Review

Pharmacology of doping agents—mechanisms promoting muscle hypertrophy

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Abstract: Doping with performance enhancing substances in professional and amateur sports has increasingly gained awareness. Furthermore, not only the number but also the variety of substances detected in sports has increased. Intending muscle growth, anabolic androgenic steroids (AAS) have been complemented by selective androgen receptor modulators (SARMs), β_2-adrenergic agonists, estrogen receptor (ER) β agonists and peptide-hormones like growth hormone (GH), insulin-like growth factor-1 (IGF-1) and insulin. However, with respect to therapeutic use, drugs which increase anabolic actions in the body are highly desired for treatment of cachexia, sarcopenia, etc.

The aim of the following review is to elaborate on the agents' similarities and differences in mechanisms inducing muscle hypertrophy while giving an overview of the relevant signalling cascades. In addition to that, potential agents for treatment of catabolic diseases are identified. Information, on which this paper is based on, has been collected from various publications which have been published in high-quality scientific journals.

The insight obtained has shown that signalling pathways mediating genomic actions of steroids have already been well-characterised, while there is still need for further research on the mechanisms of non-genomic actions and downstream pathways as well as on those of SARMs and β_2-adrenergic agonists' action. A new and promising target is the estrogen receptor β. The use of ER-β agonists, which are proposed to activate anabolic mechanisms independently from androgen receptor activation, may prevent the non-desired androgenic effects, which are associated with AAS administration. Thus, they may be considered a promising alternative for treatment of diseases, which cause loss of muscle mass and cachexia. In this context, phytoecdysteroids, such as ecdysterone etc, are considered as an interesting class of substances, where further investigations are highly desired. Furthermore, some peptide hormones also promote muscle hypertrophy. The

signalling mechanisms of growth hormone, IGF-1 and insulin are summarised. Finally, the potential of myostatin inhibition is discussed.

Keywords: muscle hypertrophy; performance enhancement; signalling cascades; doping agents; anabolic-androgenic steroids; beta-2-adrenergic agonists; peptide hormones

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Abbreviations: 4E-BP1: 4E binding protein-1; AAS: anabolic androgenic steroids; AC: adenylylcyclases; ActRIIB: activin type IIB receptor; ALK4/5: activin-like kinase 4/5; AR: androgen receptor; ARE: androgen-response-elements; ATP: adenosine triphosphates; BAD: Bcl-associated death agonist; BMP: bone morphogenetic protein; cAMP: cyclic adenosine-monophosphate; cIGF-1: circulating IGF-1; CBP: CREB-binding protein; COPD: chronic obstructive pulmonary disease; CpG: cytosine-phosphate-guanine; CRE: cAMP response element; CREB: cAMP response element binding protein; DMD: Duchenne muscular dystrophy; EGFR: endothelial growth factor receptor; Epac1: exchange protein directly activated by cAMP 1; ER: estrogen receptor; ERK: extracellular signal-regulated protein kinase; FLG: follistatin related gene; FOXO: forkhead O transcription factor; FSH: follicle stimulating hormone; GASP: growth and differentiation factor-associated serum protein-1; GH: growth hormone; GHRH: growth hormone releasing hormone; GnRH: gonadotropin releasing hormone; GPCR: G-protein coupled receptor; GR: glucocorticoid receptor; GSK-3: glycogen synthase kinase 3; HAT: histone acetyl transferase; HDAC: histone deacetylase; HMB: β-hydroxy-β-methylbutyrate; HPA: hypothalamic pituitary gonadal axis; HSP: heat-shock-protein; iAR: intracellular androgen receptors; IGF-1: insulin-like growth factor-1; IGF-1R: insulin-like growth factor-1 receptor; IL-25: interleukin 25; IR: insulin receptor; IRS: insulin-response-substrate; LH: luteinizing hormone; MAFbx: muscle atrophy F-box protein; MAPK: mitogen-activated protein kinase; mAR: membrane-located androgen receptors; MEK: MAP-Erk-Kinase; mIGF-1: muscular IGF-1; MLC: myosin light-chain; MuRF1: muscle ring finger protein 1; MyoD: myoblast determination protein; NARI: selective noradrenaline reuptake inhibitor; NFAT: nuclear factors of activated T-cells; NOR1: neuron-derived orphan receptor 1; $p70^{s6K}$: p70 ribosomal protein S6 kinase; PI3K: phosphatidylinositol-3-phosphat kinase; PIP2: phosphatidylinositol-4,5-bisphosphate; PIP3: phosphatidylinositol-3,4,5-trisphosphate; PKA: proteinkinase A; PPAR-gamma: peroxisome-proliferator activated receptor-gamma; Prmt7: protein arginine methyltransferase 7; PSA: prostate specific antigen; p/CAF: p300/CBP-associated factor; SARM: selective androgen receptor modulator; SERM: selective estrogen receptor modulators; SHBGR: sex hormone-binding globulin receptor; STEAR: selective tissue estrogenic activity regulator; SRC: steroid receptor coactivator; TGF-β: transforming growth factor β; WADA: World Anti-Doping Agency

1. Introduction

Various substance classes are misused to enhance performance in competitive sports. Current regulations in sports are based on the World Anti-Doping Code, where doping is (among others) defined as use of a prohibited substance or the application of a prohibited method [1].

The most commonly detected doping agents are anabolic androgenic steroids (AAS). They are either chemically and biologically related to the physiological male sex hormone testosterone or testosterone itself [2]. As already indicated by their name, they have anabolic effects, thus promoting muscle mass increase. These substances can be naturally occurring compounds or synthetically modified derivatives which have been chemically altered mainly with the intention of increasing anabolic properties while simultaneously decreasing androgenic adverse effects but also for altering pharmacokinetic properties [3]. Increased muscle fibre size and myosatellite cell numbers have been reported after AAS administration in rats [4]. The signalling pathways resulting from the agents' binding to androgen receptors (AR) can be divided in genomic [3,5–8] and rapid, non-genomic actions, from which mechanisms have not yet been fully understood [5,6,9–15]. In addition to that, AAS are reported to inhibit catabolic properties of glucocorticoids by blocking the binding of endogenous ligands to glucocorticoid receptors (GR) [6,16–19]. All of these mechanisms are supposed to result in muscle hypertrophy and a decrease in catabolic activities, although there is still lack of evidence for an AAS mediated increase in protein synthesis and athletic performance [3]. One scientific study reports increased performance in bench-press and squatting exercises [20]. This placebo controlled investigation reported a significant increase upon testosterone administration (testosterone enanthate 600 mg/week for six weeks, intramuscular). This effect is even more pronounced if combined with training. Adverse effects resulting from misuse of AAS include alteration of blood lipid levels and coagulation factors, related to various severe cardiovascular events such as stroke, embolism, cardiomyopathy, myocardial hypertrophy, hepatotoxicity, nephrotoxicity, and dermatological disorders like acne as well as effects regarding the central nervous system, e.g., depression and general behavioural changes [6,8,21–26]. Sex-specific side effects are virilisation in women associated with alterations in body hair, deepening of the voice, enlarged clitoris, some of them permanent, and gynaecomastia in males [6,27–29]. Alterations of sexual function and infertility is also associated with the administration of AAS [29,30]. In adolescents, accelerated bone maturation and closure of the epiphyseal growth zones with growth retardation is reported [31].

In addition to androgen receptor mediated anabolic conditions in tissues, estrogen receptor (ER) β (ER-β) has also been suggested to catalyse muscle hypertrophy and other metabolic changes [32,33], e.g., an increased immune response and activation of satellite cells [34,35], while estrogen receptor α (ER-α) does not seem to have relevant impact in mediating anabolic actions [33]. Both ER subtypes are generated from different genes and show differences in their expression pattern in various tissues [36]. Weigt et al. propose ER-α to be relevant for regulating blood lipids while ER-β is thought to mediate remodelling in muscle tissue [37]. Adipose tissue is presumed to be influenced by both receptor-subtypes [34].

The development of selective androgen receptor modulators (SARMs) was encouraged by the successful production of selective estrogen receptor modulators (SERMs) like tamoxifen, which are used in the treatment of breast cancer, and the selective tissue estrogenic activity regulator (STEAR) tibolone, which can be used to treat menopausal symptoms and endometriosis [38]. Thus, targeting the androgen receptor, also SARMs are developed intending to promote anabolic effects while not affecting reproductive tissues [39]. However, in contrast to the estrogen receptor, where two different subtypes (ER-α and ER-β) are identified, no subtypes of the AR are known. Nevertheless, tissue selectivity in SARMs could possibly be achieved, but until now none of the currently developed SARMs was found truly selective for muscle and bone tissues in comparison to the reproductive

tissues [39]. In addition to beneficial tissue selectivity, new small-molecule SARMs without steroid backbones are considered to ease the development of dosage forms and allow oral administration [40]. However, even if the intention of SARM design was to achieve muscle hypertrophy and bone consolidation while reducing the adverse effects associated with AAS use, it was observed that also after SARMs administration, hematocrit, blood pressure, blood lipid and hormone levels, i.e., total testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels, were significantly altered [26]. Until now, most of these compounds failed to advance to clinical development either due to toxicity or lack of efficacy, or other undisclosed reasons [39]. By now, a few clinical trials are ongoing, e.g., on enobosarm (GTx-024) and LY2452473 (both phase II, according to www.clinical trials.com, accessed Nov 26th, 2017).

In various animal models, β_2-adrenergic agonists like clenbuterol lead to increase in muscle growth, lipid mobilisation in fat tissue and other metabolic effects [41]. In contrast to other agents, these effects seem to depend on the route of application [42]. In animal husbandry, β_2-adrenergic agonists are known as growth promoting and repartition agents and their illegal use in meat production is reported in several publications. It is also shown that this misuse in animals, especially in case of the highly active clenbuterol, may have an impact on humans through consumption of meat, as well. It has even led to poisoning in men due to the consumption of contaminated meat products [43–51]. Signalling via β_2-adrenergic agonists is mediated through binding to a $G\alpha$-protein coupled receptor (GPCR), leading to activation of adenylylcyclase (AC) and production of cyclic adenosine-monophosphate (cAMP), which then leads to an activation of proteinkinase A and phosphorylation of various effector proteins [41,52].

Apart from the small molecule drugs discussed before also some peptide or protein drugs are listed as prohibited substances in sports according to the regulations of the World Anti-Doping Agency (WADA) as separate class S2 "Peptide Hormones, Growth Factors, Related Substances and Mimetics" [53]. Out of this class, several compounds are also involved in the promotion of muscle growth: Gonadotrophins act as stimulators of steroid hormone production, thereby increasing the endogenous production of androgens in the effector cells. Somatotropin (growth hormone, GH) is considered to act as physiological precursor of insulin-like growth factor-1 (IGF-1), inducing IGF-1 liberation into the blood stream [54]. Evidence is increasing, that IGF-1 subsequently binds to IGF-1 receptors on the surface of muscle cells, inducing growth via several pathways, including the phosphatidylinositol-3-phosphate kinase (PI3K)/protein kinase B (Akt) pathway [55]. Further growth factors are included in the list of prohibited substances in sports as well. Covered in a different class, insulin and its analogues are also prohibited in sports, mainly due to their effect of altering metabolic activities and energy supply. However, reports on pathways promoting muscle growth by insulin are also reported. A strong link between insulin and IGF-1 actions is seen in the phosphorylation of insulin response substrate (IRS) [35,56]. Sudies report β-hydroxy-β-methylbutyrate (HMB) as inducer of GH and IGF-1. However, a recent review of Holeček mentioned that the hypertrophic effect of HMB on muscles is especially relevant in elderly subjects and if combined with exercise [57].

Another strong (negative) regulator of muscle growth is myostatin, a member of the transforming growth factor β (TGF-β) superfamily [58–60]. It is reported to inhibit myoblast proliferation and differentiation [61–64]. Upregulation of myostatin results in muscle atrophy [65], which is also reported as effect after glucocorticoid administration or neuron-derived orphan receptor 1 (NOR1) inhibition [66,67]. Consequently, the inhibition of myostatin results in increases

in muscle growth [68]. Reports on myostatin deficient mice, cattle and even humans display its strong effect and underline the potential of myostatin inhibition as therapeutical option [59–74]. However, there is also evidence that myostatin inhibition may only result in early improvement, while progression of muscle degeneration still continues later on [75].

Deeper knowledge of the signalling mechanisms mediated by the different substance classes are not only important for identifying characteristics of those substances that are illegally used by athletes in order to enhance their performance. They can also be of therapeutic value in treatment of patients suffering from muscle wasting syndromes, i.e., cachexia, muscle atrophy, frailty or sarcopenia [76–82]. Patients suffering from diseases like cancer, AIDS, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, diabetes, heart or renal failure, may develop cachexia, which is also associated with increased incidence of morbidity and mortality [83,84]. Additionally, muscle wasting is associated with an increased risk of developing metabolic diseases, such as metabolic syndrome or type-2 diabetes [85]. Current therapeutic strategies mainly focus on adaptation of nutrition to high protein diet and regular physical exercises, but severe cases may also profit from pharmacotherapy involving one of the substance classes mentioned in this review.

2. Mechanism of action

2.1. Anabolic-androgenic steroids (AAS)

Signalling mechanisms, by which AAS modulate human tissues can be divided in genomic and rapid, non-genomic actions [5,14]. Genomic actions include changes in AR expression and interaction with co-activators, resulting in transcription of specific target genetic regions [6]. In addition, an anti-catabolic effect by interference with native agonists to glucocorticoid receptors, inhibiting their binding, is suggested [6,16]. Non-genomic actions are supposed to result in activation of second messenger signal transduction cascades, providing multiple changes within a few minutes [7,14].

2.1.1.   Genomic actions of AAS

Genomic actions are mediated by binding of testosterone or the respective derivative to intracellular androgen receptors (iAR), resulting in dissociation of heat-shock-protein 90 (HSP 90) and other chaperons from the receptors [5]. Subsequently, unprotected iAR dimerize and move to the nucleus, where they are phosphorylated at several serine residues and continue to recruit several co-activators like cAMP response element binding protein (CREB)-binding protein (CBP), p300/CBP-associated factor (p/CAF) and steroid receptor coactivator 1 (SRC-1), etc. [5,7,86]. In the following step, the receptor-coactivator-complexes bind to specific androgen-response-elements (ARE) on the DNA [5], leading to chromatin remodelling and recruitment of RNA-polymerase II [7]. The enzyme transcribes target DNA-regions into mRNA which, after translation, results in production of different protein products (Figure 1). These are supposed to promote relevant changes in different tissues [5]. Mechanisms of iAR activation are described more in depth by Kicman et al. [6], Li et al. [7] and Bennett et al. [5].

Results of activation in productive tissue are increased levels of prostate specific antigen (PSA) and production of probasin [5]. Muscle tissue is supposed to produce more protein while retaining

nitrogen and thereby increasing its size and strength [2,7,87]. However, only very limited numbers of studies display the finally achieved enhancement in sports performance. Evidence has been provided that bone tissue answers stimulation by increased erythropoiesis, elevated cell proliferation and increased synthesis of growth factors [23]. This signalling cascade is displayed in Figure 1.

2.1.2. Anti-catabolic effects of AAS

Evidence is increasing, that additional anti-catabolic effects of AAS are mediated through inhibition of the binding of endogenous ligands to glucocorticoid receptors [6,23]. Physiological agonists on these receptors are glucocorticoids such as cortisol, which mediate various effects in the body, mainly related to metabolism of carbohydrates and lipids. They are considered as most important stress hormones in mammals (including humans) besides adrenaline and noradrenaline [17]. The initial mechanism mediating glucocorticoid-induced processes are analogous to that described for the genomic AR response. The resulting specific mediator proteins promote amino acid degradation, nitrogen excretion and reduction of mass in muscle tissue in order to provide the energy, which might be needed in stressful situations [17–19,23].

According to Kicman et al., binding of testosterone or other AAS to the glucocorticoid receptor may inhibit binding of GR agonists, and thereby result in anti-catabolic (anabolic) effects like nitrogen retention and muscle hypertrophy (Figure 2) [6].
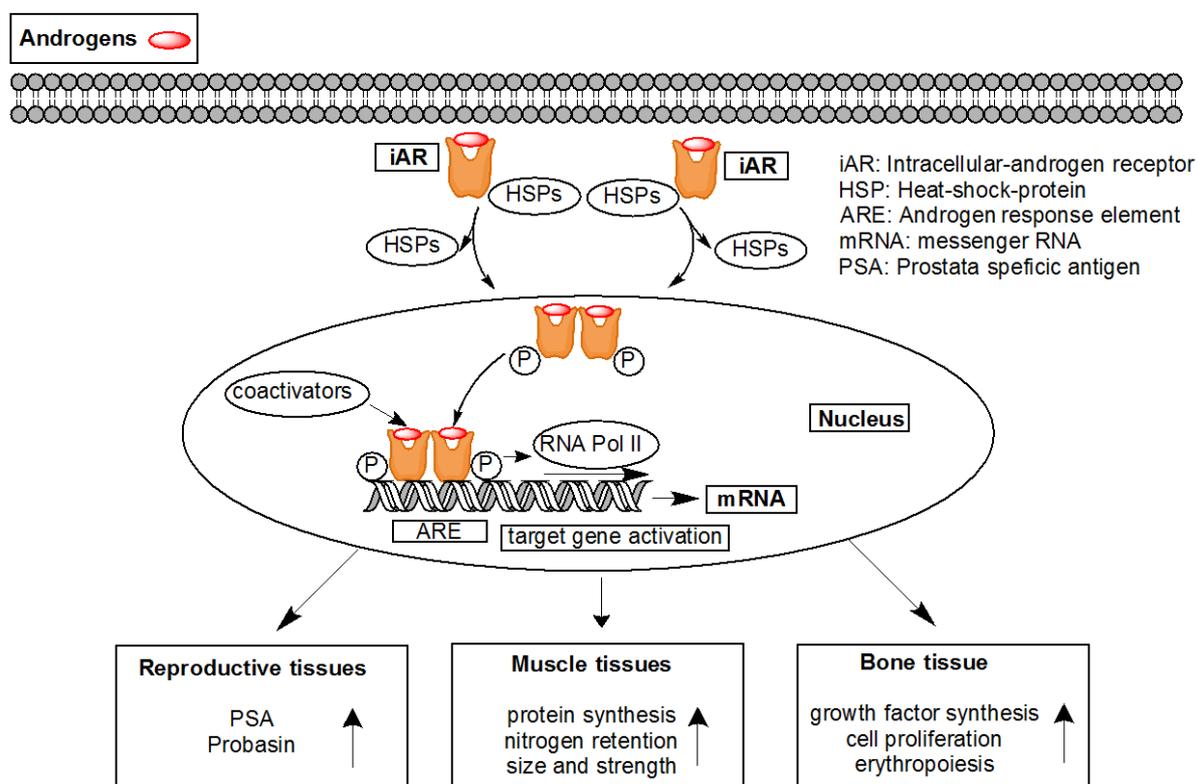

Figure 1. Genomic action of anabolic androgenic steroids mediated by androgen receptor binding.
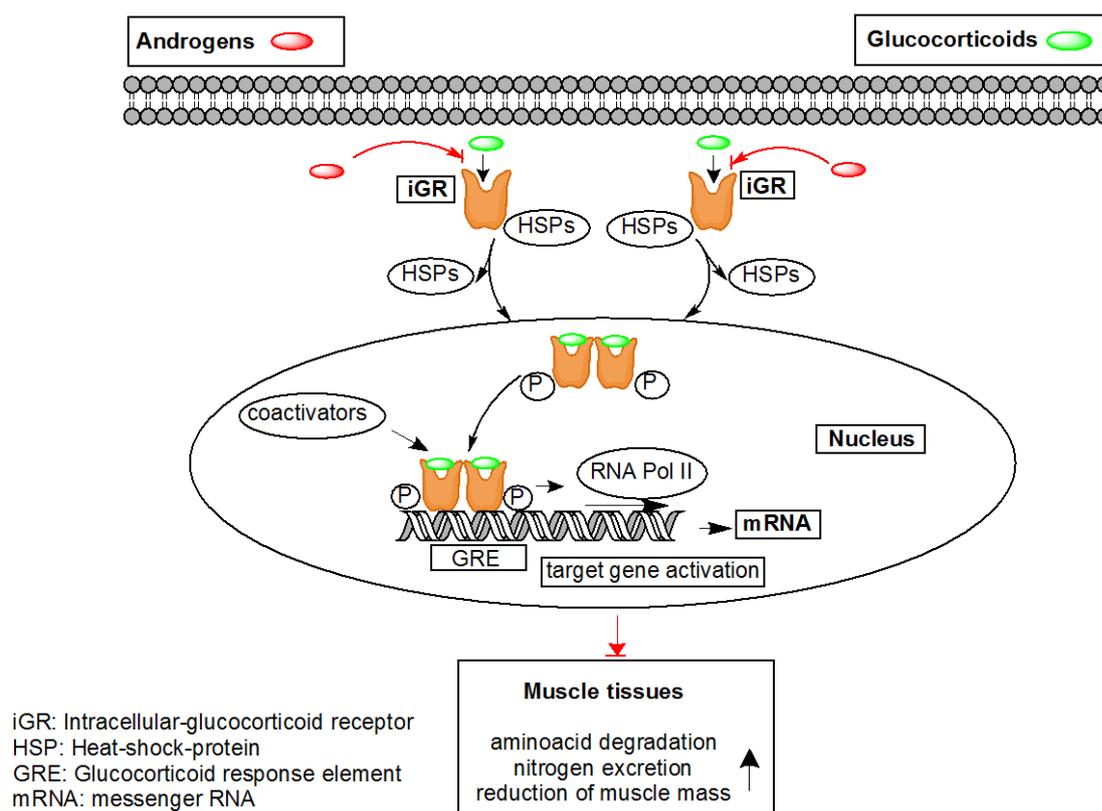
Figure 2. Anti-catabolic activity of anabolic androgenic steroids by competitive binding to glucocorticoid receptor.

2.1.3. Non-genomic actions of AAS

Rapid, non-genomic actions of testosterone are mediated through several membrane-located receptors and ion channels, including endothelial growth factor receptor (EGFR), membrane-located androgen receptors (mAR), sex hormone-binding globulin receptor (SHBGR) and calcium-ion channels [5,9,12]. Binding of AAS to these receptors leads to activation of several second messenger signalling cascade pathways (Figure 3). Those pathways result in production of mediators, which promote anabolic changes through direct interaction with cells in various tissues, with very short response times [5,7]. In addition to that, intracellular androgen receptors are also stimulated by end products of second messenger signalling cascades, facilitating an increase in transcriptional activity [9]. Protein products of activated second messenger signalling cascades are also supposed to lead to transcription of specific DNA gene regions [9].

Binding of AAS to EGFR is suggested to promote activation of Ras-Kinase [14], which itself activates MAP-Erk-Kinase (MEK) [5,9,11]. Subsequently, MEK phosphorylates extracellular signal-regulated protein kinases 1/2 (ERK 1/2), which then translocate to the nucleus and phosphorylate transcription factors like the ETS domain-containing protein ELK1 [12,14]. Bound mARs seem to stimulate the tyrosine kinase c-SRC, which can either also phosphorylate Ras, leading to ERK 1/2 activation, or interact with membrane-located adenylylcyclases (AC) [12]. AC recruit adenosine triphosphate (ATP), turning it into the second messenger cyclic adenosine-monophosphate (cAMP). cAMP activates proteinkinase A (PKA) by binding to its regulatory subunit. PKA then

phosphorylates specific target proteins with its catalytic subunit, e.g., cAMP response element binding protein-1 (CREB-1) [14].

Upon phosphorylation, CREB-1 interacts with a cAMP response element on the regulatory region of a gene and increases its transcription [52]. It should be noted that phosphorylation through PKA not necessarily leads to activation but may also result in inactivation of proteins like Ras homolog family member A (RhoA) in smooth muscle cells. Phosphorylation of RhoA by PKA promotes dephosphorylation of myosin light-chains (MLC) in smooth muscle which in turn prevents constant muscle contraction [88].
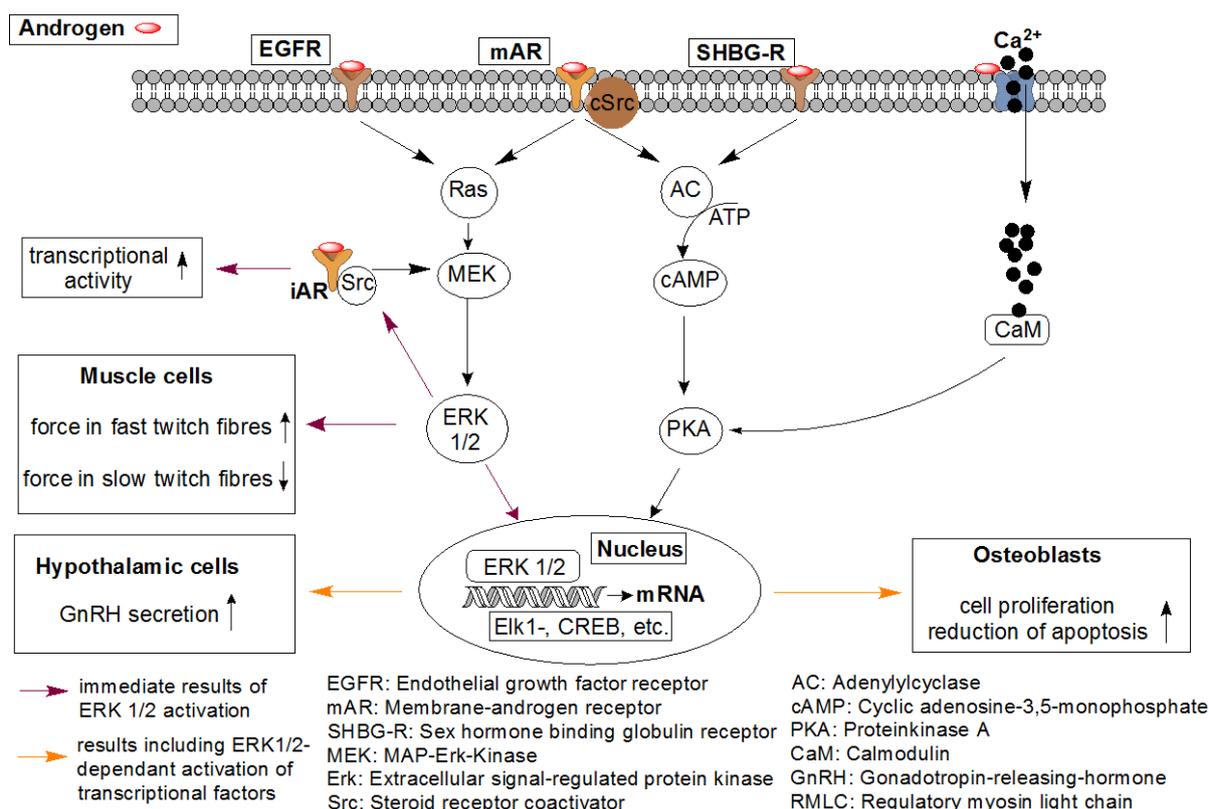

Figure 3. Signalling cascade on non-genomic action of anabolic androgenic steroids.

In addition to activation through cAMP, PKA can also be stimulated by calmodulin which itself is activated by binding of calcium ions [9]. Last but not least, sex hormone binding globulin receptors (SHBGR) have been proposed to also use the AC-PKA-signalling pathway in order to modulate target gene transcription [9,12,14,15]. Publications also propose non-genomic signalling through intracellular androgen receptors (iAR) in a kind of feedback mechanism: Phosphorylated ERK 1/2 is proposed to stimulate iAR resulting in stimulation of MEK which then again increases ERK 1/2 activation [5]. Upregulation of transcriptional activity through iAR can also be observed as an answer to ERK 1/2 stimulation [5]. Resulting reactions in tissue can be divided in those immediately mediated by ERK 1/2 and those resulting from gene transcription. Immediate changes are observed in muscle cells which increase force in fast twitch fibres and decrease force in slow twitch fibres. Gene transcription mediated modulations include increased gonadotropin releasing hormone (GnRH) secretion from the hypothalamus and metabolism changes in osteoblasts. Those

enhance cell proliferation while reducing apoptosis [9,10]. All proposed mechanisms (Figure 3) may lead to anabolic changes in tissue, promoting muscle hypertrophy [3].

There are also reports that propose AAS to activate the PI3K pathway via a non-genomic mechanism, in which stimulated AR phosphorylate the p85-subunit of PI3K [5]. Due to its confirmed relevance in ER-β mediated signalling, this pathway is presented in detail in chapter 2.3 and Figure 4.

2.1.4. Hypothalamus pituitary axis feedback loop

The administration of AAS directly affects the production of endogenous hormones via feedback mechanisms on the hypothalamic pituitary gonadal axis (HPA) [89]. The HPA is regulated by negative feedback mechanisms from AR binding in the hypothalamus [90,91]. This results in decreased GnRH synthesis in the hypothalamus, decreased gonadotropin secretion in the pituitary gland, as well as a non-steroidal feedback of inhibin B on FSH secretion in the pituitary gland [92]. Already one single dose administration of AAS was reported to result in decreased serum LH and inhibin B levels, while prolactin and IGF-1 levels increased after prolonged administration [87,93]. This also concomitantly reduced endogenous production of sex steroids including the endogenous AAS testosterone. Even after 12 weeks after withdrawal initial levels were not recovered again [93].

2.2. Selective androgen receptor modulators (SARMs)

More recently, selective androgen receptor modulators (SARMs) are under investigation for use as muscle building agents. They are developed with the intention of improving physical function and bone health without adversely affecting the prostate and cardiovascular system. The exact mechanism of their tissue selectivity still remains uncovered until now [39]. A tissue-selective modulation of signalling pathways is mainly considered to occur due to availability of coregulators [94]. Additionally, tissue-specific expression of various steroidogenic and metabolic enzymes as well as transcription factors are discussed to contribute to the tissue selectivity [39]. For further reading we recommend the reviews of Narayanan et al. and McEwan [39,95]. Different chemical classes have been investigated as non-steroidal SARMs until now. Most of them belong to the arylpropionamides or quinoline derivatives. It is reported that signalling of enobosarm (SARM S-22) is mediated through Src kinase, MEK-1/2 or MEK-3, ERK and p38 mitogen-activated protein kinase (MAPK) pathways [96]. Further research is needed to elucidate the mechanism of action in SARMs.

2.3. Estrogen receptor β (ER-β) agonists

It is reported that also an activation of the estrogen receptors (ER) results in muscle hypertrophy [97–99]. More specifically, investigations could identify the ER-β as target for mediating this effect [99,100]. Several classes of substances are reported as ER-β agonists, with lots of the recently investigated being plant derived [101–115]. To the best of our knowledge diarylpropionitril [2,3-bis (4-hydroxyphenyl)-propionitrile] and 8β-VE2 were the first selective ER-β agonists that were reported in literature [116–118]. Currently, clinical trials are performed using the ER-β agonist LY500307 as well as phytoestrogens epigallocatechin gallate and S-equol

(www.clinical trials.com, accessed Nov 29th, 2017). Furthermore, some phytoestrogens such as ecdysterone and daidzein showed strong selectivity towards the ER-β, which even exceeds the selectivity of S-equol. However, a considerably lower activity of the phytoestrogens is seen in comparison to estradiol or the synthetic ER-β agonists mentioned above [33,112,114,117,119–121]. Ecdysterone is reported to influence adipose and muscle tissue, resulting in increased muscle mass and body weight while decreasing visceral fat mass [33,37,122]. In animal studies, increase of physical performance without training, increased synthesis of myofibrillar proteins in muscle, increased protein content and growth in liver and kidney have been reported [21]. However, some phytoestrogens are proposed to mediate anabolic effects through membrane-bound ER-β receptors [33], without causing apparent adverse androgenic effects [21]. Furthermore, in a recent study, same doses of the phytoecdysteroid ecdysterone elicited stronger hypertrophic effects in mouse myoblastoma cell line C2C12 derived myotubes than the AAS metandienone, estradienedione and SARM S1 [122]. This implies a strong potency of phytoecdysteroids in inducing muscle hypertrophy and provides hope for the opportunity to evoke anti-catabolic actions already in small doses. Quercetin, reported as unselective ER agonist, was also reported to increase the cross-sectional area and minimal fibre diameter in mice. This effect was even more pronounced at co-supplementation of whey protein [123].

Depending on tissue, different signalling mechanisms may be involved (Figure 4). In adipocytes, peroxisome-proliferator activated receptor-gamma (PPAR gamma), which promotes lipid uptake and adipogenesis, is supposed to be inhibited [34,37,124,125]. This results in increased lipolysis and even apoptosis of adipose cells. Also, adipogenesis and lipid accumulation is reported to be inhibited, leading to a decrease in visceral fat, adipocyte size and an overall decrease in serum lipid concentration. Changes in muscle tissues after activation of ER-β are mediated through the PI3K/Akt pathway (Figure 4) [21,32,33,126–128]. After activation of PI3K, phosphatidylinositol-4,5-bisphosphate (PIP2) is recruited and turned into phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then acts as lipid binding site on the cell membrane, inducing the recruitment of the serine/threonine kinase Akt. After being recruited to the cell membrane, Akt is phosphorylated by the kinase PDK-1 [129]. Bodine et al. propose induction of protein synthesis through Akt via phosphorylation of glycogen synthase kinase 3 (GSK-3), resulting in its inhibition [127]. Inhibited GSK-3 releases elF2B, a transcription factor which upregulates protein synthesis [56,130]. Another important target of Akt is mTOR, which leads to activation of p70 ribosomal protein S6 kinase (p70^{S6K}) and inhibition of PHAS-1/4E-BP1 through phosphorylation [56,130]. Activated p70^{S6K} also promotes protein synthesis while PHAS-1/4E-BP1 is supposed to mediate translation initiation after release of the translation initiation factor elF4E [56,130]. After binding of elF4E to elF4G, initiation of translation is facilitated [56,130]. It is also proposed that Akt inhibits forkhead O transcription factor (FOXO), which mediates production of the ubiquitin ligases MuRF1 (muscle ring finger protein 1) and MAFbx (muscle atrophy F-box protein) [35,126,131]. Evidence is increasing that MuRF1 and MAFbx play a key role in muscle atrophy [35,126,131]. Inhibiting FOXO, and thus the transcription of MuRF1 and MAFbx, indirectly favours muscle growth by decreasing muscle atrophy [126,132]. Akt also prevents apoptosis of muscle cells by phosphorylating Bcl-associated death agonist (BAD), which is highly important for apoptotic processes [32,133]. Further information on the PI3K pathway and downstream targets of Akt is provided in other publications, e.g. [56,127,129,134–136].

Overall, resulting effects in muscle cells include increased protein synthesis, promotion of IGF-1 synthesis, increased insulin sensitivity, expanded apoptosis, and improved utilisation of glucose and lipids, while reducing protein degradation [21,33,34,37].
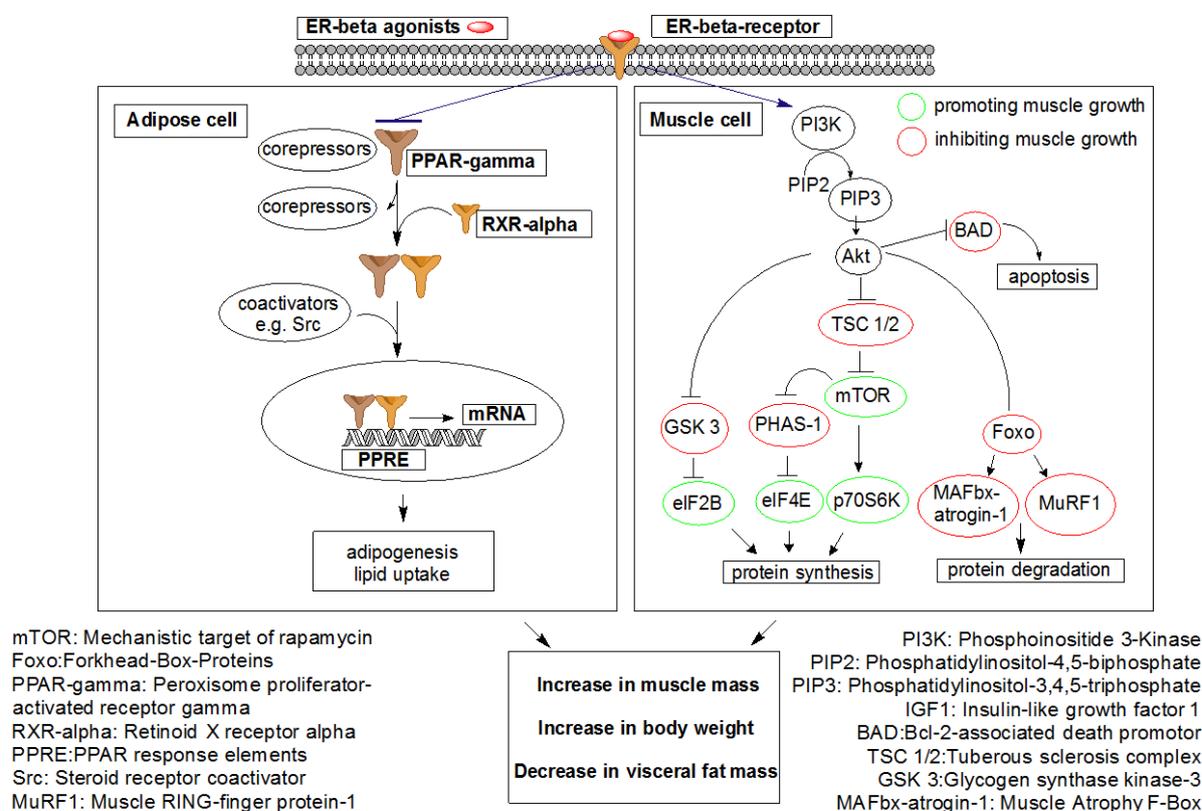

Figure 4. Estrogen receptor β mediated signalling pathways leading to muscle hypertrophy.

2.4. β₂-Adrenergic agonists

β_2-Adrenergic agonists are synthetic analogues of adrenaline that are therapeutically used as bronchodilating agents in asthma bronchiale or chronic obstructive pulmonary disease (COPD). They act as sympathomimetic agents primarily targeting the β_2-adrenoreceptor, a $G_{\alpha s}$-GPCR located in the cell membrane (Figure 5) [41,52,137]. Further information about different types of β-adrenergic receptors and their effects on tissue can be found in the publication by Mersmann et al. [52]. Stimulation of β_2-adrenergic receptors leads to AC activation and thereby increase cAMP production [52]. In smooth muscles this leads to relaxation, which is the main therapeutic concept of β_2-adrenergic agonists. However, the increased cAMP also results in promotion of the cAMP-proteinkinase A signalling pathway described in the section about non-genomic actions of AAS and actions of ER-β agonists in chapters 2.1.3 and 2.3. The Akt/mTOR signalling pathway is triggered via PI3K, mediated upon β_2-adrenergic activation by $G_{\alpha i}$-associated $G_{\beta \gamma}$ dimer [138,139]. Therapeutically desired the inhibition of PI3K reduces interleukin 25 (IL-25) mediated airway hyperresponsiveness and inflammation [140]. Downstream, it results in an increased expression of ribosomal proteins by stimulation of p70^{s6K} [141,142]. At the same time, mTOR inhibits the eukaryotic

initiation factor 4E binding protein-1 (4E-BP1), itself an inhibitor of protein translation [143]. Studies on clenbuterol-treated rats showed increased p70^{s6K} and reduced 4E-BP1 activity [144]. Since the Akt/mTOR signalling pathway (Figure 4) plays a role in the regulation of muscle growth [145], it was also possible to antagonize the anabolic effects of clenbuterol by means of rapamycin, an m-gate inhibitor [146]. A protein called exchange protein directly activated by cAMP 1 (Epac1) was introduced as important player in clenbuterol induced hypertrophy by Okumura et al [147]. Differential effects of clenbuterol on fast- and slow-twitch muscle fibres were explained by the differences in downstream regulated hydrolysis of cAMP [148], which is regulated by phosphodiesterase 4 [149]. In ractopamine fed pigs type I and IIA fibre diameters were increased [150]. However, chronic clenbuterol administration is associated with reduced contractile efficiency, especially in fast contracting muscles [151]. In addition to the activation of m-gate, Akt is also able to directly inhibit FOXO [152,153]. However, it was also reported that clenbuterol does not act via Act/FOXO but via Act/mTor pathway and IGF1 expression [154]. As already mentioned in chapter 2.3. FOXO is a transcription factor that promotes transcription of the MuRF1 (Muscle RING finger 1) and MAFbx (muscle atrophy F-box) genes. MuRF1 and MAFbx encode ubiquitin ligases that are expressed in muscle atrophy [145,155]. Thus, by β_2-receptor activation, skeletal muscle growth and a muscle-protective effect can be assumed which was successfully demonstrated for the β_2-agonist formoterol in arthritic rats [156]. Comparable effects are reported in prolonged treatment of with atomoxetine, a selective noradrenaline reuptake inhibitor (NARI) [157].

Furthermore, in non-muscular tissues, phosphorylation and de-phosphorylation of various proteins is suggested to result in changes in hormonal profile and an increase in blood flow, which enables transportation of hormones, substrates and energy sources for protein synthesis to muscular tissue [52,158]. In fat tissue PKA modulates phosphorylation, resulting in increased lipolysis and triacylglycerol hydrolysis, while lipogenesis is decreased [52]. It has to be noted that effects on adipose tissue regarding lipogenesis and lipolysis seem to vary between β_2-adrenergic drugs. For example, Peterla et al. [159] found out, that clenbuterol decreases lipogenesis but has no effect on lipolysis. They report data from an *in vitro* study on lipolysis and lipogenesis by porcine adipose tissue when treated with different β_2-adrenergic agonists [159]. Also Yang et al. [158] reported an inhibition of fatty acid synthesis concomitant with a promotion of glycogenolysis in the liver of rodents. Finally, muscle cells seem to increase protein synthesis and glycogenolysis, while reducing the rate of glycogen synthesis and protein degradation [52,137]. These hormone-mediated effects are supposed to be enhanced by direct stimulation of muscle tissue through PKA [52]. It was reported that CREB is phosphorylated in this signalling cascade. This transcription factor subsequently binds to defined cAMP response elements (CRE) and thus influences the transcription of diverse genes [160]. Among others, the expression of neuron-derived orphan receptor 1 (NOR1) is increased [161]. Inhibition of NOR1 mRNA by siRNA, in turn, led to a highly significant increase in myostatin mRNA *in vitro* [66]. Myostatin is a representative of the transforming growth factor β (TGF-β) family and is known as negative regulator of muscle growth [59,60]. Thus, β_2-adrenergic agonists most likely indirectly influence the level of myostatin and thus, also muscle growth via NOR1. Further information on myostatin is available in chapter 2.6. In summary, resulting effects of β_2-adrenergic agonists on body composition include an overall increase in body weight and muscle mass as well as a decrease in body fat (Figure 5) [52,162–164].

Not all β_2-adrenergic agonists seem to induce these effects to the same degree. It has to be considered, that the intensity of effect depends on the chemical structure of the drug and the route of application.

Intraperitoneal application is suggested to be the only efficient for salmeterol while clenbuterol also increases muscle growth when given orally [42]. Moore et al. also report that β$_2$-adrenergic agonists need a long duration of application until actually evoking these metabolic changes [23].

Finally, one has to be aware, that very few studies in humans have been conducted and that relevant studies mostly report results of animal testing or from usage in production animals. In addition to that, major differences in resulting effects have been noticed between different animal species [52]. Thus, findings in animal studies may not be directly transferable to human tissue, and the relevant metabolic changes in human tissues still need to be elucidated.
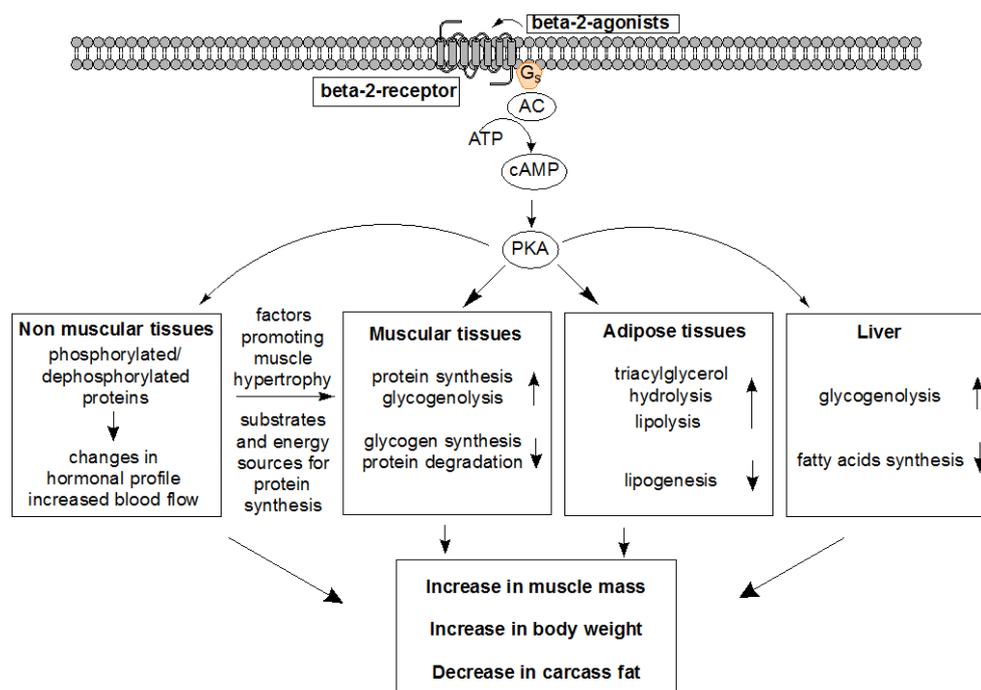

Figure 5. cAMP signalling after β$_2$-adrenergic receptor activation.

2.5. Growth-hormone, IGF-1 and insulin

Peptide-hormones like somatotropin (growth hormone, GH), insulin-like growth factor-1 (IGF-1) and insulin are also reported as essential mediators of muscle hypertrophy [35,54,55,129,165–167]. GH, physiologically secreted by the anterior hypophysis after stimulation by the hypothalamus through growth hormone releasing hormone (GHRH) [35], acts mainly as trigger for IGF-1 release from the liver [35,54,168]. Only recently, GH has been reported to increase serum decorin, a protein which interacts with various growth factors [169]. It is reported to bind to myostatin and promote hypertrophy by downstream pathways (chapter 2.6.) [170]. GH itself does not seem to be able to induce long-term increases in muscle mass without stimulation through exercise [35]. IGF-1 is mainly secreted in the liver, triggered by GH pulses that originate from the anterior hypophysis. This fraction of IGF-1 is called circulating IGF-1 (cIGF-1), indicating its separation from muscular IGF-1 (mIGF-1), which also influences body composition but is released directly from muscular tissue [35]. mIGF-1 can influence proximate muscle cells via paracrine signalling or enhance the stimulation of its cell of origin by autocrine mechanisms (Figure 6) [35,54,135,166,167,171]. Evidence is increasing,

that the mIGF-1 isoform is much more potent in inducing muscle hypertrophy than the cIGF-1, resulting from GH-stimulation [35,54,133,167,172,173]. In contrast to that, cIGF as well as GH are thought to be important in reducing catabolic activity by reducing the negative nitrogen balance when the patient is exposed to a calorific deficit [35]. Thus, Velloso et al. report that GH and cIGF are highly important in patients suffering from catabolic conditions and less important for muscle growth in healthy individuals such as athletes [35]. Upstream targets for IGF-1 also allow for induction of muscle hypertrophy. Recently, Jang et al. [174] showed that the natural flavone Apigenin enhances muscle hypertrophy via protein arginine methyltransferase 7 (Prmt7) increase, GPCR56 expression and subsequent activation of the IGF-1 pathway. Furthermore, Apigenin induced Prmt7 increase is also reported therein to induce muscle cell differentiation by regulation of p38 MAPK and myoblast determination protein (MyoD).
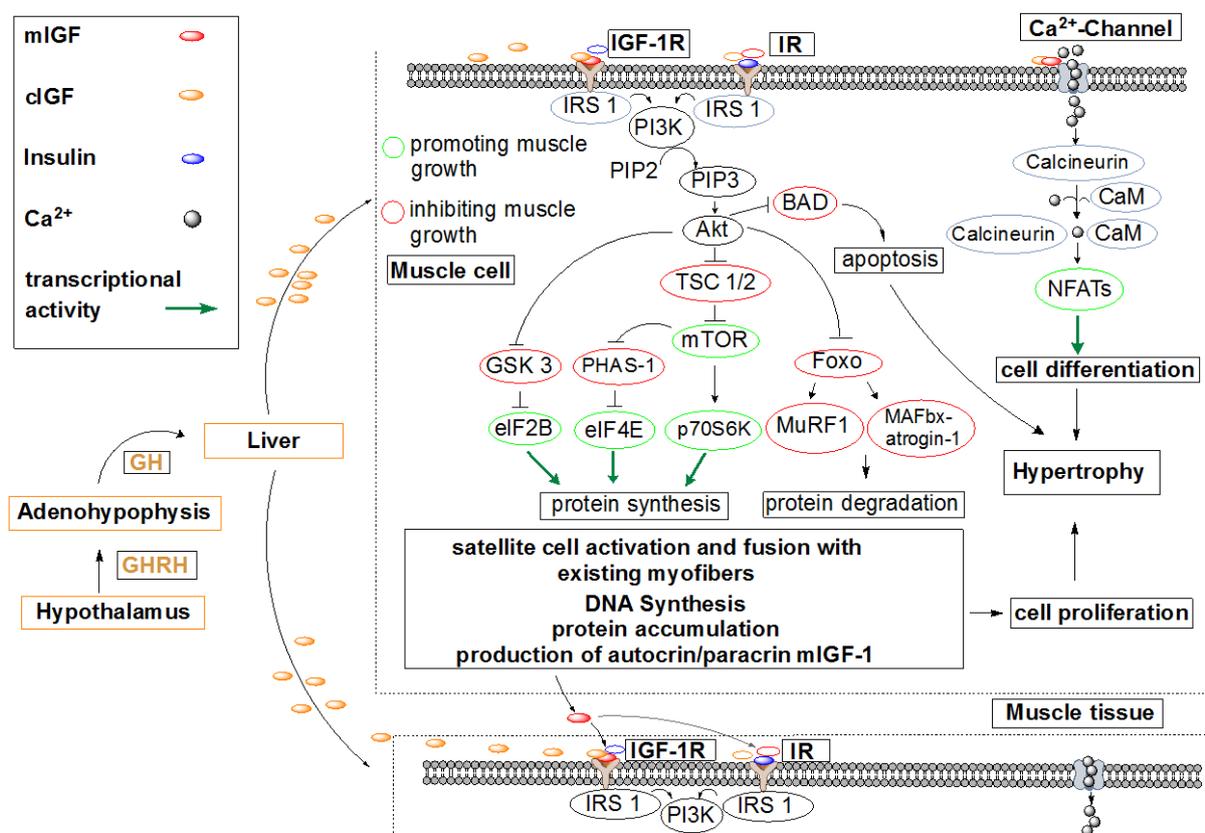

Figure 6. IGF-1 and insulin induced muscle hypertrophy involving IGF-1 receptor, insulin receptor and Ca^{2+} channel signalling.

Several signalling pathways are discussed after IGF-1 binding (Figure 6), including the PI3K/Akt pathway, that was already mentioned in the section about ER-β agonists (chapter 2.3, Figure 4) [35,54,55,129,133,166]. PI3K is activated after mIGF-1 or cIGF-1 have bound to the IGF-1 receptor (IGF-1R), resulting in phosphorylation of insulin response substrate (IRS) 1 via tyrosine kinase activity [35]. IRS-1 then phosphorylates PI3K, starting the signalling cascade [35]. In addition to activation through IGF-1, insulin is also discussed to activate the PI3K signalling cascade [35,173,175] after either binding to insulin receptors (IR) with high affinity or to IGF-1Rs with lower affinity [135]. Conversely, IGF-1 is also supposed to be able to activate IRs with low affinity [35,135]. After binding, insulin receptors phosphorylate IRS-1 and IRS-2 through their intrinsic tyrosine kinase

activity, stimulating their binding to and subsequent activation of PI3K [56]. Downstream targets of the PI3K pathway are the transcription factors elF2B, elF4E and p70^{S6K} (chapter 2.3). They are also responsible for changes in protein expression after IGF-1 or insulin administration [56,129,173]. While many researchers think of Akt as an essential mediator in activation of mTOR and p70^{S6K} as part of the PI3K pathway [129,173], other studies imply that these downstream targets are activated via mechanisms independent from Akt mediation [133].

As results of these changes in muscle cells, an increased DNA-synthesis, accumulation of protein and an increased production of mIGF, which results in amplifying the effects of cIGF through paracrine and autocrine mechanisms, are reported [35,172]. Furthermore, an activation of satellite cells, which are proposed to fuse with already existing myofibers, and thereby enlarging muscular tissue, is discussed [35,54,135,166,171,176]. In analogy to the PI3K pathway discussed in the section on ER-β agonists, IGF-1 and insulin are also reported to antagonize muscle atrophy through Akt dependent inhibition of MAFbx and MuRF1 [173]. A different approach to explaining IGF-mediated muscle hypertrophy implies involvement of the Ras-Raf signalling cascade, resulting in cell proliferation [133,135,166]. It has to be noted that the relevance of this signalling pathway is still unclear. Some researchers propose that the MAPK pathway actually has to be inhibited for achieving muscle hypertrophy in skeletal muscle [133]. Another proposed mechanism of action involves signalling through ligand-dependent calcium channels (Figure 6). This may also facilitate cell differentiation through the serine phosphatase calcineurin, resulting in muscle hypertrophy [133,167,177–180]. In analogy to signalling in ER-β stimulated cells, binding of mIGF or cIGF to these ion channels triggers their opening. Calcium influx activates calcineurin, which forms a complex with calcium ions and calmodulin. This complex then activates nuclear factors of activated T-cells (NFATs), transcription factors, promoting cell proliferation and growth of muscular tissue via dephosphorylation [133,179,181]. More detailed information regarding downstream targets of this pathway has been provided by Musaro et al. and Swoa et al. [171,182].

It has to be noted, that the importance of the NFAT pathway still remains unknown with several controversial studies being published. While some studies [179–181] find this pathway to be highly important in promoting hypertrophy especially in cardiac myocytes, other researchers find the calcineurin pathway of lower importance or even antagonised after IGF-1 stimulation [55,171,182]. Independent from effects in muscle tissue, various effects of GH on body composition and metabolism have also been reported. It is supposed, that the hormone mediates lipolytic activity and effects on collagen and bone turnover [35]. This may lead to improved performance without evolvement of muscle hypertrophy and could be a possible explanation for the widespread use of GH for doping purposes despite the lack of effects on muscular tissue.

2.6. Myostatin inhibitors

As already mentioned in the section about β$_2$-adrenergic agonists (chapter 2.4), myostatin (also called growth differentiation factor 8, GDF-8) is an important and potent regulator of muscle homeostasis (Figure 7). It is highly expressed in skeletal muscle, where it conveys its signals through the extracellular activin type IIB receptor (ActRIIB). ActRIIB is expressed as a heterodimer together with either activin-like kinase 4 (ALK4) or activin-like kinase 5 (ALK5) [183]. Both kinases belong to the group of serine/threonine kinases and lead to the phosphorylation of transcription factors Smad2 and Smad3 upon activation. Phosphorylated Smad2 and Smad3 both form heterodimers

together with Smad4 and inhibit the transcription of various genes involved in muscular proliferation and differentiation after moving to the nucleus [184,185]. As the relevance of myostatin in various diseases involving muscle atrophy and sarcopenia has become increasingly evident in recent years [186], inhibition of myostatin seems a promising approach for novel therapeutic options. Several facts contribute to myostatin being an attractive target for drug therapy: First, its presence has been clearly correlated with loss of muscle mass and muscle volume in various animal studies [184]. Second, the high abundance of myostatin in skeletal muscle, when compared to other tissue, raises hope for high specificity and few off-target effects of possible myostatin-inhibiting agents. Third, its signalling mechanism provides multiple possible options for disruption. As myostatin is secreted into the bloodstream, the circulating protein can be bound by neutralizing antibodies or soluble ActRIIB receptors (Figure 7). In addition to that, myostatin binding proteins like myostatin propetide, follistatin and the follistatin related proteins follistatin related gene (FLRG) as well as growth and differentiation factor-associated serum protein-1 (GASP-1) may be used to inactivate myostatin [184]. Increased follistatin levels were also reported to act on mTOR most likely via SMAD3, which is also found to act independently from myostatin inhibition [187] and by the recently reported bone morphogenetic protein (BMP) signalling pathway [188]. An interconnection with the IGF-1R pathway is suggested and Barbé et al. [189] found IGF-1 and insulin deficiency to attenuate follistatin induced muscle hypertrophy. While application of myostatin binding proteins or antibodies may be promising approaches, administration of soluble ActRIIB receptors should be considered with caution. Side effects like skin telangiectasias and reversible nose bleedings have been reported in a clinical trial of the human ActRIIB-Fc ACE-301 in boys suffering from Duchenne muscular dystrophy (DMD), resulting in termination of the agents development [186].
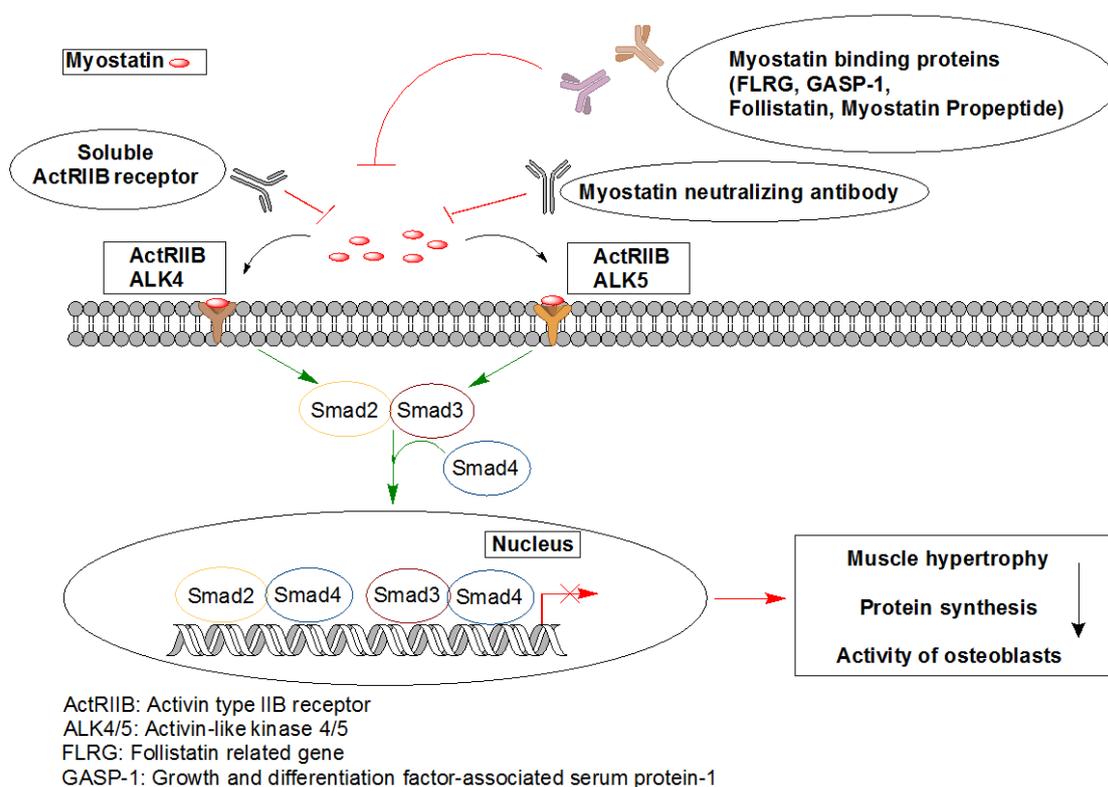

Figure 7. Myostatin signalling in muscle atrophy and possible therapeutic targets.

Furthermore, while studying the administration of an activin receptor fusion protein in a mouse model, the increase in muscle mass was associated with elevated serum levels of corticosterone and deteriorated hyperglycemia [190]. For the moment, monoclonal neutralizing antibodies targeting myostatin seem to be the most promising approach, with ongoing (PF-06252616, NCT02310763) and recently completed (LY2495655, NCT01524224) clinical trials (according to clinicaltrials.gov, accessed Nov 29th, 2017).

2.7. Future directions

Besides by small molecules and larger proteins like insulin and IGF-1, muscle composition can also be controlled by epigenetic regulation, which has gained the interest of researchers in recent years. Epigenetic regulation can be defined as heritable structural modifications of DNA and histones, resulting in modified gene activity without altering the DNA sequence [191]. Interestingly, in contrast to changes in the genetic code, changes in epigenetic structures show highly dynamic characteristics and seem to be directly influenced not only by environmental factors but also by life circumstances such as nutrition and physical activity. The most prominent epigenetic changes include DNA methylation/demethylation, histone acetylation/deacetylation and microRNA expression. DNA methylation by DNA methyltransferases within so-called CpG-islands, regions rich in cytosine-phosphate-guanine (CpG) dinucleotides, results in decreased gene-expression while demethylation allows gene activity [192]. Acetylation of lysine residues of the very basic histone proteins by histone acetyltransferases (HAT) decreases their alkaline properties and lowers their affinity to the acidic DNA strands, thus allowing proteins of the transcriptional machinery to gain access to the DNA. Conversely, deacetylation by histone deacetylases (HDACs) leads to gene silencing by providing a tightly packed chromatin structure. Last but not least, microRNAs are short single-strand RNA molecules, which can suppress the post-transcriptional processing of targeted mRNAs by binding to specific sequences in their untranslated regions [193]. It has to be noted, that one microRNA can target many different mRNAs and mRNAs can be susceptible to various microRNAs. In recent time, the epigenetic regulation of genes involved in muscle generation, differentiation and maintenance has been identified as important factor not only in embryonic myogenesis but also later in life. One example is the control of activity of muscle satellite cells, where differentiation processes have been reported to be repressed by high DNA methylation [191]. Furthermore, high activation of HATs and simultaneous inactivation of HDACs in undifferentiated myoblasts, allowing access of transcription factors for further differentiation, has been described [191]. Further information on current knowledge on the mechanisms of these DNA and histone modifications as well as thorough descriptions of the relevant enzymes involved is reviewed by Sincennes et al. [194]. In this review, the authors also mention the potential of HDAC inhibitors as therapeutic agents against muscular dystrophy. Amongst other factors, the relevant effect is supposed to result from an increased expression of follistatin after administration of HDAC inhibitors. As potent inhibitor of myostatin and other members of the TGF-β superfamily, follistatin has a direct role in compensating muscular loss. Therefore, HDAC inhibitors enter at least in part the same mechanistic pathway as myostatin inhibitors (2.6). At the moment, three clinical trials with the HDAC inhibitor givinostat are in the recruitment phase: One phase-II study for the treatment of Becker muscular dystrophy (NCT03238235) and two phase-III studies focusing on patients with DMD (NCT02851797, NCT03373968) (according to clinicaltrials.gov, accessed Mar 18th, 2018).

Another phase-II study with givinostat with patients suffering from DMD has been completed in November 2017, with results remaining to be published (NCT01761292). Finally, some miRNAs are widely expressed in muscle tissue where they are supposed to be of high importance during muscle development. The probably most relevant miRNAs in this context are miR-1, miR-133 and miR-206 [195]. While miR-1 and miR-206 are supposed to facilitate myoblast-to-myotube differentiation in skeletal muscle, miR-133 seems to promote myoblast proliferation and inhibit differentiation [191]. Further insight into the mechanisms of these miRNAs can be found in the articles by Townley-Tilson et al. [195], Proctor et al. [196] and Yu et al. [197]. There are many other tissue-specific and non-tissue-specific miRNAs involved in muscle regulation. Although the current insight into their respective role and importance is limited due to the lack of human studies, designing specific miRNAs could be a promising approach for therapeutic use after further research has been conducted.

3.   Conclusions

The substance classes discussed in this review are considered to activate several different signalling cascades, which can generally be divided in two groups: Those, which cause long-term changes and need hours to days until changes in tissue emerge, and those who mediate so-called rapid actions, with responses typically being detectable after seconds to minutes. It was observed, that the actions caused by a certain drug type normally cannot be assigned to one of these groups only. It has to be considered, that most substances affect tissue through multiple pathways, although differing in the extent to which they do so. Moreover, rapid actions may also lead to change of transcriptional activity. This happens, when second messenger molecules, which have been generated through rapid actions, stimulate transcription factors, which subsequently mediate changes in gene expression [5]. One example is the activation of the transcription factor Elk-1 through stimulated ERK1/2, which itself is considered to be activated via non-genomic signalling [5]. Another observation to be considered is that even though different substance classes bind to different receptors, they often lead to similar signalling pathways and downstream targets. For instance, the PI3K/Akt pathway, promoting protein synthesis and inhibiting protein degradation, is reported to be activated by ER-β and β_2-adrenergic agonists, IGF-1, insulin and indirectly through GH. PI3K activation through non-genomic actions of AAS, resulting in phosphorylation of the PI3K-p85 subunit by AR is also proposed [5]. Besides this, the MAPK pathway is supposed to not only be activated via non-genomic action of AAS but also through IGF-1 stimulation. In addition to that, the signalling pathways activated by one drug can also lead to activation of such downstream targets, that are normally "main-targets" of other substance types: For instance, it has been reported, that non-genomic actions of iAR can lead to increased GnRH production in the hypothalamus which in turn increases GH and IGF-1 production [9,10]. Thus, IGF-1 dependent mechanisms may indirectly be activated by administration of AAS and ER-β agonists.

Even though several signalling mechanisms have already been elucidated to great extent, there are still areas, where more research is strongly needed. Such research for instance could focus on the role of the calcineurin pathway in cells stimulated by IGF-1 or the mechanisms by which AAS influence tissues via calcium dependent non-genomic actions. Better insights into the biochemical mechanisms facilitating muscle hypertrophy may help to predict desired and adverse effects of already known but also newly emerging drugs. This will not only help to reduce the risk for athletes, which illegally use

anabolic substances to enhance their performance, and anti-doping laboratories to quickly adapt their analytical methods for detection of potentially misused drugs, but also to find possible drugs for treating cachexia and muscle atrophy while minimizing the risk of adverse effects at the same time.

With the current knowledge on different substance classes, ER-β agonists, SARMs, myostatin and HDAC inhibitors appear as most promising candidates for therapeutic use. However, finally it has to be mentioned that pharmacological intervention with the intention of performance enhancement in sports is considered as unethical and against a fair and clean competition.

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Conflict of interest

The authors declare no conflict of interest.

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