

Review article

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METABOLIC MODULATORS OF THE EXERCISE RESPONSE: DOPING CONTROL ANALYSIS OF AN AGONIST OF THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR δ (GW501516) AND 5-AMINOIMIDAZOLE-4-CARBOXAMIDE RIBONUCLEOTIDE (AICAR)

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In 2008, the team of Ronald Evans, a professor at the Salk Institute Gene Expression Laboratory, published an article about the effects of two metabolic modulators branded as GW501516 and AICAR on physical endurance of laboratory animals. Both substances, also called 'exercise pills' or 'exercise mimetics', showed the ability to cause multidirectional changes in muscle metabolism. In particular, they stimulated fatty acid oxidation and promoted muscle remodelling. These compounds were regarded as very promising drug candidates for the treatment of diseases such as obesity and type 2 diabetes. GW501516 and AICAR have received considerable attention in doping control due to assumed performance-enhancing properties and recent confiscations of illicitly distributed drugs containing AICAR. Therefore, the World Anti-Doping Agency added GW501516 and AICAR to the Prohibited List in 2009. This review covers the cellular and systemic effects of the metabolic modulators' administration with special emphasis on their role in exercise metabolism. It also presents the advancements in development of methodologies for the detection of their abuse by athletes.

Key words: *peroxisome proliferator-activated receptor; adenosine monophosphate-activated protein kinase; peroxisome proliferator-activated receptor-coactivator 1 alpha; physical performance; anti-doping; sport*

INTRODUCTION

Humans are born with the imperative and right to undertake physical activity and sport competition. Do we take advantage of this right? According to a special Eurobarometer survey on sport and physical activity, a majority of European citizens play sport or do some other form of physical exercise at least once a week, but a worrying 25% of respondents say that they are almost completely inactive (1). Globally, around 31% of adults aged 15 and over were insufficiently active in 2008 (men 28% and women 34%). In 2008, prevalence of insufficient physical activity was the highest in the Americas and the Eastern Mediterranean Region. In both of these regions, almost 50% of women were insufficiently active, while the prevalence for men was 40% in the Americas and 36% in the Eastern Mediterranean. The South East Asian Region showed the lowest percentages, i.e. 15% for men and 19% for women (2).

Physical inactivity is an important and growing major health problem and is defined as not engaging in any regular pattern of physical activity beyond that associated with daily functioning (3). What are the consequences of it? Physical inactivity seems to be associated with the development of approximately 21–25% of breast and colon cancers, 27% of diabetes and 30% of the ischaemic heart disease burden. It has been identified as the fourth leading risk factor for global

mortality, causing an estimated 3.2 million deaths each year. The number of deaths related directly to reduced physical activity will have risen by 17% by the year 2015 (2). The life expectancy is 8–10 years shorter among sedentary than physically active people (4). Are genetic engineering and pharmacogenetics the answer to the growing problem of physical inactivity? Could 'exercise pills' replace the beneficial effects of physical exercise? This review covers the cellular and systemic effects of metabolic modulators' administration with special emphasis on their role in exercise metabolism. It also presents the advancements in development of methodologies for the detection of their abuse by athletes.

METABOLIC MODULATORS AS EXERCISE MIMETICS

In 2008, the prestigious journal *Cell* published the findings of an experiment carried out by the team of Prof. Ronald Evans of the Salk Institute Gene Expression Laboratory (5). The experiment investigated the effects of two compounds identified as GW501516 and AICAR on physical endurance of mice. It was shown that both drugs regulate the muscle metabolism, in particular by stimulating glucose and fatty acid oxidation, mitochondrial biogenesis and muscle remodelling to a fibre-type composition that is metabolically more oxidative (5-7). In interviews, Prof. Ronald

Evans explained that GW501516 and AICAR (aminoimidazole carboxamide ribonucleotide) work by reprogramming the contracting muscle fibres in such a way that they are able to work longer without fatigue. For this reason, the two compounds were called ‘exercise pills’ or ‘exercise mimetics’.

The first compound to be tested was GW501516 - an agonist of the peroxisome proliferator-activated receptor δ (PPAR δ). This receptor belongs to the nuclear PPAR receptor family activated by numerous natural and synthetic substances such as fatty acids, steroids, thyroxine, retinoids, fibrates and thiazolidinediones (Table 1) (8-12). The PPAR δ receptor was described in the 1990s as the key regulator of lipid metabolism, the main source of energy during long-term exercise (13). An increased level of transcripts for PPAR δ receptors was observed in skeletal muscles after constant and interval endurance exercise. Additionally, the level of PPAR δ in muscle fibres was found to be 10-fold and 50-fold higher than those of PPAR α and PPAR γ , respectively (14).

In 2004, Evans and his colleagues generated transgenic mice with a PPAR δ transgene expressed solely in skeletal muscles. Remarkably, these modified mice ran nearly twice as long as the genetically non-modified littermates. At the same time studies with a PPAR δ agonist, termed GW501516, were commenced (15). Treating mice with GW501516 (10 mg/kg/d) for 5 weeks in conjugation with physical training increased their performance time by approx. 70% compared to their non-drugged counterparts. Moreover, it was shown that GW501516 enhanced the influence of exercise on the whole body (5).

However, the results of studies with AICAR, the analogue of adenosine monophosphate, were far more striking. After 4 weeks of AICAR application (500 mg/kg/d) the sedentary mice were running by 23% faster and by 44% further than untreated and untrained mice (5). This report confirmed former studies that had shown that 5 days of AICAR treatment may lead to the increased levels of glycogen, glucose transporter GLUT4 and mitochondrial enzymes in mice (16).

Publication of the results in *Cell* sparked off a debate in scientific media whether ‘exercise in a pill’ will indeed replace physical activity, and whether ‘exercise pills’ are physical exercise mimetics. Warden and Fuchs claimed that physical exercise has multisystem effects and it is highly unlikely to be replaced with just a single pill (17). Hawley and Holloszy pointed out that exercise pills are characterised by too short half-life, comparable to lactic acid, to induce long lasting effects (18). Prof. Goodyear of Harvard Medical School described exercise pills as “too good to be true” (19). We must bear in mind that even though numerous metabolic alterations have been observed by Evans and his colleagues, exercise pills will probably not bring about the positive effects of physical exercise on mental condition, personality, ability to cope with stress, etc. It is an aspect of our lives equally as important as physical health.

Although exercise pills seems not to be the answer to the growing problem of physical inactivity in activity, the nuances the chances of using GW501516 and AICAR in situations when performing exercise is not possible or limited may not be rejected. According to Evan’s team, the drugs would help combat the high risks of obesity, including heart and cardiovascular disease, in people with sedentary lifestyles. They stress that AICAR can partly replace exercise, whereas GW501516 may enhance health effects of physical exercise *via* improvement in blood lipid profile and glucose uptake, transport and oxidation of fatty acids, immune cells activity, etc. (5, 19-21). Such use of GW501516 or AICAR is not a novelty, as some medicines used to date in the treatment of type 2 diabetes act in a similar way to exercise pills (22, 23).

GW501516

GW501516 (also known as GW-501,516, GW1516, and GSK-516) is an agonist of the nuclear receptor PPAR δ that regulates transcription of over 100 housekeeping genes, products of which are engaged in the fundamental tissue metabolism (5). PPARs heterodimerize with retinoic X receptor (RXR) and alter the transcription of target genes upon binding of the resulting complexes to their respective response elements. Upon activation by fatty acids (FA) or drugs such as GW501516 that affect lipid metabolism, PPARs control the expression of genes implicated in intra- and extracellular lipid metabolism and oxidative capacity (22). In skeletal muscles, PPAR δ regulates fatty acid transport and oxidation, thermogenesis, and the formation of slow-twitch muscle fibres, which altogether result in enhanced endurance performance. It likewise activates thermogenesis and fatty acid transport and oxidation in adipose tissue, retarding weight gain. PPAR δ regulates the availability of BCL-6 (B-cell lymphoma 6 protein), an inflammatory suppressor protein released upon binding of PPAR δ , thereby functioning as an ‘anti-inflammatory switch’ to control macrophage-elicited inflammation and atherogenesis. In the liver, PPAR δ activation suppresses glucose production by upregulating the pentose phosphate shunt. PPAR δ activation also improves atherogenic dyslipidaemia by raising serum HDL cholesterol levels *via* unclear mechanisms. Additionally, PPAR δ activation in the heart enhances contractile function and may improve cardiomyopathy (20) (Fig. 1).

During study on fat metabolism GW501516 was reported to increase physical performance in animal experiments at a dosage of 2–5 mg/kg/day used in conjugation with exercise. Additionally, the induction of oxidative genes transcription as well as modified substrates of skeletal muscles that caused a shift from carbohydrate to lipid consumption was observed. As a result, an overall improvement of endurance performance by approx. 70% was measured (5, 15).

Table 1. Tissue distribution, ligands and main biological effects of PPAR isoforms; FA - fatty acids, PUFAs - polyunsaturated FA, TZD - thiazolidinediones, 15dPGJ2 - 15-deoxy-prostaglandin J2 (8-11).

PPAR	Ligand	Tissue expression	Function
PPAR α	FA, fibrates	heart, liver, skeletal muscle	FA oxidation, anti-inflammatory
PPAR γ	PUFAs, 15dPGJ2 TZD	adipocytes	adipogenesis, anti-proliferating, anti-angiogenic
PPAR β/δ	FA	various tissues	organogenesis (prenatal period), synergistic with PPAR α function-lipid metabolism regulation (in mature organism)

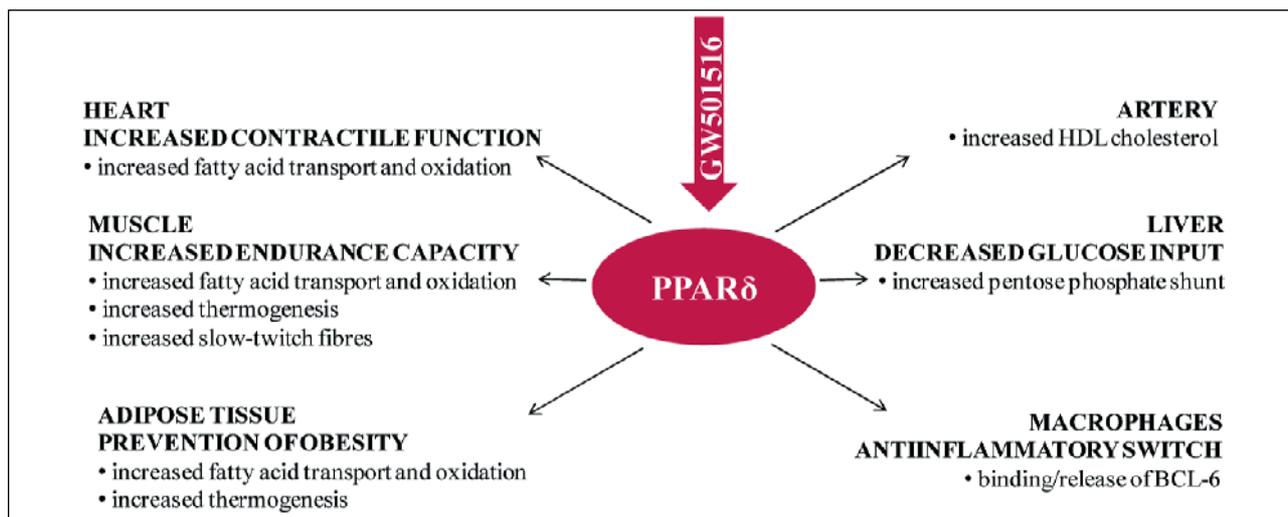


Fig. 1. Effects of GW501516 on the nuclear receptor PPAR δ (19); GW501516, as an agonist of PPAR δ , can reveal multiple tissue- and cell-specific effects. In heart, GW501516 can enhance contractile function *via* increase in fatty acid transport and oxidation. In skeletal muscle, it can regulate fatty acid transport and oxidation as well as thermogenesis and the formation of slow-twitch muscle fibers resulting in enhanced endurance performance. In adipose tissue, it can activate fatty acid transport and oxidation as well as thermogenesis retarding weight gain. It can also improve atherogenic dyslipidemia by raising serum HDL cholesterol levels. In the liver, it can suppress glucose production by upregulating the oxidative pentose phosphate shunt. GW501516, as an agonist of PPAR δ , can regulate the availability of B-cell lymphoma 6 (BCL-6), an inflammatory suppressor protein released upon ligation of PPAR δ , thereby reducing the risk of inflammation and atherogenesis.

AICAR

AICAR, which stands for 5-aminoimidazole-4-carboxamide ribonucleotide, is an active agent of acadesine, a medicine developed by PeriCor Therapeutics and licensed to Schering-Plough. The substance reached phase III studies as a prevention of reperfusion injury in coronary artery bypass graft surgery, but the trial was terminated in 2010 after proving that the drug was insufficient (24). During the last 10 years, AICAR has been tested for various applications, including mainly studies on and treatment of metabolism disorders, induced sudden death, diabetes, cancer and other pathologies associated with muscle wasting. It was also noted that most purine metabolism-associated diseases result in AICAR accumulation in the patient cells (25-33).

AICAR is an endogenous substance and an intermediate metabolite in the purine *de novo* synthesis pathway (34). It activates the AMP-sensitive enzymes such as adenosine monophosphate (AMP)-activated kinase (AMPK), glycogen phosphorylase and fructose-1,6-bisphosphatase and thus contributes to oxidative metabolism and mitochondrial biogenesis. AICAR can compete with released adenosine for reuptake into cells and consequently it allows adenosine to accumulate in the medium and exerts its effects *via* adenosine receptors (35, 36).

AMPK has been described as a “low fuel sensor” or a “metabolic master switch”, which reflects AMPK’s central role in cellular energy metabolism. AMPK switches on catabolic pathways that generate ATP such as glycolysis and fatty acid β -oxidation. At the same time, it switches off ATP-consuming processes such as biosynthesis and cell growth and proliferation (36) (*Fig. 2*). AMPK consists of 3 subunits, with the α subunit being catalytic, the β subunit containing a glycogen-sensing domain, and the γ subunit containing 2 regulatory sites that bind the activating and inhibitory nucleotides AMP and ATP, respectively. Although it may have evolved to respond to metabolic stress at the cellular level, hormones and cytokines such as insulin, leptin, and adiponectin can interact with the

system. Hence, it appears to play a key role in maintaining energy balance at the whole body level (35, 36).

AMPK is also implicated in the induction of mitochondrial biogenesis. A transcription factor, peroxisome proliferator-activated receptor-coactivator 1 α (PGC-1 α) has emerged as a key orchestrator of transcriptional pathways that induce mitochondrial biogenesis and has been associated with exercise training adaptation and muscle fibre transformation. Similarly to exercise, AMPK increases in expression of mitochondrial genes encoding cytochrome c, aminolevulinic acid synthase, malate dehydrogenase, and succinate dehydrogenase in glycolytic muscle. Because AMPK activation through exercise is hypothesized to initiate a coordinated sequence of events that favour lipid oxidation, enhanced mitochondrial function, and a more energy-efficient state of metabolic fitness, its influence on PGC-1 α activity has naturally been addressed. More recently, AMPK has been shown to directly interact with PGC-1 α , indicating that many AMPK-induced changes in mitochondrial gene expression occur through PGC-1 α action (37, 38). Further details regarding the interaction between AMPK and PGC-1 α will undoubtedly become evident in the near future.

METABOLIC MODULATORS AND ENDURANCE CAPACITY

The experiment of Evans’s team showed that GW501516 and AICAR enhance the response to exercise through AMPK, PGC-1 α and PPAR δ the molecular system, which regulate expression of target genes for the endurance adaptation (5) (*Fig. 3*).

These gene products are engaged in lipid and carbohydrate metabolism, signal transduction, transcription, membrane transport, proliferation and apoptosis, oxygen transportation, steroid biosynthesis, angiogenesis, and synthesis of cytokines, heart shock proteins and other molecules whose function could not be identified by Evans’s team (5). By DNA microarray technology, it was shown that application of GW501516 accompanied by

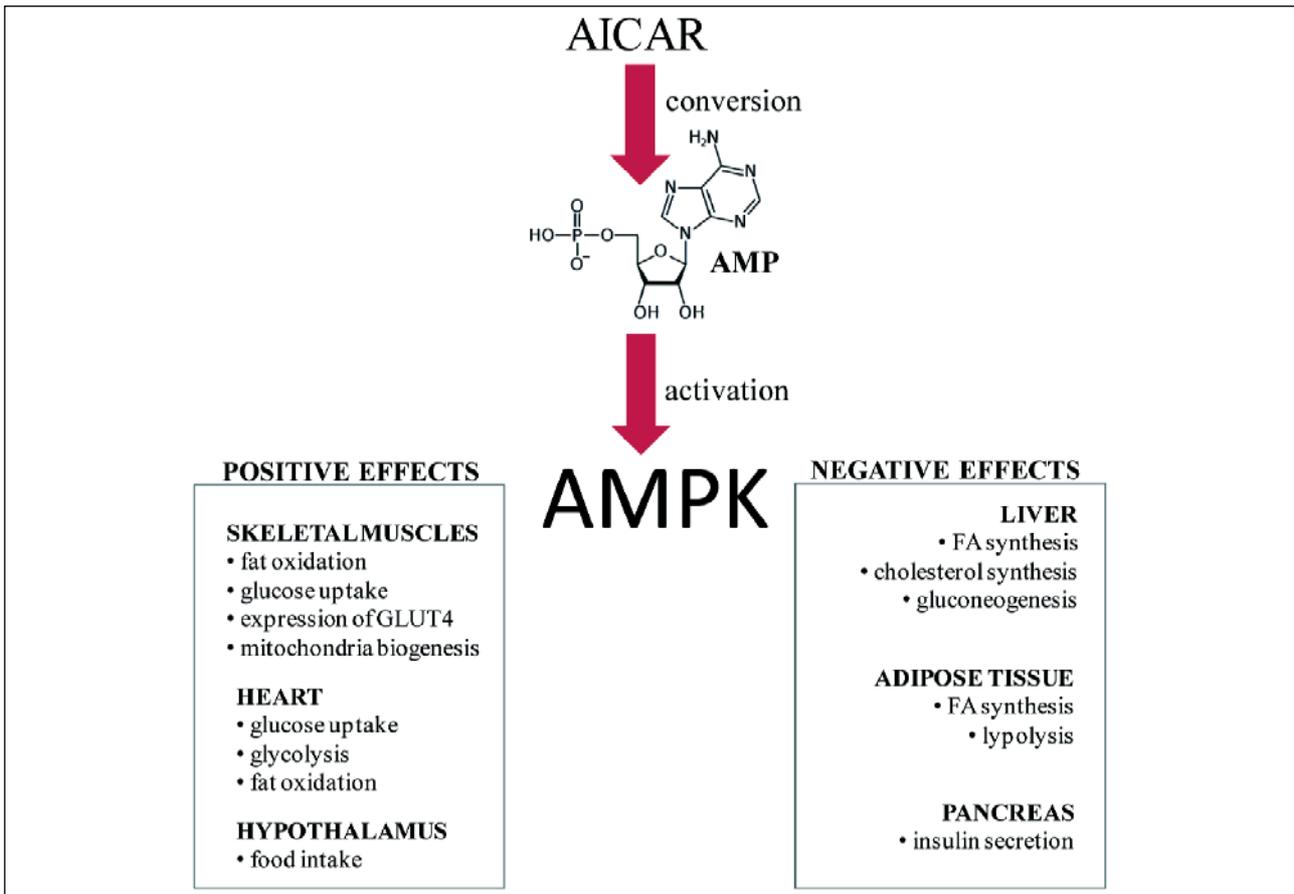


Fig. 2. The effects of AICAR on AMP-activated protein kinase (AMPK) activity (35); Activation of AMPK in many tissues switches on catabolic processes that generate ATP while switching off ATP-consuming processes. The adipocyte-derived hormones leptin and adiponectin, as well as exercise, activate AMPK in skeletal muscle, stimulating fatty acid oxidation. Leptin's activation of AMPK in skeletal muscle involves the hypothalamic-sympathetic nervous system (SNS) axis. Adiponectin also activates AMPK in liver, increasing fatty acid oxidation and reducing gluconeogenesis, and in adipocytes, where the downstream biologic pathway has not been studied. Resistin inhibits AMPK in liver. AMPK inhibits insulin secretion from pancreatic β cells.

exercise training induces expression of 118 to 130 genes in muscle cells, which is 48 more than in the case of application of GW501516 or exercise only. A comparable result was observed with AICAR. The effect of the increased number of activated genes *via* synergistic activity of GW501516 and exercise or AICAR was enhanced speed of the run and longer distance compared to non-drugged mice (5). Such a response was observed after 4–5 weeks treatment with exercise pills while single administration of AICAR triggered short lasting enhancement of lipid oxidation (39).

METABOLIC MODULATORS AND HUMAN HEALTH RISKS

In March 2013, the World Anti-Doping Agency (WADA) published on its website a warning concerning health risks associated with the use of GW501516. It stated that this substance, once a developmental drug, was withdrawn from research and terminated when serious toxicities were discovered in animal subjects (40). The long-term carcinogenicity studies (104-week long) at high doses showed that administration of GW501516 to rats (40 mg/kg/day) and mice (80 mg/kg/day) leads to neoplastic findings in multiple tissues (41, 42). Mortality of female Han Wistar rats was increased at all doses mostly due to development of uterine endometrial adenocarcinoma. Other types of identified neoplasms affected

such organs as stomach, liver, and urinary bladder, and their prevalence seemed to be a confluence of such factors as dose and gender (41). Survival of CD1 mice also significantly decreased at doses ≥ 30 mg/kg/d and led to combined squamous cell tumours (at all doses) and neoplastic changes in liver and stomach (42). Exposure to GW501516 has also been shown to cause a significant increase in the number and size of intestinal polyps in *Apc^{min}* mice that are predisposed to intestinal polyposis (43). Interestingly, a few clinical trials have been performed on GW501516 safety and its effects on lipid and lipoprotein metabolism (44–46). In contrast to the animal toxicity studies, no significant adverse effects were reported, which may reflect the considerably lower doses administered (up to 10 mg/day) for much shorter periods of time (up to 12 weeks) (43, 45–48). Nevertheless, the available data on GW501516 safety are insufficient to assess the long-term health risks associated with its intake by human subjects.

AICAR has been proved to have only a single negative side-effect. A group of researchers under the leadership of Prof. Goodyear scrutinized the effects of AICAR treatment on glycogen metabolism in skeletal muscle, showing that it inevitably leads to substantial increase of lactic acid in muscles (49). Nevertheless, it would be incorrect to neglect the fact that only limited tests were done on humans, so there might be further, especially long-term consequences that have not been discovered yet.

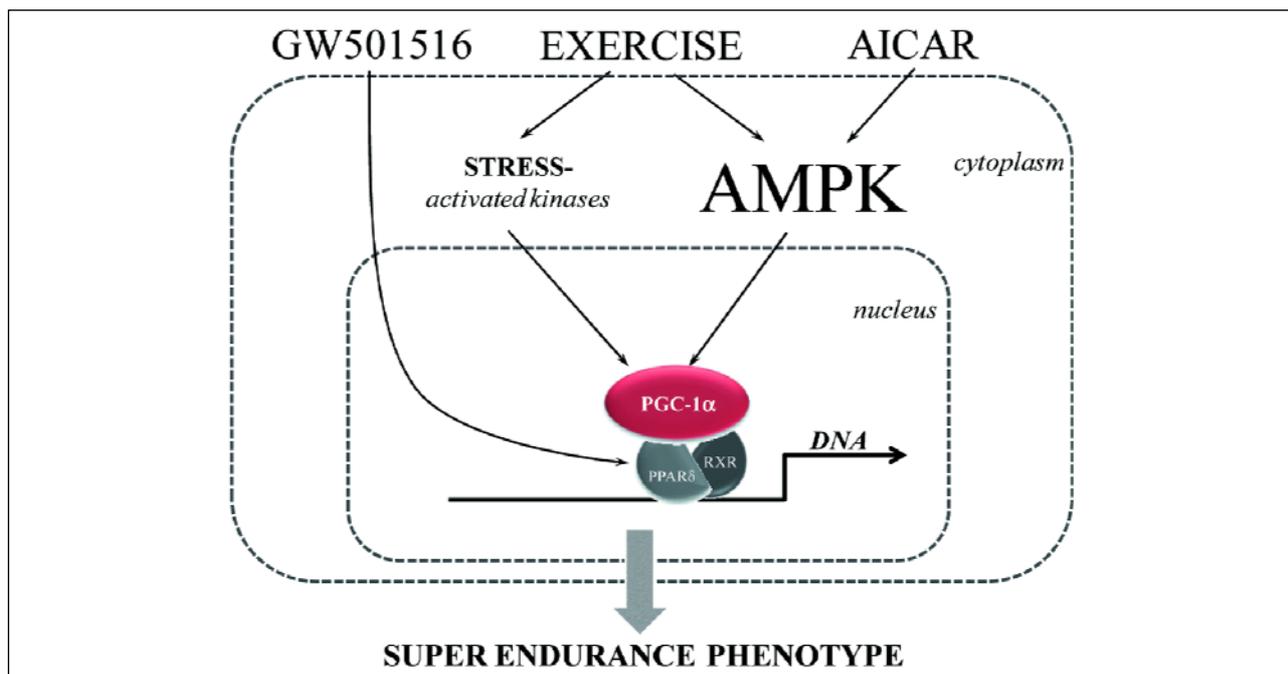


Fig. 3. Interaction between exercise and PPAR δ -AMPK agonists (5). The molecular partnership between AMPK and PPAR δ in reprogramming skeletal muscle transcriptome and endurance can be readily exploited by orally active AMPK drugs to replace exercise.

METABOLIC MODULATORS AND DOPING CONTROL

WADA added PPAR δ agonists such as GW501516 to the Prohibited List that became effective in January 2009 (50, 51). Similarly, AICAR has received considerable attention in doping controls due to its assumed performance-enhancing properties and recent confiscations of illicitly distributed preparations (52). Due to these facts, it has also been listed as a banned substance since 2009 (53). In the 2011 list version, GW501516 and AICAR were placed in class M3, “gene doping”, based on the following annotation: “The use of agents that directly or indirectly affect functions known to influence performance by altering gene expression. For example, peroxisome proliferator activated receptor δ (PPAR δ) agonists (e.g. GW 501516) and PPAR δ -AMP-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are prohibited”. In 2012, GW501516 and AICAR were moved to class S4, hormone and metabolic modulators, and gene doping was more precisely defined as “the transfer of polymers of nucleic acids or nucleic acid analogues” (54).

The methods for detection of doping substances, or substances of abuse in general, require a detailed understanding of the processes of their metabolic transformation and elimination. This knowledge is necessary in order to target a correct form of a prohibited substance in biological samples and allows, therefore to prove its intake, ideally over an extended period of time (wide window of opportunity). There was very little known about the disposition of GW501516 in the body at the moment of its inclusion in the Prohibited List. Hence, the first methods targeted the parent compound in blood (51), often the most abundant form of any xenobiotic in this matrix. However, this method had a very limited applicability as the most frequently provided doping control samples are urine specimens. Additionally, urine as a test material usually provides a longer window of opportunity than blood.

The first insights into the GW501516 metabolism were obtained by *in vitro* studies performed with the use of human

liver microsomes. They led to the identification of two oxygenated phase I metabolites, namely GW501516-sulfoxide and GW501516-sulfone, in the reaction mixture (55, 56), and later, in the excretion urine samples (57). Further studies showed that the metabolites are excreted in urine both unchanged and in the form of glucuronide conjugates. The latter fraction seems to prevail as the enzymatic hydrolysis of the conjugates increased the abundance of the unchanged metabolites by a factor of 2 to 5. The GW501516 metabolites demonstrate very slow elimination rates. GW501516-sulfone was found to be the best marker of GW501516 abuse as it could be detected for approx. 40 days after the intake of a single oral dose of 15 mg; GW501516-sulfoxide and unchanged GW501516 could be traced for 25 days and 72 hours, respectively (57). The methods developed based on the pharmacological data gathered led finally to the reports of first doping cases regarding GW501516 in cycling (58).

Importantly, PPAR δ agonists seem to be very attractive ingredients to manufacture “highly effective” sport supplements, and indeed, GW501516 was quickly introduced to the market in a form of research or not-for-human consumption products (55). Moreover, an emergence of new substances of this class may be anticipated in the near future. This is due to the fact that the supplement industry often employs clandestine laboratories to develop and manufacture designer substances; they have proven to be particularly effective in supplying numerous derivatives and isomers of well-established stimulants and anabolic agents (59-64). In recognition of this problem, a bioassay for untargeted detection of PPAR δ agonists in different matrices, including food supplements, have been developed (65). Not only is it sensitive, but it also allows to assess the activity of the detected substance in relation to the well-known activators of PPAR δ receptor, including GW501516. In combination with other techniques such as mass spectrometry and NMR, this bioassay may be used by antidoping laboratories as a tool for identification of new, designer PPAR δ agonists offered on the

market. The detection of AICAR abuse is a more complex problem as it is an endogenous substance. So far, the best method for AICAR quantitation in urine for doping control purposes was developed by the WADA-accredited laboratory in Cologne (66). The group demonstrated that the urinary AICAR levels were higher in the samples taken from men (2141 ng/mL) than women (1433 ng/mL), and similarly in the samples collected in-competition (2144 ng/mL) than between competitions (1503 ng/mL). Moreover, the athletes representing endurance or team sports manifested higher AICAR levels (1912 ng/mL) than the strength sport athletes (1319 ng/mL). Based on the collected data, a baseline value for endogenous AICAR in urine was established at 2186 ng/mL with the standard deviation of 1655 ng/mL. Even though the studies on its elimination after infusion (25, 50 or 100 mg/kg) to healthy humans revealed that the renal excretion of unchanged AICAR ranges only from 5–8% of the dose, its urinary concentrations post-administration are expected to peak at more than 100 µg/mL, at least up to 10 hours after the intake. This is approximately ten-fold higher than the highest concentration found by Thomas *et al.* (66) in the athletes' samples. Despite that AICAR seems to be abused by the athletes (based on the confiscations) and the presented method has sufficient detection capabilities, no athlete has tested positive for this performance-enhancing drug so far. This may be due to the fact that the method has a narrow window of opportunity (hours). On the quest to extend it, researchers turned to blood samples. AICAR can be easily quantified in blood by liquid chromatography coupled with mass spectrometry (53, 66). Additionally, it has been shown to accumulate in red blood cells in which AICAR levels are considered to be conserved for the lifetime of erythrocyte (up to 120 days). Thus, the LC/MS/MS method for AICAR quantitation in the RBC fraction seems to offer a possibility for a long-term detection of AICAR abuse, perhaps up to several days post-administration (53). In general, blood sampling is a growing field in doping control. Beside the athlete biological passport (ABP) and growth hormone analysis, more and more assays are being developed each year. Testing blood samples for more parameters seems meaningful, also to justify the significantly higher costs of analysis of a single doping control blood sample (53).

Another approach for the detection of AICAR abuse is based on the GC/C/IRMS technique which allows to assess whether a studied substance is of the exogenous or endogenous origin. The rationale is based on the fact that synthetic compounds differ in the content of ¹³C isotope when compared to endogenously secreted compounds. This technique has been used successfully for over 10 years by anti-doping laboratories to detect the abuse of synthetic steroids which can also be produced endogenously (67, 68). The usefulness of ¹³C/¹²C ratio determination for exo- and endogenous AICAR differentiation was tested by using excretion urine samples. This new method seems to be a promising tool for confirmatory analysis (69).

CONCLUSIONS

Even though GW501516 and AICAR have revealed to have beneficial effects in many studies, they have not yet reached the status of pharmaceutical products. The findings of positive influence of 'exercise pills' on endurance capacity come mainly from the studies on mice and rats; no such data is available for healthy humans. Additionally, the metabolic and skeletal muscles' responses to 'exercise pills' were observed only in combination with exercise and not with an acute GW501516 or AICAR infusion. Nevertheless, an illicit market and distribution of GW501516 and AICAR grows rapidly. The unrestricted access to GW501516 on the internet already

resulted in first doping cases regarding its misuse. This underlines the necessity for rapid development and implementation of new methods that target novel designer doping agents in the routine antidoping testing.

The work was performed in the laboratory of Department of Anti-Doping Research, Institute of Sport, 2/16 Trylogii Street, 01-982 Warsaw, Poland.

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