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ORIGINAL ARTICLE

Treatment with sera from Water Polo athletes activates AMPKα and ACC proteins In HepG2 hepatoma cell line

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Abstract

Purpose Physical activity and professional physical activity such as water polo (WP) sport, has numerous beneficial effects to fight metabolism-related disorders through several mechanisms, including the promotion of liver metabolic adaptations, and the modulation of cytokine production. The aim of this study was to investigate the effects of different types of physical activity on AMPKα and ACC, two proteins involved in liver metabolism; therefore, we treated the hepatoma cell line Hep G2 with sera from elite WP athletes and amateur (basket) players. As control, we used serum from both sedentary and obese subjects.

Methods Help G2 cells were treated with 5% of human sera from the different subjects; after 24 h and 48 h, HepG2 cell viability was verified through MTT assay and activation status of AMPKα and ACC through western blotting. Cytokine's serum levels were measured through ELISA assay.

Results After 72 h, the treatment of HepG2 cells with sera from the different subjects produced no effect on cell viability. Furthermore, after 48 h of treatment, both AMPKα and ACC phosphorylation statistically increases in HepG2 cells treated with sera from WP athletes. Furthermore, IL-4, IL-6 and IL-10 levels resulted statistically increased in WP athlete's sera than in sedentary subjects.

Conclusion The specific activation of AMPKα and ACC by WP sera confirms that professional sport activity carried out by WP athletes can be considered as a physiological activator of these two proteins also in HepG2 liver cells. In addition, the increase of anti-inflammatory cytokines in WP sera confirms the ample evidence for multiple anti-inflammatory activities carried out by WP discipline.

Keywords Hepatoma cells · Physical activity · Obesity · Cytokines · Water polo

Introduction

Nutrition and physical activity affect general health status [1]. In addition, physical exercise is considered to be the most potent non-pharmacological intervention to manage hepatic alterations. Indeed, regular physical activity beneficially impacts on the risk of hepatic chronic diseases onset and progression. However, research regarding the effects of exercising on chronic liver diseases is relatively recent and not fully explored. Increasing clinical and experimental data focused on liver diseases finding that there is a cross-talk among skeletal muscle, adipose tissue and liver that regulates intrahepatic fat storage [2, 3]. In this setting, physical activity allows an effective decrease of intrahepatic lipid component, and, despite that evidence is not conclusive, several studies suggest that a vigorous activity might be more effective than moderate activity in improving steatohepatitis

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[2–5]. On the other hand, evidence regarding the effects of exercise on the risk of hepatocellular carcinoma is scarce; some epidemiological studies indicate a lower risk in patients regularly and vigorously exercising. Among metabolic diseases, obesity is a chronic disease mainly due to excessive fat accumulation that may seriously impair health [2, 3]. During the last 20 years, obesity has rapidly become a global pandemic health problem, often associated with insulin resistance, diabetes mellitus and dyslipidaemias. In addition, several data demonstrated that concomitant presence of obesity and insulin resistance might be associated with the development of liver diseases [4]. Indeed, visceral fat mass is a predictor not only of hyperinsulinaemia and insulin resistance but also of hepatic steatosis [5]. Furthermore, insulin is involved in the regulation of liver free fatty acids (FFA) metabolism and can inhibit hepatic mitochondrial beta-oxidation of FFA while, on the other hand, FFA accumulation in the liver may influence insulin clearance and insulin resistance initiating an unhealthy vicious circle [6]. For these reasons, a sedentary lifestyle is closely associated to an increased risk of mortality and morbidity for dysmetabolic diseases such as liver diseases and physical activity has an important role in its prevention [7–9]. Indeed, regular exercise promotes favourable muscle construction and metabolic adaptations [10]. However, it is important to distinguish between regular physical activity and athletic and professional physical activity. Water Polo (WP) is an aquatic team sport characterized by a combination of both aerobic and anaerobic efforts that requires intensive training and a high metabolic demand on the athletes [11]. Increased energy expenditure, reduced fat depots and promotion of beta-oxidation are at basis of professional sport, such as WP, beneficial effects [12]. Given the skeletal muscle cross-talking to the adipose tissue and the liver, professional physical activity has numerous beneficial effects not only on metabolic pathways but also on inflammatory processes, regulating cytokines response. In this contest, a key role is played by the pro-inflammatory cytokines, especially tumor necrosis factor-alpha (TNF-α) and IL-6 [7, 8, 13]. In particular, physical exercise is also able to reverse the imbalance in pro- and anti-inflammatory cytokines, phenomenon at the basis of many inflammatory processes, and to contribute to an overall decline of systemic inflammation [14, 15]. Several studies, dealing with the association between exercise and inflammation, affirm that physical activity has an anti-inflammatory effect in various pathological and physiological states characterized by low-grade inflammation, such as ageing, metabolic disease, obesity, etc. [16–18]. The anti-inflammatory response induced by physical activity is mediated by the muscle endocrine functions; indeed, during contraction, muscle-derived cytokines, named myokines, are released to regulate both muscle metabolism [19]. Literature data report that circulating levels of anti-inflammatory cytokines rise and those of the pro-inflammatory adipokines such as tumor necrosis factor alpha (TNFα Interferon gamma INFγ and interleukin (IL)-2 decrease as a result of the regular and moderate physical activity [16]. Indeed, the pattern of cytokines induced by physical activity is typically anti-inflammatory, with a marked increase in serum levels of several cytokines, such as IL-10, IL-4, and IL-6 [20–22]. Furthermore, the release of IL6 after intense physical exercise as in athletes is reported to create an anti-inflammatory environment [13, 23, 24].

In this scenario, this study investigated the effects of sera from professional, amateur, inactive and obese subjects on hepatic cells. To this aim, we treated the hepatoma cell line, Hep G2, to find out if sera from different trained subjects could induce a different biological response. In particular, we aimed at clarifying if the treatment with the above-mentioned sera affects cell viability and/or the activation status of AMPKα and ACC, two proteins involved in different molecular pathways linked to metabolism [25, 26]. Considering that our data show that the treatment with Water Polo player's sera induces relevant effects on AMPKα and ACC, we analysed the serum cytokine levels (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN γ in these professional athletes' respect to control subjects.

Materials and methods

Participants

22 unrelated professional international level men's Water Polo players were recruited from Circolo Nautico Posillipo, Napoli, Italy. At investigation time, the athletes performed 2 sessions of training/day with a competition taking place during the week end. Generally, the sessions of training consisted of technical–tactical drills, swimming, and strength training (60–120 min/session) as previously reported [27]. Three male basket players were recruited from an amateur team in Naples and three obese subjects were recruited as previously reported [28]. 20 age- and sex-male matched sedentary healthy volunteers were recruited from CEINGE-Biotecnologie Avanzate staff. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University of Campania "L. Vanvitelli" (Prot. n. 587/18). All participants signed informed consent. WP samples were collected after an overnight fast (12 h) before training session.

Cell culture and treatments

Cell lines derived from liver, Hep G2 (ATCC) were kindly provided from the Bank of Human and Animal Continuous Cell Lines CEINGE-Biotecnologie Avanzate. Cells were

cultured in RPMI medium supplemented with 10% FBS, L-glutamine (1%), penicillin/streptomycin (1%).

Human sera were decomplemented at 95 °C for 30 min before the treatments.

Viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 4×10^6 Hep G2 cells were seeded in 96-well plates and incubated with:—5% sera from—Water polo athletes,—basket amatorial players,—obese subjects, and—healthy controls. After 24, 48, and 72 h of incubation, cells were stained with MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphe-niltetrazolium-bromide/PBS) as previously reported [29]. All data are presented as mean ± SE of three independent experiments.

Protein extraction and western blotting

Hep G2 cells were incubated for 24 and 48 h with: − 5% sera from—Water polo players,—basket players,—obese subjects, or—healthy controls. After incubation times, cells were lysed and homogenized in RIPA buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM Na_3OV_4, 1 mM NaF, 1% Triton X100, 0.1% SDS, 1% Na deoxycholate) containing 1 mM PMSF and protease inhibitors. Proteins were quantified by the Bradford method. 25 µg of proteins were dissolved in 1X Laemmli buffer and separated using 10% SDS-PAGE gel and the electrophoresis was performed as previously described [30]. The membranes were incubated with p-ACC, ACC, p-AMPKα, AMPKα (Cell-Signaling Technology, MA) and GAPDH primary antibody (Sigma–Aldrich, MO, USA) according to the manufacturer's instructions. Enhanced chemiluminescence (ECL) (Euroclone) was employed to detect the HRP secondary antibody signal. To conclude, protein bands were detected with ChemiDoc XRS (Bio-Rad) and quantified with Image J software (http://rsbweb.nih.gov.ij/). Each treatment was performed two times in triplicate.

Cytokines quantification

Sera from Water Polo athletes and sedentary healthy controls were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ. Measurements were performed with the Bio-Plex human cytokine kit from BioRad (BioRad, Hercules, CA, USA), according to the manufacturer's protocol.

Statistical analysis

Data are means ± SE. Two groups were compared with 2-tailed unpaired Student's *t* test. Multiple comparisons were performed by ANOVA test. All statistical analyses were performed using the StatView software 5.0.1.0. Differences were considered statistically significant when $P < 0.05$.

Results

Anthropometric and biochemical features measurements

The anthropometric and biochemical characteristics of Water Polo players have been previously reported [27]. The anthropometric and biochemical characteristics of basket players, obese patients and sedentary healthy control subjects are shown in Table 1. Compared to controls, WP have significant reduced levels of total cholesterol ($P < 0.05$) (Table 1). No significant differences were found in HDL, triglycerides, and glucose. Obese

Table 1 Biochemical features of Obese patients, water polo players, basket players and controls

Parameters	Controls	Obese	WP players	BK Players	Controls *vs.* Obese *P value*	Controls *vs.* Water Polo players *P value*	Controls *Vs.* Basket Players *P value*
Sex male/female	20/0	3/0	22/0	3/0	–	–	–
Age (years)	34 ± 3	31 ± 6	27 ± 5	31 ± 1	0.84	0.08	0.40
BMI (kg/m^2)	26 ± 2	46 ± 4	25 ± 1	23 ± 0.9	**0.01**	0.29	0.06
Waist circumference (cm)	< 94 ± 7	> 110 ± 4	< 94 ± 5	< 94 ± 6	**2.33E-05**	0.56	0.62
Total Cholesterol (mg/dl)	198 ± 34	164 ± 35	174 ± 25	171 ± 7	0.06	**0.04**	0.64
HDL (mg/dl)	53 ± 0.4	35 ± 2	57 ± 12	55 ± 2	**0.01**	0.34	0.48
LDL (mg/dl)	90 ± 1	100 ± 34	101 ± 21	98 ± 7	0.06	**0.06**	0.21
Triglycerides (mg/dl)	55 ± 5	144 ± 39	89 ± 39	93 ± 13	**0.02**	0.09	0.12
Glycemia (mg/dl)	92 ± 6	97 ± 4	84 ± 8	95 ± 6	0.12	0.06	0.40
Fibrinogen (mg/dl)	337 ± 24	354 ± 13	–	–	0.27	–	–

subjects compared to controls have significant higher weight, BMI, waist circumference, triglycerides and lower HDL levels ($P < 0.05$). No statistically differences were found in LDL, glucose and fibrinogen. No differences were found between controls and basket players.

Effects of sera treatments on HepG2 Cell viability

To investigate the effects on cell viability induced by sera from sedentary, active subjects and professional athletes, we treated Hep G2 cells for 24 h, 48 h and 72 h with 5% sera from Water Polo athletes, amateur basket players, obese and healthy control subjects. Our results showed that cell viability was not affected by the treatment with sera from the different trained subjects, both after 24 h, 48 h and 72 h of incubation (Fig. 1).

AMPKα and ACC analysis by Western blot

We investigated the activation status of AMPKα and ACC enzymes following cell treatment with sera from sedentary healthy controls, obeses, active subjects or from WP athletes in Hep G2 cells after 24 h and 48 h of incubation (Fig. 2). After 48 h of incubation, the phosphorylation of AMPKα and ACC statistically increased in the cells treated with WP players' sera, compared to that treated with the sera from the other used sera.

Cytokine levels in water polo players and sedentary healthy controls sera

Considering that the treatment of Hep G2 cells with Water Polo players' sera induces the activation of AMPK and ACC, and as it is known that exercise modulates cytokine production, we analysed the levels of IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ cytokines in sera from Water Polo players and compared them to sedentary healthy controls. Serum levels of IL-4, IL-6 and IL-10 were statistically increased in WP players compared to controls (Fig. 3, panels A, B and C). Also, we found that IL-2, and IL-8 and GM-CSF levels are increased in athlete's sera than in control subjects even if the difference is not statistically relevant (data not shown). Finally, TNF-α and INF-γ were not differentially expressed between athletes and controls (data not shown).

Discussion

Healthy habits such as correct nutrition and physical activity are the first-line approach for prevention and treatment of liver disorders. Indeed, it is well known that physical activity could work as a potent anti-inflammatory factor, counteracting liver inflammation typical of metabolic disorders and ameliorating lipid metabolism. On the contrary, bad habits such as overeating and a sedentary lifestyle lead to metabolic disorders which are negatively affect liver health [13]. Starting from this evidence, in the present study we collected sera from 22 WP players, 3 amatorial basket players, 3 obese subjects and 20 control subjects and studied the

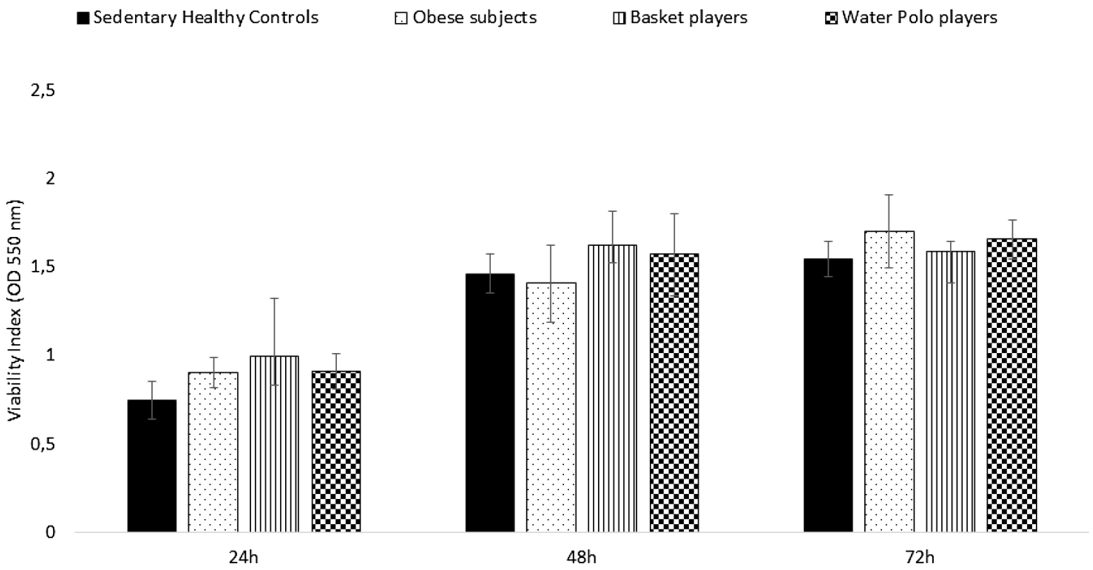

Fig. 1 Treatments with sera from the different trained subjects do not affect cell viability of HepG2. Hep G2 cells were treated with sera from Water Polo players, Basket players, obeses and healthy controls and checked for the viability. No statistically relevant variations were found in cell viability after 24 h, 48 h and 72 h of incubation

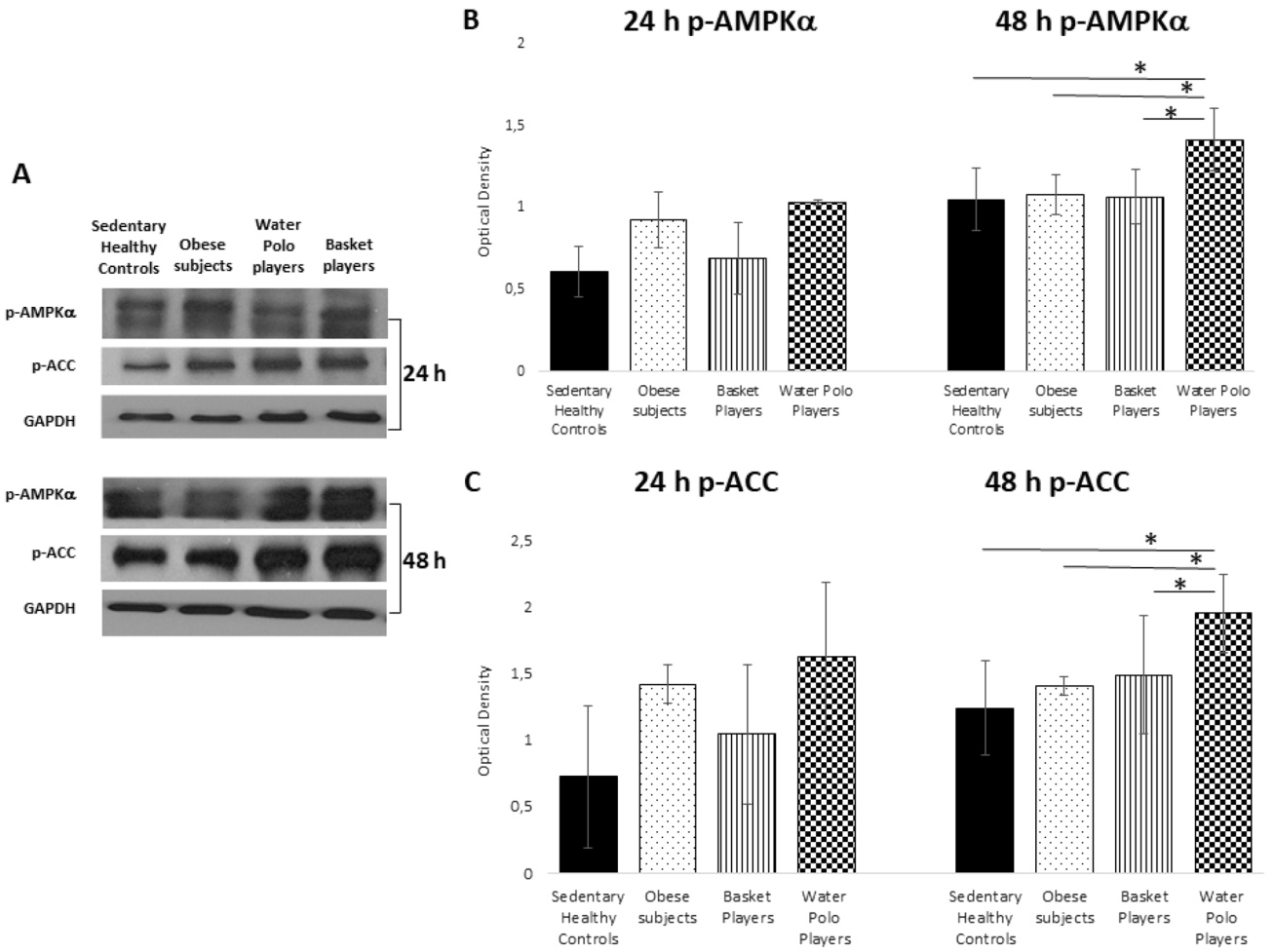

Fig. 2 Treatment of Hep G2 cells with sera from professional WP athletes induces the phosphorylation of AMPKα and ACC. Treatment of Hep G2 cells with WP players' sera increases the phosphorylation of AMPKα and ACC compared to cells treated with basket players, obese subjects and sedentary healthy controls sera after 48 h of incubation. **a** representative images of three replicate experiments. **b, c** Quantitative data with graphs showing the densitometric intensity of pAMPKα/GAPDH, pACC/GAPDH bands ratio. Values represent means ± SE of experiments performed two times in triplicate. The intensities of signals were expressed as arbitrary units. $*P < 0.05$

effects of treatment with these sera of HepG2, a hepatoma cell line [31]. We first investigated the effects in terms of cell viability founding that all sera do not modulate cell growth. Accordingly, Conti et al. [32] demonstrated that the treatment with sera from different type of athletes does not alter cell viability of endothelial cells, but, interestingly, induced an increase of cell survival; we did not observe any increase in cell survival but this discrepancy may be explained by the difference in the cell models utilized and/or by the physical activity of the athletes recruited in the two analysed studies.

Then, we investigated the activation status of two key molecules involved in both metabolism and energy balance, AMPKα and ACC. Activation of AMPKα in liver leads to the stimulation of fatty acid oxidation and inhibition of lipogenesis through inhibition of ACC [33]. Regular physical exercise has been described as the most powerful non-pharmacological therapeutic approach in the prevention and treatment of disorders associated with metabolic dysfunctions through the regulation of metabolic and inflammatory processes [34]. The molecular pathways beyond these effects are mainly mediated by the activation of AMPKα [35, 36]. Our results showed that WP athlete's sera increase the phosphorylation of AMPKα and ACC respect to sera of obese and of control subjects. It has been shown that, in skeletal muscle, AMPKα modulates the phosphorylation with the resulting inhibition of ACC, the limiting enzyme in the malonyl CoA synthesis [37, 38]. AMPKα plays an important role in regulating lipid metabolism in multiple tissues after exercise. Chen et al. [39] reported that in lean mice performing exercise, the phosphorylation levels of AMPKα strongly increase compared to obese and controls mice. In accordance with our study, Gibala et al. [40] reported that intense interval exercise induces metabolic remodelling through AMPKα activation.

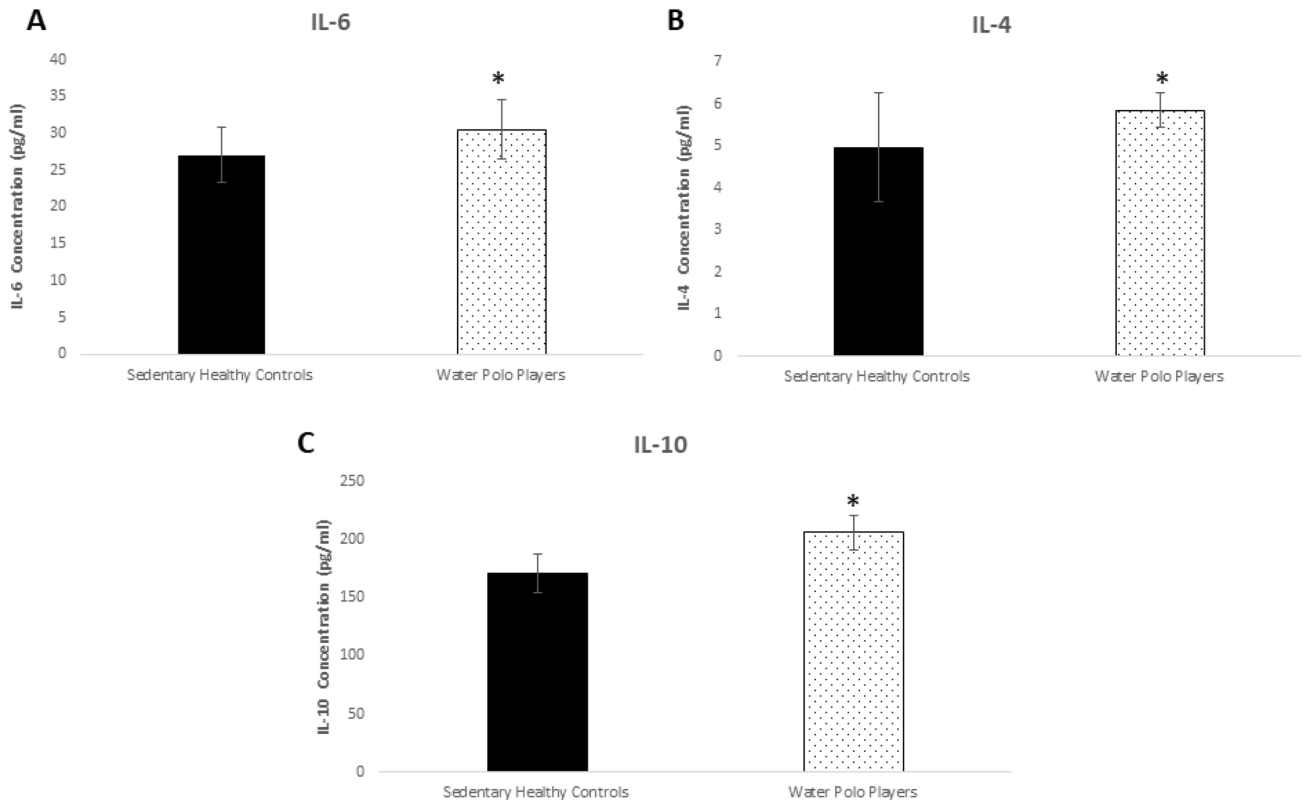

Fig. 3 The expression of IL-4, IlL6, IL-10 cytokines is statistically increased in WP player's sera compared to sedentary healthy controls: Serum levels of IL-4, IL-6 and IL-10 were statistically increased in WP players compared to controls (**a, b, c**)

Considering that in our study WP player's sera induce a relevant effect in terms of AMPK/ACC activation, we next investigated cytokine levels in the sera from the athletes assuming that an increased and/or decrease of cytokines might have been responsible for the effects of sera on Hep G2. Indeed, cytokines have important roles in many physiological and pathological conditions [13, 14]. In particular, professional sports can induce the release of pro- and anti-inflammatory cytokines among of these, the IL-6 plays a dominant role [41] but several authors found increased levels also of TNF-α, IL-1, IL-4,, IL-10, and IL-8 [42–45]. In the sera from the athletes we found a statistical increase of IL-4, IL-6, and IL-10 levels compared to controls. In accordance with our data, Nemet et al. found increased levels of IL-6 in a group of WP players [46]. In addition, several studies suggest that IL-6 is induced by exercise and involved in mediating exercise-related metabolic changes. Although to our knowledge there are no available data about IL-4 and IL-10 in WP, a beneficial role for the anti-inflammatory cytokines IL-4 and IL-10 has been suggested by many studies in different cohorts of athletes [43, 45]. Our data are indicative that an anti-inflammatory environment is induced by exercise in WP resulting in cytokine production that in turn might participate in stimulating AMPKα pathway in cells.

Conclusions

We demonstrated that professional sport activity such as WP can induce an in vivo anti-inflammatory milieu and that possibly determines an increase in the phosphorylation status of AMPKα and consequently of ACC in Hep G2 cells. This activation, together with the modulation of cytokine production and release, may be, at least in part, at the basis of the beneficial preventing effects exerted by exercise towards disorders associated with the metabolism.

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Author contributions RP and EN performed the experiments, MLM conducted the statistical analysis. MM and AE recruited the participants. EN and AD wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflict of interest.

Ethical approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University of Campania "L. Vanvitelli" (Prot. n. 587/18).

Informed consent All participants signed informed consent.

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