in LNCaP cell growth was noted (Fig. 2). Apoptosis (Fig. 3) was significantly higher ($P < 0.05$) after 2 (368%) and 4 (772%) days of exercise serum stimulation, with the apoptosis after 4 days being significantly greater ($P < 0.05$) than that after 2 days for the exercise samples.

Lymph node cancer of the prostate p53 and p21 protein

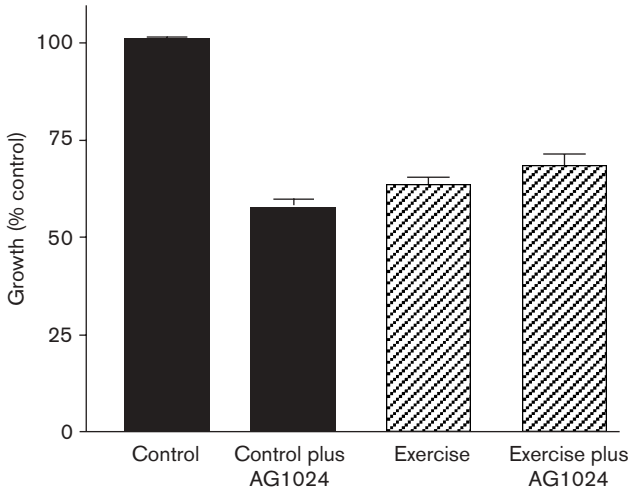
Serum-stimulated cell lysate p53 protein was increased from 38.2 ± 2 pg/μg protein for the controls to 75.2 ± 2 pg/μg protein for the exercise samples after 2 days of growth ($P < 0.05$) and from 41.0 ± 2 to 62.5 ± 4 pg/μg protein after 4 days of cell growth ($P < 0.05$) (Fig. 4). Figure 5 shows the p21 protein data. Serum-stimulated cell lysate p21 protein was increased from 0.42 ± 0.01 units/μg protein for the controls to 1.79 ± 0.08 units/μg protein for the exercise samples after 2 days of growth. At 4 days of growth, there was no

Fig. 1



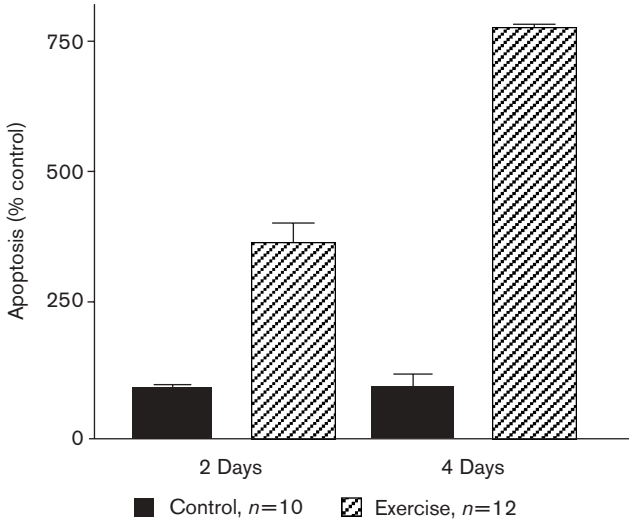
Lymph node cancer of the prostate cell growth following 2 or 4 days of sedentary control or exercise participants' serum stimulation. The differences between the control and exercise groups were significant ($P<0.05$) at both 2 and 4 days.

Fig. 2



Effect of insulin-like growth factor-I receptor/insulin receptor tyrosine kinase inhibition with tryphostin (AG 1024) on serum-stimulated lymph node cancer of the prostate (LNCaP) cell growth. Blocking the receptor kinase activity in the control group significantly reduced LNCaP growth but had no affect on the exercise group.

Fig. 3



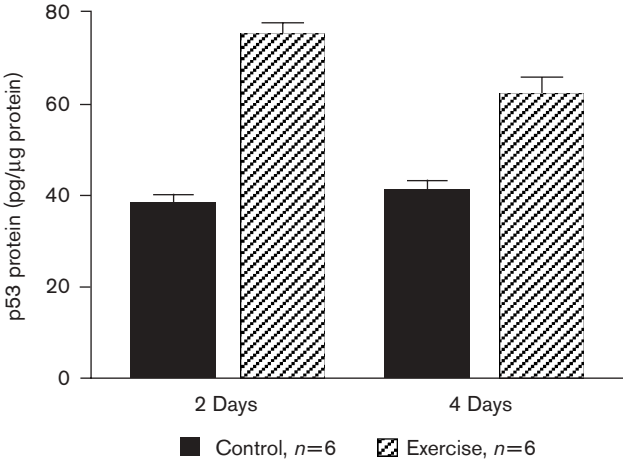
Lymph node cancer of the prostate cell apoptosis following 2 or 4 days of control or exercise participants' serum stimulation. Apoptosis was significantly increased ($P<0.05$) in the exercise samples compared with the control, and the 4-day values for the exercise group were significantly higher than the 2-day values.

significant difference between the groups (0.45 ± 0.01 vs. 0.43 ± 0.01 units/μg protein).

Apoptosis pathway

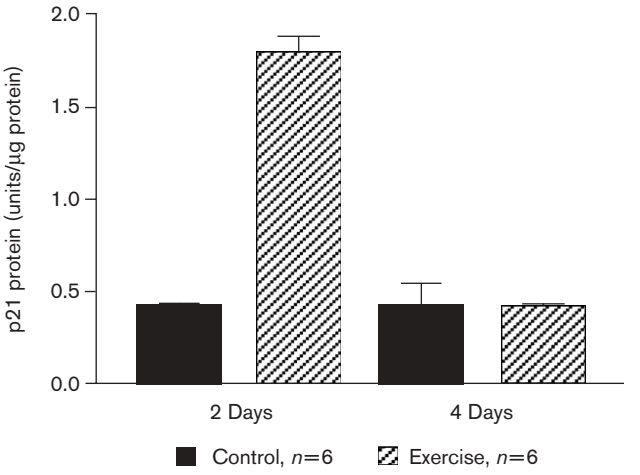
Figure 6 shows the Bcl-2 data and the western blot. The Bcl-2 optical density units normalized for HSP-60 protein

Fig. 4



Lysate p53 protein content in lymph node cancer of the prostate cells following 2 or 4 days of control or exercise participants' serum stimulation. At both 2 and 4 days, the p53 values were significantly ($P<0.05$) elevated in the exercise group compared with the controls.

Fig. 5



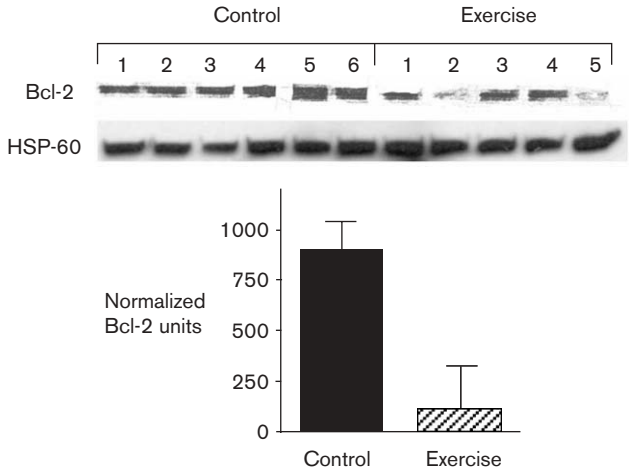
Lysate p21 protein content in lymph node cancer of the prostate cells following 2 or 4 days of control or exercise participants' serum stimulation. The p21 level was significantly ($P<0.05$) elevated at 2 days in the exercise samples but was not different at 4 days.

for the exercise serum-stimulated samples were significantly decreased ($P < 0.05$) from those of control samples after 2 days of growth. No difference was observed in HSP-60 protein optical density units between the control and exercise groups (data not shown).

Discussion

In previous studies (Barnard *et al.*, 2003; Leung *et al.*, 2004), we reported that men who participated in regular exercise had alterations in the serum IGF axis including a

Fig. 6



Lysate Bcl-2 protein in lymph node cancer of the prostate cells following 2 days of control or exercise participants' serum stimulation. Heat shock protein-60 (HSP-60) was run as the control and the optical density readings for the Bcl-2 protein were normalized for the HSP-60 optical density readings. The normalized values for the exercise group were significantly ($P<0.05$) less than those of the control group.

reduction in IGF-I and an increase in IGFBP-1 that were associated with an in-vitro reduction in LNCaP cell growth with increased apoptosis relative to a sedentary control group of obese men. These results should reduce the risk for clinical PCa. The results of the present study confirm our earlier reports of reduced growth and increased apoptosis with exercise serum-stimulated LNCaP cells. In the present study, we extended growth of the LNCaP cells from 2 to 4 days and found no significant further reduction in growth compared with controls, but a significant increase in the amount of apoptosis. The importance of the IGF-I receptor/insulin receptor kinase activity for LNCaP cell growth was documented by the reduction of growth when the kinase inhibitor was added to the control serum. When the kinase inhibitor was added to the exercise serum, no further reduction in LNCaP cell growth was noted, which indicates the importance of the reduction in serum IGF-I and increase in IGFBP-1 noted in the exercise group participants. The reduction in LNCaP growth with exercise serum stimulation was associated with an increase in p53 protein and one of its downstream effectors p21 protein. The increase in apoptosis after 2 days of LNCaP growth was associated with a decrease in Bcl-2 a protein known to block the mitochondrial apoptotic pathway.

The large international variation in PCa mortality between westernized and developing countries might be explained, in part, by differences in physical activity. Indeed, epidemiological studies from westernized countries report a 10–70% reduction in PCa with increased occupational or leisure time activity (Thune and Furberg, 2001). The results of the present study provide a mechanism that supports the value of regular physical activity to reduce the risk for clinical PCa. Furthermore, the cell culture studies with serum from the sedentary control participants showing robust growth of LNCaP cells with almost no apoptosis and very low levels of p53 protein support the hypothesis that a western lifestyle leads to a hyperproliferative state stimulating the promotion of latent to clinical PCa.

In our previous study (Barnard *et al.*, 2003) with serum from the same participants, we reported that the exercise program reduced serum insulin and IGF-I while increasing IGFBP-1with no change in IGFBP-3. In an earlier study (Ngo *et al.*, 2003b), we reported that adding IGFBP-1 to control human serum reduced growth and induced apoptosis of LNCaP cells. In our earlier exercise study (Barnard *et al.*, 2003), we reported that adding IFG-I to the exercise serum eliminated the difference between the growth of LNCaP cells in exercise vs. control serum. These data, along with the tyrosine kinase inhibitor experiments in the present study, all suggest that the IGF axis plays an important role in the development of PCa that is supported by prospective (Chan *et al.*, 1998; Harman *et al.*, 2000; Stattin *et al.*, 2000, 2004; Li *et al.*, 2003) and case–control (Mantzoros *et al.*, 1997; Wolk *et al.*, 1998; Chokkalingam *et al.*, 2001) studies. Some studies (Lacey *et al.*, 2001; Woodson *et al.*, 2003), however, reported no relationship between IGF-I and the risk for PCa and one study found it to be only predictive for advanced PCa (Chan *et al.*, 2002).

The underlying mechanism by which exercise alters the IGF axis to reduce growth and induce apoptosis in LNCaP cells is likely the result of decreasing insulin resistance and lowering serum insulin. It is well documented that regular exercise has an insulin-like effect, enhances insulin sensitivity and reduces serum insulin (Goodyear and Kahn, 1998). IGF-I is produced in many tissues in the body including tumors, but 75–80% of the circulating IGF-I comes from the liver where IGFBP-1 is also produced (Yakar *et al.*, 1999; LeRoith and Roberts, 2003). IGF-I is important during growth and development and is controlled primarily by growth hormone. With aging, growth hormone levels fall as do IGF-I levels (Benbassat *et al.*, 1997). We previously speculated, however, that the drop in IGF-I that should normally be associated with aging is blunted by the increase in serum insulin normally associated with insulin resistance in older individuals (Barnard *et al.*, 2002). Insulin has been shown to stimulate liver production of IGF-I (Shimizu *et al.*, 1991). Higher levels of IGFBP-1 have been reported in older participants; however, an inverse relationship between insulin and IGFBP-1 was noted in both the young and old participants indicating

that insulin suppresses IGFBP-1 (Benbassat *et al.*, 1997). This is consistent with our data showing a higher IGFBP-1 with lower insulin in men who exercise (Barnard *et al.*, 2003). In addition to the effects of insulin on the liver to alter the IGF axis, it also suppresses the production of sex hormone-binding globulin that would increase the amount of free testosterone available to interact with androgen-dependent PCa cells like LNCaP (Plymate *et al.*, 1988, 1990). We previously reported that lowering insulin through a low-fat diet and exercise intervention was associated with an increase in sex hormone-binding globulin and reduction in free testosterone (Tymchuk *et al.*, 1998, 2001).

Although different pathways have been identified that might control tumor cell growth, the results of this study implicate the $p21^{WAF1}$ pathway resulting from an increase in p53 protein. It is well known that one of the downstream effectors of p53 is p21, a protein that is known to bind and inhibit cyclin-dependent kinases to reduce cell cycle progression at both the G1 and G2 checkpoints (Balint and Vousden, 2001). Thus, the increase in p21 protein in the exercise serum-stimulated LNCaP cells is probably responsible for the noted reduction in cell growth.

The induction of apoptosis is known to occur by different pathways. The results from this study indicate that the increase in p53 protein may activate the mitochondria pathway to increase caspase 9/3 activity. The observed decrease in Bcl-2 protein would allow Bax and Bad to insert into the mitochondria membrane to release cytochrome C and other factors known to activate the caspase system (Tsujimoto, 2003). The decrease in Bcl-2 and increase in apoptosis after 2 days of LNCaP growth in exercise serum suggests an increase in caspase 9/3 activity. The fact that incubating the cells for 4 days resulted in no further major reduction in cell growth but a major increase in apoptosis is probably due to the decrease in p21 protein going from 2 to 4 days of serum stimulation. These results are similar to those reported for lung carcinoma cells expressing wild-type p53 (Lehman *et al.*, 1991). It has been reported that caspase 3 activity mediates cleavage and inactivation of p21 that converts cancer cells from growth arrest to undergoing apoptosis (Zhang *et al.*, 1999). This may account for the decrease in p21 protein we observed at 4 days of incubation compared with that observed at 2 days. Thus, the dramatic increase in apoptosis between 2 and 4 days of exercise serum stimulation may be due to the drop in p21 protein. The significantly higher p53 protein levels in the exercise serum-stimulated lysates are consistent with other reports showing that IGF-I increases p53 degradation via Mdm-2 ubiquitination (Heron-Milhavet *et al.*, 2001; Heron-Milhavet and LeRoith, 2002). Thus, reducing serum IGF-I by exercise leads to an increase in p53 protein in the tumor cells allowing it to activate downstream factors (i.e. p21). The increase in apoptosis and decrease in Bcl-2 protein in the exercise serum-stimulated LNCaP cells is consistent with reports for other cell lines showing that IGF-I increases Bcl-2 protein and prevents apoptosis (Fernandez *et al.*, 2004; Yin *et al.*, 2005).

If the results reported here for the in-vitro experiments also occur *in vivo*, then regular exercise should be important not only for prevention but also for the treatment of early stage PCa by inducing apoptosis via p53. While p53 defects have been identified in many cancers, early PCa has intact p53 while end-stage PCa may have p53 defects (Gurumurthy *et al.*, 2001). We previously reported that androgen-independent PC-3 cells showed little response to diet and exercise (Tymchuk *et al.*, 2001). Recently, the initial results from a randomized study of men with PCa on 'watchful waiting' treated with a low-fat diet and regular aerobic exercise have been reported (Ornish *et al.*, 2005). Serum samples obtained from the patients showed a dramatic reduction in LNCaP cell growth with increased apoptosis in the bioassay. The clinical results after 1 year showed that six of 49 patients in the control group required treatment for raising prostate-specific antigen compared with none of 44 in the diet and exercise group. These results suggest that the serum changes that reduce cell growth and induce apoptosis in the bioassay *in vitro* may also be affecting the tumors *in vivo*.

A limitation to the present study is that it is a case–control study and that we did not have serum samples from the exercise group participants before they started the exercise program. In earlier studies (Tymchuk *et al.*, 2001; Ngo *et al.*, 2002) with diet and exercise intervention, however, we obtained serum samples from the participants before initiating the intervention and showed results similar to those seen in this case–control study.

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