

Research article

IGF-1 gene expression in rat colonic mucosa after different exercise volumes

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Abstract

The evidence is increasing for a close link between the insulin/insulin-like growth factor (IGF) system and colon cancer prevention by physical exercise. To reveal exercise-induced alterations in colon mucosa, gene expression of IGF-1 and related genes and serum IGF-1 were investigated. Twenty male Wistar rats performed a 12 week voluntary exercise program. Nine rats served as the control group. Gene expression of IGF-1, IGF-1 receptor (IGF-1R) and IGF-binding protein 3 (IGF-BP3) were quantified by real-time RT-PCR. Circulating IGF-1 was analyzed exercise volume-dependent. Based on 3 distinguished groups with low (L-EX, <2629 m·night⁻¹), medium (M-EX, 3003-7458 m·night⁻¹) and high exercise volume (H-EX, >8314 m·night⁻¹), we observed lower serum IGF-1 levels ($P < 0.05$) in all exercise groups as compared to the control group and IGF-1 levels declined proportional to the increase in exercise volume. A significant ($p < 0.05$) positive correlation was found between IGF-1 concentration and body mass ($r = 0.50$) and a significant negative correlation exists between body mass and exercise volume ($r = -0.50$). Significant differences in colonic mRNA levels of IGF-1, IGF-1R and IGF-BP3 could not be observed. Based on our data we propose that the exercise as well as the body mass reduction leads to a decrease in circulating IGF-1 and this might represent a prime link to colon cancer prevention.

Key words: Cancer prevention, IGF-1R, IGF-BP3, real-time RT PCR, physical exercise.

Introduction

Studies on the effects of exercise on colon cancer development in experimental animals have revealed more controversial results. Exercise was shown to reduce the development of colonic adenocarcinomas or tumors in rats when those were induced by chemical carcinogens (Andrianopoulos et al., 1987; Reddy et al., 1988; Thorling et al., 1993; 1994) whereas in APC^{Min} (multiple intestinal neoplasia) mice, which develop primarily polyps in the terminal region of the small intestine, exercise failed to have an effect on the constitutive intestinal hyperproliferation (Colbert et al., 2000; 2003). Even an increase in numbers of aberrant crypt foci in colon was reported based on exhaustive forced exercise (Demarzo and Garcia, 2004).

In contrast to these animal studies, human epidemiological data provide convincing evidence for a protective effect of physical activity on colon cancer development and even indicate that there might be a dose-

response relationship between exercise intensities and protective effect (Colditz et al., 1997; Friedenreich and Orenstein, 2002; Quadrilatero and Hoffman-Goetz, 2003). Only one human randomised controlled trial has so far assessed the role of physical activity in primary prevention of colon cancer (McTiernan et al., 2006) and here it was found that moderate-to-vigorous exercising in men had a significant effect in decreasing colon crypt cell proliferation indices after a 12 months intervention period. As far as the mechanisms underlying these protective actions are concerned, the following pathways and factors have been proposed a) an enhanced immune function, b) a reduced gastrointestinal transit time, c) altered prostaglandin levels, d) lowered bile acid secretion, e) decreased insulin/insulin-like growth factors (IGFs) /glucose level, f) altered cholesterol concentrations, g) changes in intestinal/pancreatic hormone profiles, h) improved antioxidative defence system activities and i) a reduction of body weight (Friedenreich and Orenstein, 2002; Kaaks and Lukanova, 2002; Slattery, 2004; Quadrilatero and Hoffman-Goetz, 2003). Quadrilatero and Hoffman-Goetz (2003) have recently summarized the current evidence on physical activity and the putative underlying mechanisms and concluded that the strongest evidence comes from physical activity effects on the IGF-system. This might occur by an enhanced level of circulating IGF-binding protein 3 (IGF-BP3) caused by physical exercise that could antagonize IGF and reduce its mitogenic activity (Quadrilatero and Hoffman-Goetz, 2003). Although tissue-specific effects in the IGF-system by physical activity have not been assessed yet in colon *in vivo*, but *in vitro* studies with prostate cancer cells revealed a close association of altered exercise-induced serum IGF-1, increased apoptosis rates and decreased cell growth (Leung et al., 2003; Ngo et al., 2002; Tymchuk et al., 2001; 2002).

As the expression of the genes encoding IGF-1 and other proteins mediating its action in colon mucosa has not been studied *in vivo* in the context of physical activity, we analyzed the effect of different volumes of voluntary physical exercise on serum IGF-1 and colon mucosa IGF-1, IGF-1 receptor (IGF-1R) and IGF-BP3 mRNA expression levels in rats.

Methods

Animals

Twenty-nine male Wistar rats were purchased at the age of 6 weeks from Charles River Laboratories (Sulzfeld, Germany) and were housed under controlled environmental conditions (21°C, 12:12-h light-dark cycle). Food (standard rodent chow from Ssniff; Soest, Germany) and water were given ad libitum. The animals were maintained according to the policy statement with respect to the Declaration of Helsinki. The study design was approved by the Regional Administration of the City of Cologne (Bezirksregierung Köln).

Voluntary running model

Our model was described previously in detail (Matsakas et al., 2004). In brief, after 3 days of acclimatization the animals were randomly divided into two groups: an exercise (n = 20) and a control (n = 9) group. The exercising animals were housed individually in cages with free access to a running wheel to assure stress-free exercise. The exercise training period lasted 12 weeks. During this period, the control group was housed individually in plain cages. The body mass of all animals was monitored weekly.

Tissue collection

After completion of the 12 week period all animals were decapitated at the same time window (9:00-11:00 a.m.). Wheels and food had been removed from the cages 12h earlier to minimize the influence of the last exercise bout and the last feeding on the (molecular) targets of interest. For details of the serum extraction see Matsakas et al. (2004). The whole colon was separated, rinsed with Ringer's solution and prepared to be free of fat and faeces. Afterwards the colon was turned inside out to scrape off the mucosa. The isolated mucosa was directly put into liquid nitrogen and stored at -80°C for further analysis. Finally, the heart (without the great vessels) was removed and weighed immediately.

Serum IGF-1 analysis

Serum insulin-like growth factor 1 (IGF-1) was detected via enzyme immunoassay (DRG, Marburg, Germany) in an Anthos 2000 photometer (Salzburg, Austria). The sensitivity was about 30 ng ml⁻¹ and the intra- and inter-assay coefficient of variation were 7.4 and 9.5 %, respectively (Matsakas et al., 2004).

mRNA analysis

Frozen mucosa was homogenized in 2 ml Lysing Matrix D tubes (Q-BIOgene, Irvine, CA) filled with provided 1.4 mm ceramic spheres and additionally filled up with buffer

for cell lysis (Macherey-Nagel, Düren, Germany). For the pulverizing process a FastPrep[®] FP120A Instrument (Q-BIOgene, Irvine, CA) was used. Total RNA was isolated using the NucleoSpin[®]RNA II-Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. A DNA digestion step was included. Isolated RNA was diluted in 60 µl of RNase-free water. RNA concentration was measured photometrically and purity was checked through the ratio of optical density at 260 and 280 nm (Biophotometer Eppendorff; Hamburg, Germany). The quality of the isolated RNA was checked by gel electrophoresis (1 % agarose, formaldehyde containing) through interpretation of 18 S and 28 S bands. Only high quality material was accepted and used for further analysis.

cDNA-synthesis: A mix of 0.5 µg RNA, 8 µl 5x MMLV reaction buffer (Promega, Mannheim, Germany), 6µl dNTP's (300 µM; Fermentas, St. Leon-Rot, Germany) and an adequate amount of nuclease-free water was prepared to a final volume of 30 µl. The mixture was denatured by incubation at 65°C for 5 minutes. Afterwards, 0.4 µl of random hexamers (0.2 µg·µl⁻¹; Fermentas, St. Leon-Rot, Germany), 0.63 µl RNase inhibitor (20 U·µl⁻¹; Fermentas, St. Leon-Rot, Germany), 1 µl MMLV Reverse Transcriptase (200 U·µl⁻¹; Promega, Mannheim, Germany) and 7.37 µl nuclease-free water were added. Finally, the samples were incubated at 37°C for one hour followed by 1 minute at 99°C.

Primer design: Highly purified salt-free primers from MWG (Ebersberg, Germany) were used. The final concentration of the primer working solution was 20 µM. The primer sets described in Table 1 were designed based on the LightCycler Probe Design Software, Version 1.0 (Idaho Technology Inc., Salt Lake City, USA). All primer pairs were set at an exon-intron barrier. Optimal annealing temperature (AT) and quantification temperature (QT) were established by using the LightCycler (Roche, Germany). Melting curve analysis and final agarose gel electrophoresis were used to set the experimental values. The quantification temperature was set below the specific melting curve of the product.

Quantitative real-time RT-PCR: For the final gene expression step the SYBR Green I - Kit (Roche, Mannheim, Germany) was used. After denaturation of the cDNA at 65°C for 5 minutes, a standard reaction mix for each sample was prepared: 1 µl cDNA (12.5 ng), 6.4 µl nuclease-free water, 1.2 µl MgCl₂ (final concentration: 4 mM), 0.2 µl of each primer (final concentration: 4 pmol) and 1 µl 10x LCM-reaction mix (Roche, Mannheim, Germany). Samples were analyzed with the LightCycler

Table 1. Primer sequences used in quantitative real time RT-PCR.

GenBank Acc. No.	Gene name	PS forward 5' to 3'	PS reverse 5' to 3'	Amplicon size (bp)	AT	QT
NM 001101	β-actin	CTA CGT CGC CCT GGA CTT CGA GC	GAT GGA GCC GCC GAT CCA CAC GG	385	60°C	79°C
NM 012495	AldolaseA	ATGCCCCACCCATACCCAGCACT	AGCAGCAGTTGGCGGTAGAAGCG	191	64°C	79°C
NM 002046	GAPDH	GTC TTC ACC ACC ATG GAG AAG G	TCA TGG ATG ACC TTG GCC AG	198	60°C	79°C
NM 001082477	IGF-1	AAG CCT ACA AAG TCA GCT CG	GGT CTT GTT TCC TGC ACT TC	166	58°C	79°C
NM 052807	IGF-1R	AAA ACC ATC GAT TCT GTG ACG	GGT TCT TCA GGA AGG ACA AGG	199	60°C	79°C
NM 012588	IGF-BP3	CGC TAC AAA GTT GAC TAT GAG	CGT CTT TCC CCT TGG T	292	60°C	79°C

Abbreviations: GenBank Acc. No.= GenBank Accession number, PS = primer sequence, AT = annealing temperature, QT = quantification temperature, bp = base pairs, GAPDH = glycine aldehyde 3-phosphate dehydrogenase, IGF-1 = insulin-like growth factor-1, IGF-1R = IGF-1 receptor, IGF-BP3 = IGF-binding protein 3.

Table 2. Serum IGF-1 of the subgroups: For details of the whole group see Matsakas et al. (2004). Control group (CO), low exercise volume group (L-EX), medium exercise volume group (M-EX), high exercise volume group (H-EX). Data are means (\pm SD).

	CO (n = 9)	L-EX (n = 5)	M-EX (n = 10)	H-EX (n = 5)
Serum IGF-1 level ($\text{ng}\cdot\text{ml}^{-1}$)	2039 (218)	1463 (106) ^a	1369 (300) ^a	1277 (283) ^a
Body weight (g)	499 (48)	477 (31)	445 (31) ^b	442 (39) ^b

^aSignificant different from the control group, adjusted for body weight. ^bSignificant different from the control group.

(Roche, Mannheim, Germany). A standard protocol was used for the specific gene amplification: the initial polymerase activation step was at 95°C for 190 seconds. For further denaturation (95°C, 15 seconds), annealing (AT, 10 seconds), elongation (72°C, 20 seconds), and quantification (QT, 5 seconds) steps the above mentioned AT and QT were used individually for each primer pair. To evaluate the specific amplification a final melting curve analysis (from AT up to 99°C) was added under continuous fluorescence measurements. Relative quantification was performed using sample crossing points as described (Rasmussen, 2001) and analyzed by LightCycler Software 3.5 (Roche, Mannheim, Germany). The method of choice was the “second derivative maximum” method (Rasmussen, 2001). Following data analysis was performed by two different Excel based applications: Best-Keeper (Pfaffl et al., 2004) and Rest© (Pfaffl et al., 2002). Using these two programs the data were checked for statistical significance, normality and reliability.

Calculations and statistics

Values are presented as mean \pm standard deviation. IGF-1 values were adjusted for body weight by regression analysis. One-way analysis of variance (factor: group) was used to compare the subgroups with the control group in the cases of serum IGF-1 and body mass. A post-hoc test was used to assess significant differences at the $p < 0.05$ level. mRNA data were analyzed using the BestKeeper program (Pfaffl et al., 2004) based on the calculation of a housekeeping gene index from among several housekeeping genes (AldolaseA, β -actin, GAPDH). A second program, Rest©, (Pfaffl et al., 2002) was used for transformation of raw gene expression data into a normalized x-fold expression ratio of a target gene compared to the housekeeping genes. The examination of statistical significance ($P < 0.05$) in this case was done by a Pair Wise Fixed Reallocation Randomisation Test© as described (Pfaffl et al., 2002). Correlation analysis was performed by Pearson product moment correlation test. The level of statistical significance was set at $\alpha = 0.05$. The statistical analysis was performed with SigmaStat 3.0 (SPSS Inc.) software if not described otherwise.

Results

Body mass

Table 2 shows the final body mass of the animals after the experimental period. For details see Matsakas et al. (2004) and Buehlmeier et al. (2007).

Running activity

The exercise group was divided into a low (L-EX; <2630 m per night; $n = 5$), a medium (M-EX; 3000 - 7460 m per night; $n = 10$) and a high (H-EX; >8310 m per night; $n =$

5) exercise volume group. For additional information see Matsakas et al. (2004) and Buehlmeier et al. (2007).

Serum IGF-1

Serum IGF-1 concentrations (adjusted for body weight) were significantly lower in the L-EX, M-EX and H-EX groups as compared to the control group after the exercise period (Table 2). In addition, there was a negative, yet not significant, trend in the relationship between mean exercise volume and serum IGF-1 concentrations.

Heart mass

For the heart mass data see Buehlmeier et al. (2007).

Correlations between exercise volume, body mass, heart mass and serum IGF-1

In all rats, heart mass per kg body mass showed a significant negative correlation with serum IGF-1 levels ($r = -0.66$; $p < 0.001$) and body mass ($r = -0.55$). IGF-1 serum levels showed a significant positive correlation with body mass ($r = 0.50$). Exercise volume revealed a significant negative correlation with body mass ($r = -0.50$) and a highly significant positive correlation with heart mass per kg body mass ($r = 0.77$; $p < 0.001$).

mRNA of IGF-1, IGF-1R and IGF-BP3 in colon mucosa

Figure 1 displays the normalized data of IGF-1, IGF-1R and IGF-BP3 mRNA levels as the ratio of the target to the housekeeping gene by assigning the control group a factor of 1. Even though we could not find any significant differences or correlations, the following results are worth mentioning: First, a linear negative trend between exercise volume and IGF-BP3 mRNA was observed. Second, IGF-1R mRNA tended to be lower in the L-EX and M-EX groups. Third, in comparison to the control group, we observed 1.7 to 3.0 fold higher expression of IGF-1-mRNA in colon mucosa of L-EX and M-EX groups.

Discussion

In our rat model of voluntary exercise over a 12 week period, we assessed the effects of increasing exercise volume on serum IGF-1 concentrations and gene expression levels of the IGF-system in colonic tissue.

The exercise volume of the animals changed over time in a fashion as described by others in similar experiments (Allen et al., 2001; Nikolaidis et al., 2004; Reddy et al., 1988) with a rapid increase in exercise volume during the first experimental weeks, followed by a plateau phase and a decrease of mean exercise volume per day. These data have been previously described (Buehlmeier et al., 2007; Matsakas et al., 2004). Based on our grouping (Buehlmeier et al., 2007) we assessed the exercise volume-dependent variations of serum IGF-1 levels. The

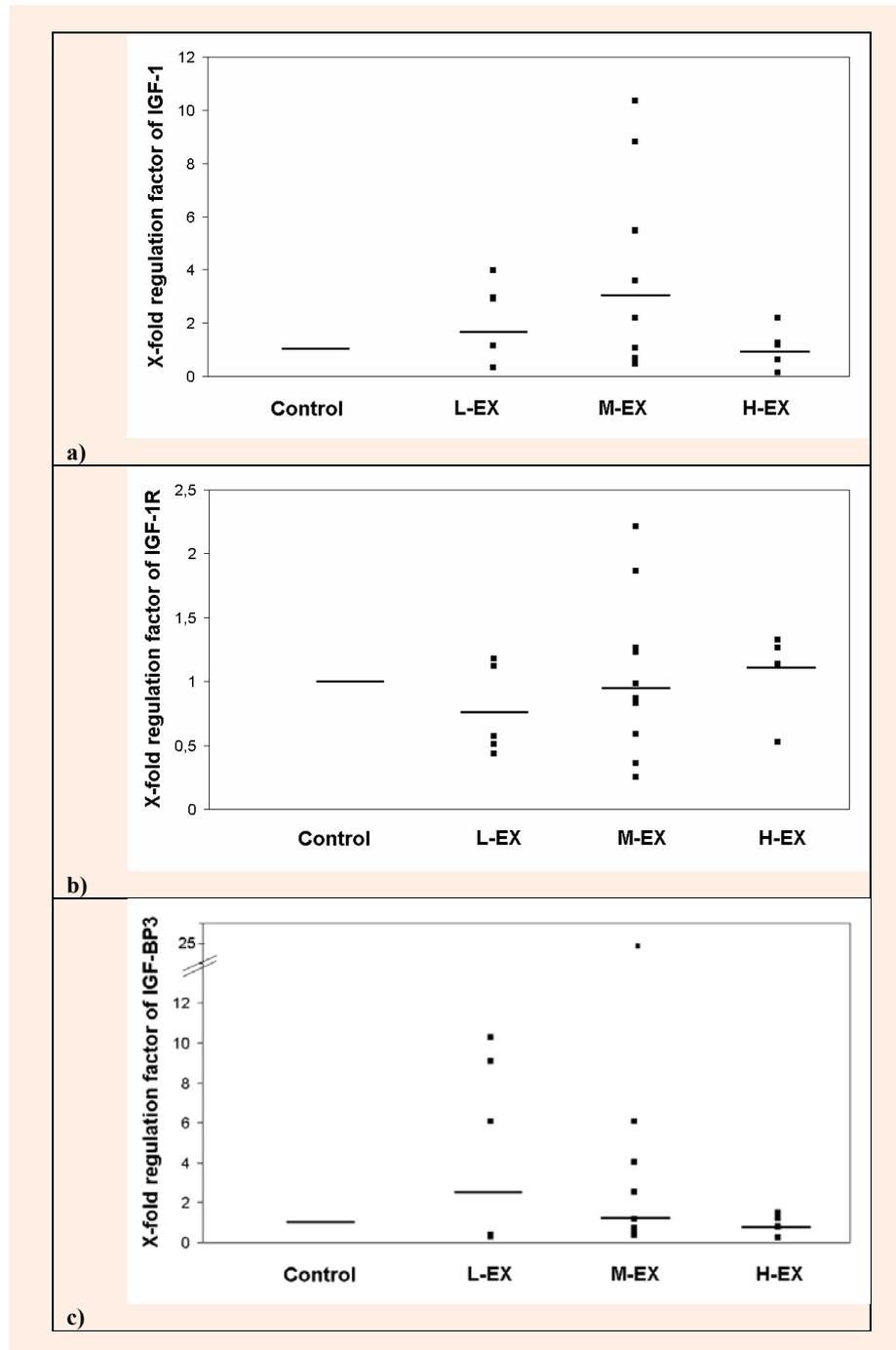


Figure 1. Gene expression of IGF-1 (a), IGF-1R (b) and IGFBP3 (c) at the mRNA level in the colon mucosa at the end of the experiment, shown as x-fold regulation factors relative to the control group. Single normalized values of control group (CO, n=9), low exercise volume group (L-EX, n=5), middle exercise volume group (M-EX, n=10) and high exercise volume group (H-EX, n=5) are shown as dots – normalized means are indicated as lines.

observed increase of heart mass per kg body mass (Buehlmeier et al., 2007) reflected an expected adaptation to exercise in the high exercise volume and the medium exercise volume groups in accordance with previously reported data (Allen et al., 2001; Kingwell et al., 1998; Matsakas et al., 2004). Concerning the serum IGF-1 concentration, there were exercise volume-dependent alterations with lower values at higher exercise volumes independent of body weight influences (Table 2). Previous studies reported predominantly increased or unchanged circulating IGF-1 concentrations in rats (Anthony et al., 2001; Bravenboer et al., 2001; Cooper et

al., 1994; Yeh et al., 1994) or humans (Chadan et al., 1999; Kraemer et al., 2004; Ngyen et al., 1998, Wallace et al., 1999) after various exercise periods. However, studies in human also indicate that long-term exercise decreases circulating IGF-1 (Koistinen et al., 1996; Nehmet et al., 2002; Suikkari et al., 1989). In addition, several animal experiments showed constant or modestly decreased serum IGF-1 levels following physical exercise (Banu et al., 1999; Colbert et al., 2003; Matsakas et al., 2004). These inconsistent findings appear to be due to the different exercise models, ages, rat strains and sexes studied and make it almost impossible to compare the studies

and findings. There have also been differences between the time points of blood collection. It is well known that total IGF-1 concentration in serum rapidly increases after moderate exercise (Bang et al., 1990; Kostka et al., 2003; Schwarz et al., 1996) but this elevation is transient. Other investigations with more extensive exercise models showed an IGF-1 alteration lasting up to 24 hours after the bout (Raastad et al., 2000) and Anthony et al. (2001) showed a second peak of IGF-1 plasma concentration 12 hours after the exercise bout (2 hours, 26m/min., 1,5% slope). Nevertheless, our data with a significant positive correlation between circulating IGF-1 and body mass are in accordance with data obtained in a human study that also detected significantly lower body mass indices and serum IGF-1 levels in the exercise group when compared to the control group (Barnard et al., 2003; Leung et al., 2003). The negative correlation between physical exercise and development of body mass is well documented (Tou and Wade, 2002) and a close link is also found for the effect of caloric restriction and circulating lower IGF-1 levels (Kritchewsky, 1999). Since it is also known that pathophysiological conditions with elevated IGF-1 concentrations (acromegaly) increase the risk for colon cancer (Giovannucci, 2001; Kaaks and Lukanova, 2002; Sandhu et al., 2002) and increased body size also correlates with the malignancy of colorectal cancers (Gunter and Leitzmann, 2005), IGF-1 appears to represent a prime target molecule that transmits the protective effects of exercise and low body mass in the colon. Regarding that nearly 90% of IGF-1 is bound to IGF-BP3 (Grimberg and Cohen, 2000), it would be useful to measure this circulating protein in further studies.

Given the role of the IGF-system in control of colon tumour growth (Grimberg and Cohen, 2000), we studied the mRNA steady-state levels of IGF-1, IGF-1R and IGF-BP3 in rat mucosa by quantitative real-time RT-PCR. To avoid variations because of different sample amounts, standardization with housekeeping genes was the method of choice. Many housekeeping genes do show different expression patterns under different experimental conditions and the influence of physical exercise on housekeeping gene expression is not well studied (Jemiolo and Trappe, 2004; Mahoney et al., 2004; Murphy et al., 2003). We therefore normalized gene expression based on β -actin, ALDA, and GAPDH levels by using the BestKeeper tool (Jemiolo and Trappe, 2004; Pfaffl et al., 2004). To our knowledge, this is the first study that has examined the influence of exercise on the expression of the genes encoding IGF-1 and proteins mediating its action in rat colon mucosa. However, no significant effects could be found for any target gene in the different exercise classes. These findings match with those obtained in muscle (Matsakas et al., 2004, 2005) and other tissues (Eliakim et al., 1997; Zanonato et al., 1994). As over 80% of the circulating IGF-1 is derived from the liver (Kaaks and Lukanova, 2002; Quadrilatero and Hoffman-Goetz, 2003) the colon-specific IGF-1 secretion does presumably not contribute to alterations in circulating IGF-1. Recent studies with mice confirm the prime role of the liver by showing that a liver-specific IGF-1 gene deletion reduces circulating IGF-1 by about 75% (Yakar et al., 1999; 2001). Therefore gene expres-

sion analysis in the liver appears attractive for further studies. The gene expression analysis in our model revealed ambiguous steady-state levels of IGF-1, IGF-1R and IGF-BP3 mRNA but no significant differences in the expression of these genes between the exercise and control groups were found.

Conclusion

In summary, we observed a significant inverse relationship between exercise volume and serum IGF-1 concentrations in our voluntary long-term exercise rat model that also revealed a negative correlation between body mass and physical exercise. Differences in colonic mRNA levels of IGF-1, IGF-1R and IGF-BP3 could not be observed. Both the body mass reduction and the exercise in turn decreases circulating IGF-1 levels which is known to enhance colonic tissue growth. This might be a suggestive link to colon cancer prevention by physical exercise.

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Key points

- There were significantly lower serum IGF-1 levels in all exercise groups as compared to the control group.
- IGF-1 levels declined proportional to the increase in exercise volume.
- A significant positive correlation was found between IGF-1 concentration and body mass and a significant negative correlation was found between body mass and exercise volume.
- Significant differences in colonic mRNA levels of IGF-1, IGF-1R and IGF-BP3 could not be observed.

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