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REVIEW

Unraveling the mechanisms underlying the rapid vascular effects of steroids: sorting out the receptors and the pathways

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Aldosterone, oestrogens and other vasoactive steroids are important physiological and pathophysiological regulators of cardiovascular and metabolic function. The traditional view of the cardiovascular actions of these vasoactive steroids has focused on their roles as regulators of transcription via activation of their 'classical' receptors [mineralocorticoid receptors (MR) and oestrogen receptors (ER)]. However, based on a series of observations going back more than half a century, scientists have speculated that a range of steroids, including oestrogen and aldosterone, might have effects on regulation of smooth muscle contractility, cell growth and differentiation that are too rapid to be accounted for by transcriptional regulation. Recent studies performed in our laboratories (and those of others) have begun to elucidate the mechanism of rapid steroid-mediated cardiometabolic regulation. GPR30, now designated as GPER-1 (http://www.iuphar-db.org/DATABASE/FamilyIntroductionForward?familyId=22), a newly characterized 'orphan receptor', has been implicated in mediating the rapid effects of estradiol and most recently those of aldosterone. Studies to date have taught us that to understand the rapid vascular mechanisms of steroids, one must (i) know which vascular 'compartment' the steroid is acting; (ii) know which receptor the steroid hormone is activating; and (iii) not assume the receptor specificity of a steroid receptor ligand based *solely* on its selectivity for its traditional 'transcriptional' steroid receptor. Our newfound appreciation of the rapid effects of steroids such as aldosterone and oestrogens opens up a new vista for advancing our understanding of the biology and pathobiology of vascular regulation.

LINKED ARTICLES

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Vasoactive steroid hormones such as aldosterone and oestrogens have been increasingly appreciated as important physiological and pathophysiological regulators of cardiovascular functions. These (and other) steroid hormones were thought to associate with ligand-specific nuclear receptors and function as transcriptional regulators. However, it is now known that steroid hormones can mediate their actions both via 'classical' transcriptional mechanisms as well as via 'rapid' (previously denoted as non-genomic) mechanisms, and that some of these steroid hormones can interact with multiple receptor types. The purpose of this brief review is to provide an update on advancing knowledge regarding the receptor basis of the rapid vascular effects of steroids and their importance physiologically and potentially pathophysiologically.

What is the basis for the accelerating interest in elucidating how steroid hormones like estradiol and aldosterone mediate their cardiovascular effects?

A cardioprotective role of oestrogens has been long surmised – based on the observed lower rates of cardiovascular disease in premenopausal women compared with men and the rapid rise postmenopausal rise in cardiovascular disease prevalence – which was suggested to be preventable by postmenopausal oestrogen replacement. Indeed, the early observational studies such as the Nurses' Health Study suggested that oestrogen hormone replacement therapy was associated with a reduction in cardiovascular disease in postmenopausal women, suggesting the potentially 'beneficial' cardiovascular effects of oestrogen (Miller and Duckles, 2008). However, subsequent randomized clinical trials such as the Women Health Initiative (WHI) (Rossouw *et al.*, 2002) and the Heart and Estrogen/Progestin Replacement Study (HERS) (Grady *et al.*, 2002) demonstrated no benefit of postmenopausal oestrogen replacement and a trend towards increased thrombotic events. These findings led to the speculation that oestrogens might have competing cardiovascular effects – both beneficial and detrimental. These findings have intensified the efforts to elucidate the range of cardiovascular effects mediated by estrogens. For example, oestrogen has been shown to be important in the regulation of cardiac hypertrophy (Pelzer *et al.*, 1997). In addition, oestrogen has been shown to regulate endothelial vasodilator function, promote angiogenesis and modulate autonomic function (Miller and Duckles, 2008).

For aldosterone, several clinical trials uncovered evidence of its effects not anticipated by actions mediated via their 'classical renal-specific transcriptional pathways'. For example, the Randomized Aldactone Evaluation Study (RALES) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) studies demonstrated the 'positive' effects of aldosterone receptor *antagonists* in patients with congestive heart failure or following myocardial infarction (Pitt *et al.*, 1999; 2003). These findings suggested that by inference, aldosterone must have been mediating detrimental cardiovascular effects – beyond its effects on renal sodium balance. Over the past decade, these clinical trial findings have stimulated an accelerating effort to elucidate the non-renal effects of aldosterone and the impact of these mechanisms in the development of cardiovascular disease. Effects of aldosterone on cardiac hypertrophy (Duprez *et al.*, 1993) and on cardiac and vascular fibrosis (Brilla *et al.*, 1990; Weber and Brilla, 1991; Duprez *et al.*, 1998) have been reported. Similarly, aldosterone has been shown to regulate vascular reactivity (Liu *et al.*, 2003), single cell contractility (Gros *et al.*, 2007) and growth regulatory mechanisms (Liu *et al.*, 2003) (see below).

Rapid vascular effects of steroids

The traditional view of genomic actions of the vasoactive steroids, like aldosterone and oestrogen, is that these hormones bind to hormone-specific receptors such as the mineralocorticoid and oestrogen receptors (MR and ERs, respectively), which acted as intracellular transcription factors that increased or decreased the expression of target genes. However, a growing body of experimental evidence supports the importance of effects of these steroids over a time course too rapid to be explained by their classic 'transcriptional' actions. These so-called 'non-genomic' steroid effects were primarily characterized by their rapid onset of action, usually within seconds to minutes, and/or the by lack of effect of transcription and protein synthesis inhibitors (Levin, 2008; Vinson and Coghlan, 2010; Wendler *et al.*, 2010). In the remainder of this review, we will focus on three key considerations in order to gain a better understanding of the rapid vascular effects of steroid hormones. In particular, we will focus on what we see as the key learnings, viz., that to understand the mechanisms of rapid steroid effects in the vasculature, (i) one needs to know in which vascular 'compartment' the steroid is acting; (ii) one needs to know which receptor the steroid hormone is activating; and (iii) one cannot assume the receptor specificity of a steroid receptor ligand based *solely* on its selectivity for their traditional 'transcriptional' steroid receptors.

The importance of knowing which vascular cell type is the target for mediating the actions of any specific steroid hormone

For more than 50 years, it has been speculated that a number of steroids including aldosterone and oestrogen might have rapid effects contractile reactivity and regulation of cell growth/death pathways (Streeten *et al.*, 1957; Klein and Henk, 1963; Szego and Davis, 1967; Pietras and Szego, 1975, 1977). However, until recently there was wide-spread scepticism regarding the significance of these rapid effects – in part based on what was viewed as the contradictory findings of the published studies. The initial report of the rapid effects of aldosterone was made by David Streeten and colleagues in 1957 (Streeten *et al.*, 1957). These rapid effects studied in intestinal smooth muscle were constrictor in nature. Subsequently, rapid vascular actions of aldosterone on reactivity were studied in a range of vascular beds and species. These were variably characterized as vasodilator (Schmidt *et al.*, 2001), vasoconstrictor (Wehling *et al.*, 1998; Schmidt *et al.*, 1999; Farquharson and Struthers, 2002; Arima *et al.*, 2003; Romagni *et al.*, 2003) and/or without effects (Gunaruwan *et al.*, 2002).

In retrospect, the confusion raised by the directionally divergent findings in these studies of the effects of aldosterone was undoubtedly related to the failure to appreciate that the overall vascular action of aldosterone was dependent on the vascular cell it was acting on (i.e. endothelial vs. smooth muscle cell) and perhaps dependent on the receptor upon which it was acting (i.e. mineralocorticoid receptor vs. GPER-1; see below). The variation in aldosterone-mediated contractile response dependent on the cell type being regulated is reminiscent of the effects of acetylcholine in endothelium-intact versus endothelium-denuded models – a

divergence in directionality of effects that led to the discovery of the nitric oxide (NO) pathway of vascular regulation. Similar to the effects of acetylcholine, we now appreciate that aldosterone regulates vascular reactivity via acting on different vascular 'compartments' (i.e. endothelium vs. media). In our initial studies, using endothelium-intact rat aortic rings, we demonstrated that aldosterone attenuated alpha adrenergic-mediated vasoconstrictor responses (Liu *et al.*, 2003). These effects paralleled potent stimulation of NO synthase (NOS) activity via a phosphoinositide 3 (PI3) kinase-dependent pathway. However, removal of the endothelium had the completely opposite effect where aldosterone *enhanced* alpha adrenergic-mediated vasoconstriction. Subsequent studies in isolated vascular smooth muscle cells demonstrated that this vasoconstrictor effect paralleled a rapid enhancement of myosin light chain phosphorylation, which also occurred via a PI3 kinase-dependent pathway (Gros *et al.*, 2007). Similarly in humans, aldosterone caused vasodilation as assessed by changes in forearm blood flow measured by venous occlusion plethysmography. However, the directionality of this effect reversed (i.e. aldosterone became a vasoconstrictor hormone decreasing forearm blood flow) following inhibition of NOS with L-NG-nitroarginine methyl ester (Schmidt *et al.*, 2003). Notably, in the setting where aldosterone's overall effect in intact vessels is vasoconstrictor (cf. the studies of Arima and colleagues in rabbit preglomerular afferent arteriole assessed by microperfusion techniques; Arima *et al.*, 2004), inhibition of endothelial NOS activity enhanced the potency of aldosterone's vasoconstrictor effects. These findings were interpreted as implying that even in vascular beds where aldosterone acted (in aggregate) as a vasoconstrictor, that an endothelium-mediated vasodilator effect of aldosterone could still be detected. Thus, the net effect of aldosterone on vascular reactivity appears to be highly dependent on the balance between its endothelial-dependent vasodilatory effects and its vascular smooth muscle-dependent vasoconstrictor effects. Interestingly both vasodilator and vasoconstrictor components are commonly dependent on PI3 kinase activation. How this balance is shifted with vascular disease and aging has yet to be clarified. However, given the opposing effect of PI3 kinase activation (Budzyn *et al.*, 2005) on vascular reactivity in endothelial (vasodilator) versus smooth muscle (vasoconstrictor) cells, one might speculate that with endothelial dysfunction aldosterone's net effects on vascular reactivity would shift towards the vasoconstrictor side of the balance.

The importance of knowing which receptor a steroid hormone is activating

For the steroid hormone oestrogen and aldosterone, many of their genomic 'transcriptional' actions are known to occur via the interactions with their respective 'classical' steroid receptors, the ERs and MR (Miller and Duckles, 2008). Most of the effects of oestrogen are adequately explained by the activation of classical cytoplasmic/nuclear oestrogen receptors, which act as transcription factors. These ERs (ER_α, ER_β and ER_γ) are all members of the steroid hormone nuclear receptor subfamily. The binding of these receptor complexes with oestrogen response elements regulates the target genes mediating the many of the reproductive, developmental, behavioural, skeletal, neural and cardiovascular effects of steroids. The 'transcriptional' effects of oestrogen have been linked for the most part to the activation of the 'classical' oestrogen receptor activation. However, the role of ERs in mediating the rapid (non-genomic) effects of oestrogen is less certain. A number of intracellular mechanisms have now been identified to be mediated by so-called non-genomic (also called rapid) effects of oestrogen including activation of mitogen-activated protein, raf, cSRC kinases as well protein kinase A (PKA), PI3 kinase, NOS and many others important in cardiovascular regulation (Miller and Duckles, 2008). Although these rapid effects have in part been linked to 'traditional' oestrogen receptor activation, a previously unappreciated G protein coupled receptor (GPCR), GPER-1, has been suggested to be the (or at least a) principal actor in mediating the rapid effects of oestrogens via 'non-classical' receptor systems (Revankar *et al.*, 2005).

Many of the transcriptional events associated with the actions of aldosterone are related to MR activation, its consequent dissociation from its chaperone proteins, nuclear translocation and ultimate interaction with a range of molecular partners resulting in transcriptional regulation (Viengchareun *et al.*, 2007). However, like oestrogen, aldosterone has also been reported to stimulate a number of more rapid signalling pathways including calcium mobilization, cAMP generation, phosphoinositide hydrolysis, extracellular signal-regulated kinase (ERK) and cSRC activation (Falkenstein *et al.*, 2000; Grossmann *et al.*, 2005). These have been linked to both MR- and non-MR-dependent pathways (Grossmann *et al.*, 2005). Additionally, non-MR-dependent mechanisms have been linked to longer-term effects of aldosterone. For example, a non-mineralocorticoid receptor-mediated effect of aldosterone to increase elastogenesis has been described (Bunda *et al.*, 2009). This conclusion was based on the use of small interfering RNA-driven elimination of MR. The identity of this non-MR receptor was unknown. However, our recent studies (see below) have suggested that GPER-1 may have a role in at least some of these non-MR-dependent effects of aldosterone.

GPER-1 was cloned and reported independently by several different groups (Carmeci *et al.*, 1997; Takada *et al.*, 1997; O'Dowd *et al.*, 1998). GPER-1 remained an 'orphan GPCR' until recently, when a series of publications suggested that GPER-1 contributed to the rapid effects of oestrogen in several model systems (Filardo, 2002; Filardo and Thomas, 2005; Hasbi *et al.*, 2005; Revankar *et al.*, 2005; Vivacqua *et al.*, 2006a; Filardo *et al.*, 2007; Revankar *et al.*, 2007; Wang *et al.*, 2008). Some of these initial studies demonstrating the signalling of oestrogen through GPER-1 resulted in the renaming of the receptor from its original name, GPR30. GPER-1 is a widely expressed GPCR (expressed in heart, arteries, breast, lung, central nervous system and leukocytes) (Hasbi *et al.*, 2005) and is detectable by reverse transcription polymerase chain reaction in freshly isolated vascular smooth muscle cells as well as in vascular endothelial cells (Ding *et al.*, 2009; Gros *et al.*, 2011).

GPER-1 has been studied predominantly in the context of reproductive biology and cancer biology. Initial studies primarily focused on GPER-1's growth regulating effects (Ahola

et al., 2002; Filardo, 2002; Vivacqua *et al.*, 2006b; Albanito *et al.*, 2007), the impact of its expression on cancer survival (Filardo *et al.*, 2006; Vivacqua *et al.*, 2006b; Smith *et al.*, 2007) and GPER-1's actions on oocyte maturation (Kolkova *et al.*, 2010; Pang and Thomas, 2010; Wang *et al.*, 2007). Interestingly GPER-1 activation has been linked to either anti-proliferative/pro-apoptotic (Ariazi *et al.*, 2010; Chan *et al.*, 2010) or pro-proliferative/anti-apoptotic (He *et al.*, 2009; Lin *et al.*, 2009; Pandey *et al.*, 2009) consequences.

In the vasculature, GPER-1 is expressed in both veins and arteries (Haas *et al.*, 2007). GPER-1 has been demonstrated to mediate vasodilation in human and rat vessels and lowers blood pressure in normotensive rats (Haas *et al.*, 2009). Additionally, in various mouse knockout models, GPER-1 deletion has had differential effects ranging from sex-specific increased blood pressure (Martensson *et al.*, 2009) to no alteration in blood pressure (Haas *et al.*, 2009). Furthermore, common to the effects of oestrogen in vascular cells, the GPER-1 signalling pathway mediates rapid PI3 kinase activation and ERK activation as well as EGFR transactivation (Filardo and Thomas, 2005). Therefore, to further appreciate the rapid vascular actions of oestrogens, one would need to understand the nature of the balance between the classical ER and GPER-1 receptor pathways.

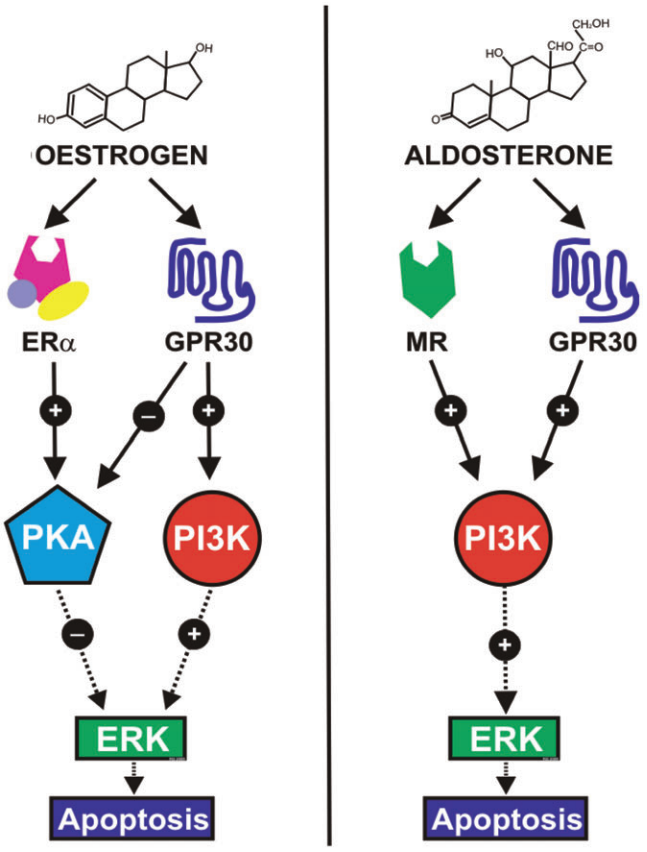

Figure 1

Schematic depiction of divergent pathway activation by oestrogen and aldosterone acting through ERα/MR and/or GPR30 (GPER-1)-linked pathways resulting in the activation or inhibition of ERK activity ERK-dependent apoptosis.

Studies from our laboratories have highlighted the role of GPER-1 versus ER in mediating a balance of opposing signal transduction pathways that can be concurrently activated by estradiol. We demonstrated the opposing actions of GPER-1 versus ER activation on the regulation of growth regulatory pathways in isolated rat vascular smooth muscle cells (Ding *et al.*, 2009) (Figure 1). The rapid effects of oestrogen acting via the classical ER pathway (ERα) were shown to be anti-apoptotic, mediating inhibition of ERK activity via PKA-dependent pathway. In contrast, activation of the GPER-1 pathway by oestrogen resulted in diametrically opposed effects (i.e. ERK activation and apoptosis). These actions were mediated by PI3 kinase activation and PKA inhibition via a G protein-dependent pathway. The rapid actions of oestrogens follow a recurring pattern in receptor biology, where one hormone, acting via 2 co-existing receptor systems, mediates opposing actions. These antithetical effects of estradiol might be viewed as analogous to the directionally divergent effects of catecholamines (i.e. being both vasodilator and vasoconstrictor) reported more than 100 years ago. This duality of function of catecholamines was subsequently understood appreciated to dependent in part on which adrenergic receptor the catecholamine was activating. The physiological and pathophysiological implications of these receptor-specific opposing effects of oestrogens have yet to be established. However, a shift in the balance between ER and GPER-1 receptor expression and/or action might be expected to significantly alter the pattern of oestrogen-mediated responses in the vasculature. Whether this is important in settings such as menopause, hypertension or atherosclerotic disease remains to be established.

The importance of NOT assuming the receptor specificity of a steroid receptor ligand based *solely* on its selectivity for its traditional 'transcriptional' steroid receptors

As discussed above, it is now understood that steroid hormones such as oestrogen can interact with many different receptors to mediate its effects (i.e. ER_α, ER_β and ER_γ and GPR30/GPER1). However, characterization of the selective effects of steroid receptor ligands (agonists and antagonists) has primarily been based on their relative selectivity for 'traditional' steroid receptors mediating 'transcriptional' actions. With the appreciation of the rapid pathways for steroid effects and the recognition of the existence of 'non-traditional' steroid receptors like GPER-1 (and perhaps others; Pi *et al.*, 2010), we will need to rethink the basis of our definitions of ligand–receptor selectivity.

Rethinking our view of agonist selectivity

The promiscuity of 'physiological' steroid hormones for multiple 'traditional' steroid receptors has long been appreciated. For example, corticosteroids are potent agonists for the MR (Sheppard and Funder, 1987), and given their much higher circulating concentrations (vs. aldosterone), are the dominant ligands directing MR activation in cells in the absence of

high levels of 11β-hydroxysteroid dehydrogenase activity (Molnar *et al.*, 2008). However, we are only beginning to appreciate that at least several of these ligands also interact with GPER-1. In a recent series of experiments, we demonstrated that in vascular smooth muscle cells aldosterone can utilize both GPER-1 and MR, leading to activation of signalling pathways such as PI3 kinase, ERK and subsequently apoptosis as well as myosin light chain phosphorylation. These GPER-1-dependent effects occurred at concentrations of aldosterone far less than those required for estradiol-mediated GPER-1 effects (pM vs. nM respectively). Thus, GPER-1 can no longer be considered solely an oestrogen-specific receptor (Gros *et al.*, 2011).

These studies raise important questions regarding the consideration of which hormones are most important for the activation of GPRER-1 physiologically (or pathobiologically) and the importance of sexual considerations in understanding its actions. In the setting where oestrogens were considered the principal regulator of GPER-1-mediated effects, the characterization of its vascular actions in rats studies almost exclusively restricted to male animals might have been viewed as problematic (cf. Ding *et al.*, 2009; Haas *et al.*, 2009). However, when the effect of GPER-1 activation has been examined in both males and females, as in the study of its cardioprotective effects, no sexual differences were seen (Deschamps and Murphy, 2009). Additionally, in our studies, the potency of aldosterone for activating GPER-1 was up to 1000-fold greater than that of estradiol. Thus, at least based on potency considerations and in the setting of comparable circulating hormone concentrations, aldosterone might well be the predominant ligand responsible for GPR30 activation. Because circulating aldosterone concentrations are not different in males and females (Miller *et al.*, 1999), sexual considerations relating to the actions of GPR30 may be somewhat less important than initially presumed. In contrast, sexual considerations would remain important in regards to the countervailing effects of ER receptor activation in mitigating the effects of GPER-1 activation (as discussed above).

Rethinking our view of antagonist selectivity

Many of the early studies characterizing the rapid actions of steroids utilized previously believed steroid receptor-specific antagonists. However, with the appreciation of the role of GPER-1, the selectivity of these agents has been questioned. Initial studies suggested the previously believed ER-specific antagonist, ICI-182780, could also act as a GPER-1 agonist (Revankar *et al.*, 2005; Meyer *et al.*, 2010), although these findings have not been universal (Gingerich *et al.*, 2010). Our recent studies using both heterologous expression and knockdown of steroid receptors have demonstrated that the previously believed MR-specific antagonist eplerenone could also act as a GPER-1 antagonist. In aggregate, these studies should rightly undermine our confidence in ascription of the receptor specificity of a steroid receptor ligand based solely on the use of pharmacological approaches (i.e. by the assessment of its modulatory effects on other steroid receptor agonists and antagonists). The characterization of receptor specificity of ligands should rather be based on a combination of pharmacological and genetic approaches, viz. by utilization of shRNA/knockdown and heterologous expression models.

Future perspectives

Our newfound appreciation of the rapid effects of steroids such as aldosterone and oestrogens opens up a new vista for advancing our understanding the biology and pathobiology of vascular regulation. However, the delineation of the receptor basis for these vascular effects is just beginning to be elucidated. As is usual in science, we have much more to learn. Delineating which steroids interact with which receptors in the vasculature and the functional consequences of these interactions will be expected to be an important step in our understanding of the role of these hormones physiologically and pathologically and the first step in developing novel therapeutic approaches to selectively modulate their pathobiological effects. Furthermore, determination of the importance of changing the balance of rapid steroid-mediated responses with aging, menopausal status and atherosclerotic disease/risk factors may be of critical importance in understanding the role of these systems in the development of vascular disease.

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