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REVIEW

Steroid Conjugates and Their Physiological Role

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Summary
While there are hundreds of synthetic steroids conjugates with acids, sugars, proteins and other molecules, only two types of conjugates occur in living organisms, namely sulfates and glucuronides. Steroid glucuronidation in the human liver is the main mechanism controlling the levels and biological activity of unconjugated hormones, and glucuronides are their main excretion products. This process is generally irreversible. On the other hand, sulfates possess their own biological activity that differs from that of the unconjugated steroid, emphasizing the importance of steroid sulfatases and sulfotransferases. Due to their negative charge, steroid sulfates cannot cross the blood-cell barrier and have to use transporters. Their efflux is mediated by specific transporters of the ATP binding cassette protein group, which thus are further factors controlling their physiological effects. Steroid sulfates, especially dehydroepiandrosterone sulfate (DHEAS) are neuroactive steroids, with well-known effects as allosteric modulators of some neurotransmitter receptors, functioning as ion channels, such as gamma-aminobutyric acid, type A ($GABA_A$) receptors or N-methyl-D-aspartate (NMDA) receptors. In this minireview, we highlight some recent findings of non-genomic steroid sulfate actions through specific G-protein coupled receptors (GPCR), which we believe show the way of further research. A few studies have even indicated that sulfates such as DHEAS may even indirectly regulate gene expression via ligand binding to the membrane receptor and, through G-protein and second messenger formation, activate proteins like cAMP Regulated Elements Binding protein (CREB), which then binds to regulated DNA elements of the expressed gene, in a "classical" genomic effect.

Keywords
Steroid • Conjugate • Sulfates • Glucuronides • Action

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Introduction

Steroid conjugates are generally dual compounds of steroids with other molecules. Since the recognition of the multifunctional properties of steroids, especially those that are hormonally active, along with their therapeutic potential, a vast number of steroid conjugates have been synthesized. These conjugates were often designed to facilitate the transport of steroids to target sites such as steroid receptors in order to prolong their biological time in the organism and affect their metabolism. Thanks to the presence of easily derivatized oxygen groups, thousands of conjugates were prepared with amino acids, various sugars, proteins and other molecules [1]. Steroid conjugates have also been prepared with haptens to be used as immunogens for immunoassays [2].

In contrast to synthetic conjugates, there are only two types of steroid conjugates occurring in living organisms, namely with sulfuric acid (sulfates) or conjugated with glucuronic acid (glucuronides). In the past conjugated steroids were believed to be primarily excretion products, but newer investigations have shown that they possess their own biological activities, differing from free hormones and their metabolites. This is especially true of steroid sulfates, since the main role of glucuronides remains in controlling the actual levels of biologically active steroids [3]. However, the importance of glucuronidation enzymes and changes in their activity during human development are also key issues [4].

Advances in analytical techniques enabling the determination of steroid sulfates in various biological fluids (see e.g.[5]) revealed associations with various pathologies, first of all in the brain [6-8]. This led to detailed investigations of sulfation enzymes [9] and of the

molecular mechanisms of their action. One of the most interesting recent findings has been the discovery of a non-genomic action of DHEAS through a membrane G-protein bound receptor (see further). These effects, mostly based on rodent studies, have been detected not only in the central nervous system but also in many other tissues [10].

Until the introduction of immunoassays and more advance chromatographic techniques in the late sixties, the only available analytes were urinary steroid conjugates, the concentration of which gave little insights into their levels in biological fluids (blood plasma, interstitial fluid, cerebrospinal fluid) or concentrations in tissues and cell compartments. Modern analytical approaches based on a combination of advanced chromatographic techniques in tandem with mass spectrometry have enabled us to simultaneously measure unconjugated steroids and their conjugates in multiple biological materials, shining new light on their importance and action.

Steroid glucuronides

Biosynthesis and transport of steroid glucuronosides

Steroid glucuronidation is the main process controlling the levels and the biological activity of unconjugated hormones. Glucuronidation of hormonal steroids and their catabolites consists in the addition of a polar glucuronosyl group from uridine-5′-diphosphoglucuronic acid (UDPGA) to the hydroxy groups of steroids. This reaction takes place primarily in the liver, but also occurs in other steroid metabolizing tissues such as the kidney, skin, uterus, mammary gland, prostate and even in the brain [3,11]. The respective enzymes are members of the uridine-diphosphate glucuronosyltransferase (UGT) family, consisting of products of as many as 24 human genes divided in two families, UGT1 and UGT2, based on sequence homology. They differ in substrate selectivity and chromosome location [11]. As for the steroids, the most important are UGT2B7, UGT2B15 and UGT2B17, with large differences in UGT2B polymorphisms between Asians and Caucasians [12]. The glucuronidation of steroids in the human body is generally thought to be irreversible, with the exception of β-glucuronidase activity in certain gut bacteria [13]. Various drugs known to act as inhibitors or activators of steroid biosynthetic and metabolizing enzymes were tested as for their effect on steroid glucuronidation, see e.g. [14,15].

As hydrophilic compounds, glucuronides cannot freely cross the cell membrane, and thus their efflux from cells in which they are synthesized is provided by transporter proteins such as multidrug resistance protein 4, belonging to the large family of ATP-binding cassette transporters (ABC) responsible for pumping a wide variety of organic anionic compounds out of the cell [16,17]. Related to their metabolic role in the inactivation of androgens, the glucuronidation of these steroids is also an important mechanism in androgen deprivation therapy for prostate cancer [18,19].

Steroid glucuronides in physiology and pathophysiology

Glucuronidation of various substrates by UDP glucuronyl transferases is a common mechanism of elimination/detoxication of many biologically active molecules as hormones including steroids or drugs. One of especial role of these enzymes is metabolic inactivation of compounds used in drug therapy of cancer [19]. In males androsterone glucuronide and its 5 α-reduced metabolite sandrostane-3α,17β-diol glucuronide reflect the rise in testosterone during puberty showing that glucuronidation is a major pathway of steroid metabolism in steroid target tissues [4]. It is also the important further mechanism of androgen deprivation therapy [18]. From the point of view of their therapeutic application, of interest are thermogenic 7-oxygenated derivatives of DHEA inducing liver thermogenic enzymes. In order to obtain their more active, longer lasting forms, glucuronides and other derivatives of 7-oxo- and 7-hydroxy steroids have been prepared and tested [20]. With respect to importance of glucuronosidation as a deactivation/detoxification pathway in steroid (and not only steroid) metabolism, attempts were performed to find out a suitable probe which would overcome overlapping substrate specificity of (UDP)-glucurosyltransferase enzymes. Desacetyl-cinobufagin (DACB) containing a steroid skeleton wih hydroxygroups at various postitions were found to be a suitable compound for such purpose [21].

Steroid glucuronides as such, however are involved in various physiological/pathological processes and may serve as biochemical markers. One of such marker is already mentioned 5α-androstane-3α,17β-diol glucuronide, a dihydrotestosterone-reduced metabolite, which is markedly enhanced in women with idiopathic hirsutism due to increased 5α-reductase activity [22].

Steroid sulfates

Biosynthesis and transport of steroid sulfates

Recent reviews exist on steroid sulfation/desulfation and their physiological role [8,21]. Here we provide only a brief summary, while in the next text we would like to highlight newly revealed hormonal actions, focused on steroid sulfates.

The main sites of steroid sulfation are inner zones of the adrenal cortex, but sulfation occurs in many other tissues such as the liver, colon, kidney, breast, ovary, testis, and prostate [23]. Of particular importance is the brain, representing a unique site of action for some steroid sulfates. Steroid sulfation proceeds in two steps:

1. Activation of the sulfate group using the common coenzyme 3´phosphoadenosine-5´-phosphosulfate (PAPS) by PAPS synthase. Sulfate is an obligatory nutrient obtained from food and drinking water, taken up from the gut by several sulfate transporters.
2. Transfer of the activated sulfate on a hydroxyl group of the steroid by sulfotransferase (SULT) Both sulfotransferases and sulfatases are broad families of enzymes differing in substrate specificity, cellular and tissue localization; see [23,24].

Like glucuronides, sulfated steroids cannot pass through the cell membranes due to their negative charge at physiological pH, and to cross the blood-cell barrier and have to use transporters. Their efflux is mediated by specific efflux transporters (ATP binding cassette protein transporters (ABCs)). Their role is especially interesting in tissues where steroid sulfates act as signal molecules, such as in the brain or testis (see further). Another interesting mechanism how DHEAS may influence the transport of molecules including steroid sulfates themselves: DHEAS may stimulate the expression of tight junction proteins through a putative DHEAS membrane-bound G-protein-coupled receptor [25].

Ionotropic action of steroid sulfates

The nongenomic, ionotropic actions of steroid sulfates, in particular but not only DHEAS, are well-known, especially their effects in the brain. In brief, steroid sulfates bind to allosteric binding sites of various neurotransmitter receptors and thus modulate their function as ion or voltage-gated channels. These binding sites differ from those of unconjugated steroids and their effects are also different, often opposite (positive/negative). This influences the ion entry into the cell and finally the neural signal. The balance between sulfated and desulfated steroids is thus an important factor for regulating neural function and stresses the role of *in situ* sulfotransferases and sulfatases. As steroid sulfates are concerned, the most important are glutamate receptors, namely the N-methyl-D-aspartate (NMDA) receptors, serving as a calcium voltage-gated channel, and gamma-aminobutyric acid, type A ($GABA_A$) receptors, functioning as a chloride channel [8,26,27,28]. Among naturally occurring steroid sulfates let us mention pregnenolone sulfate, a potent endogenous NMDA agonist [29]. NMDA and $GABA_A$ are by far not the only neurotransmitter receptors allosterically binding steroids and their sulfates, and a list can be found in Ref [8]. It concerns also saturated naturally occurring neurosteroids as pregnanolone and its sulfate, which potentiate glutamate release downstream of presynaptic Ca^{2+} influx [30].

Non genomic action of steroid sulfates

Among more recent findings belong the discovery of steroid sulfate actions through specific G-protein coupled receptors (GPCR), with binding to putative membrane receptors triggering or inhibiting various signalling pathways. It should be said however, that no membrane-bound non-classical receptors have not been identified thus far [7]. On the other end, several reports show that DHEAS is capable to initiate such effects. That DHEAS and not DHEA was responsible for these effects was indirectly evidenced by the fact that inhibitors of sulfatase did not influence them. Similarly abrogation of G-protein prevented the effect of DHEAS [7]). Non genomic effect of DHEAS were thoroughly studied in rodent gonads. in murine spermatic cells DHEAS induced significant phosphorylation (activation) of the extracellular signal-regulated kinases (ERKs) or classical MAP kinases absence of sulfatase (see Papadopoulos *et al.* [7]) and the literature therein. Another example of a non-genomic nature utilizing G-proteins for mediating the functional link between the membrane bound ligand-receptor complex and intracellular effector was demonstrated by degranulation of mast cell line RBL-2H3 using the Gq/11 protein-coupled neurosteroid receptor and phospholipase C as an effector. The inhibitors of the latter enzyme completely blocked the effects [31]. The examples of non-genomic effects in various human and animal model systems and cell preparations of DHEAS may be also found in the review of Clark *et al.* 2018 [10].

Steroid sulfate actions via membrane-G-protein- linked mechanism resulting in regulation of gene expresssion

Unconjugated DHEA, in contrast to DHEAS, may bind to some nuclear receptors including sex steroid- and retinoid receptors, and is responsible for their genomic action. The affinities are very low, however, on the order hundreds of nM or μM, and the physiological importance is negligible. A few studies, however, have indicated that sulfates, namely DHEAS, may regulate gene expression indirectly. This "mixed" mechanism begins by binding of the ligand to the membrane receptor and through G-protein and a second messenger formation triggers a cascade of events, leading to activation (e.g. phosphorylation) of a protein like cAMP Regulated Elements Binding protein (CREB), which is then bound to respective regulated DNA elements of the expressed gene, as in the "classical" genomic effect [7]. Let us mention here the already-cited expression of tight junction proteins claudin 3 and 5 forming a blood-testis barrier by DHEAS, which may be considered a kind of a feed-back mechanism since it regulates the entry of the steroid into the cells. Another example is the effect of DHEAS on the expression of transcription factors involved in spermatogenesis in the mouse line GC-2 [32]. The latter authors established a research group called "Sulfated Steroids in Reproduction" to investigate the biological significance of sulfated steroid hormones for reproductive processes in humans and animals, under the German Research Foundation DFG (FOR1369), and interesting new results may be expected [33]. Blood-testis barrier is not only example where DHEAS stimulates expression of tight junctions proteins: DHEAS used this "mixed mechanism" also by stimulation of expression of claudin in endothelial cells forming blood-brain barrier in mouse [34].

Steroid sulfates in physiology and pathophysiology

Neuroprotective effects of steroids including their sulfates as supporting neurogenesis, neuronal survival, and their overall positive effects on memory, and, on the other hand, reducing apoptosis and oxidative stress, inspired studies of their concentrations in patients with most common neurodegenerative disorders as Alzheimer dementia (AD), Parkinson´s disease (PD) and multiple sclerosis (MS) [35,36]. Among potentially neuroprotective steroids sulfates belong among others above mentioned saturated pregnanolone and its sulfate [30,37]. Circulating levels of sulfated steroids are result of interplay between sulfotransferases and sulfatase activities. They may differ from their actual tissue concentrations due to existence of blood-tissue barriers. It concerns primarily the brain. Better insight to actual situation provide data of sulphated steroids in cerebrospinal fluid.

Several studies dealt with assessment of DHEAS and other sulphated steroids in patient suffering from the above diseases, in view to use them as biomarkers for the early detection of the disease and follow up of its progression. The main results have been summarized in our recent review [8]. In summary, the levels of sulphated steroids in AD and PD were reduced in comparison with otherwise healthy subjects, while ambiguous data were obtained in MS. The similar results were obtained from cadaverous tissues from brain autopsies

Conclusion

Glucuronides are the final degradation product of hormonal steroids, and glucuronidation is the basic process maintaining a balance between biologically active and inactive steroids. Sulfates, on the other hand, serve as a pool for biologically active steroids, but also possess their own biological function. Their effects as neurosteroids are well-known, modulating the entry of ions into neurons through their allosteric binding sites on neurotransmitter receptors. A number of reports and reviews have dealt with changes of neurosteroids in neuropsychiatric disorders. In this minireview, we highlight some newer findings on the actions of steroid sulfates on both the non-genomic and genomic levels, which we hope may pave the way for further research.

Conflict of Interest

There is no conflict of interest.

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