RESEARCH

20-Hydroxyecdysone activates the protective arm of the RAAS via the MAS receptor

René Lafont[1,2], Maria Serova[1], Blaise Didry-Barca[1], Sophie Raynal[1], Louis Guibout[1], Laurence Dinan[1], Stanislas Veillet[1], Mathilde Latil[1], Waly Dioh[1] and **Pierre J Dilda[1]**

[1]Biophytis, Sorbonne Université – BC9, Paris, France
[2]Sorbonne Université, CNRS -Institut de Biologie Paris Seine (BIOSIPE), Paris, France

Correspondence should be addressed to P J Dilda: pierre.dilda@biophytis.com

Abstract

20-Hydroxyecdysone (20E) is a steroid hormone that plays a key role in insect development through nuclear ecdysteroid receptors (EcR/RXR complex) and at least one membrane GPCR receptor (DopEcR). It also displays numerous pharmacological effects in mammals, where its mechanism of action is still debated, involving either an unidentified GPCR or the estrogen ERβ receptor. The goal of this study was to better understand 20E mechanism of action in mammals. A mouse myoblast cell line (C2C12) and the gene expression of myostatin (a negative regulator of muscle growth) were used as a reporter system of anabolic activity. Experiments using protein-bound 20E established the involvement of a membrane receptor. 20E-like effects were also observed with angiotensin(1–7), the endogenous ligand of MAS. Additionally, the effect on myostatin gene expression was abolished by Mas receptor knock-down using siRNA or pharmacological inhibitors. 17β-Estradiol (E2) also inhibited myostatin gene expression, but protein-bound E2 was inactive, and E2 activity was not abolished by angiotensin(1–7) antagonists. A mechanism involving cooperation between the MAS receptor and a membrane-bound palmitoylated estrogen receptor is proposed. The possibility to activate the MAS receptor with a safe steroid molecule is consistent with the pleiotropic pharmacological effects of ecdysteroids in mammals and, indeed, the proposed mechanism may explain the close similarity between the effects of angiotensin(1–7) and 20E. Our findings open up many possible therapeutic developments involving stimulation of the protective arm of the renin–angiotensin–aldosterone system (RAAS) with 20E.

Key Words

- 20-hydroxyecdysone (20E)
- MAS receptor
- renin–angiotensin–aldosterone system (RAAS)
- ecdysteroid
- G protein-coupled receptor (GPCR)
- estrogen
- muscle
- myoblast

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Introduction

Steroids in animal and plant kingdoms

Steroid hormones are (chole)sterol derivatives widespread in animals and plants, where they are involved in the control of many physiological processes. They include, for example, vertebrate sex hormones (progestagens, estrogens, androgens), insect moulting hormones (ecdysteroids), as well as plant growth hormones (brassinosteroids). The rigid carbon skeleton of sterols is particularly suitable to generate a very large number of derivatives, which differ in carbon number and/or the position of various substituents (mainly hydroxyl or keto groups) (Karlson 1983). In addition to hormones, sterols give rise to bile acids/alcohols, initially considered as emulsifiers facilitating lipid digestion but nowadays also known to be important signaling molecules acting on specific receptors (Chiang 2013).

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Diversity of steroid mechanisms of action

Our concepts of (steroid) hormone mechanism of action have evolved. In the classical scheme, steroid hormones interact with nuclear receptors and the complex formed regulates the transcriptional activity of target genes, the promoters of which contain specific sequences (hormone-responsive elements). However, steroids also act at cell membrane level, where they elicit rapid non-transcriptional effects. Among the identified steroid membrane receptors, we may mention vertebrate GPER1/GPR30 (a membrane estrogen receptor (Prossnitz *et al.* 2008)), TGR5 (a bile acid membrane receptor (Thomas *et al.* 2009)), MARRS (a calcitriol receptor (Khanal & Nemere 2007)) or drosophila DopEcR (a dopamine and ecdysteroid membrane receptor (Evans *et al.* 2014)). All these receptors belong to the family of GPCR/7TD receptors. Moreover, intact or truncated forms or the steroid nuclear receptors are bound to the plasma membrane and do not act there as transcription factors (Meitzen *et al.* 2013, Schreihofer *et al.* 2018).

Ecdysteroid effects on vertebrates

We are especially interested by the pharmacological effects of ecdysteroids on mammals. Ecdysteroids are a large family of steroids initially discovered in arthropods (zooecdysteroids) and later in plants (phytoecdysteroids) (Dinan 2009). They are present in many plant species where they can reach concentrations of up to 2–3% of the plant dry weight and where they are expected to protect plants against phytophagous insects.

With the prospect of using these molecules for crop protection, toxicological studies were performed on mammals, which unexpectedly concluded as to both their lack of toxicity (oral LD_{50} >9 g/kg) and their ‘beneficial’ effects, for example, anti-diabetic and anabolic properties (Dinan & Lafont 2006). Such effects have been linked with the presence of large amounts of phytoecdysteroids in several plants used worldwide in traditional medicine. Up to now, numerous positive effects have been reported, such that ecdysteroids can be considered as some kind of ‘universal remedy’ (Sláma & Lafont 1995). While many effects have been described on animals (Sláma & Lafont 1995, Báthori *et al.* 2008, Seidlova-Wuttke *et al.* 2010), the clinical evidence for 20E effectiveness in humans remains limited at the moment (Simakin *et al.* 1988, Wuttke & Seidlova-Wuttke 2015, Isenmann *et al.* 2019).

How do ecdysteroids work?

In spite of more than 40 years of research, the mechanism(s) of action of these molecules in mammals/humans has not been clearly elucidated, as diverging hypotheses are currently available. Several studies favor an action on membranes through a GPCR receptor (Gorelick-Feldman *et al.* 2010), whereas others suggest the involvement of a nuclear receptor, the estrogen receptor ERβ (Parr *et al.* 2014, Parr & Müller-Schöll 2018).

There is in fact no direct evidence for the binding of 20E to nuclear estrogen (or androgen) receptors (Báthori *et al.* 2008, Seidlova-Wuttke *et al.* 2010). The evidence of ERβ involvement in 20E action is based on the use of specific pharmacological activators or inhibitors of ERs, the former being able to mimic and the latter to inhibit the effects of 20E on target cells such as osteoblasts (Gao *et al.* 2008) or myoblasts (Parr *et al.* 2014). These studies, however, do not provide proof for direct 20E binding, as ER receptor could be activated indirectly, and even in the absence of ligand, for example, by phosphorylation (Sanchez *et al.* 2010). Some supporting evidence for direct binding was provided by *in silico* modeling (Parr *et al.* 2015), but this certainly does not represent a definite proof, as the result may strongly depend on the model used, and an opposite conclusion was drawn by Lapenna *et al.* (2015).

Evidence for membrane effects of 20E is based on early studies showing the rapid modulation of several second messengers (cAMP, cGMP, IP3, DAG, Ca^{2+}) in target cells (Kotsyuruba *et al.* 1995, 1998, 1999) and on the fact that 20E bound to metallic nanoparticles, preventing its entrance in target cells, is still active (Mykhaylyk *et al.* 2001). More recently, Gorelick-Feldman *et al.* (2010) used a pharmacological approach with various inhibitors (e.g. pertussis toxin). They concluded that the membrane 20E receptor belongs to the GPCR family and proposed a mechanism of transduction involving an unidentified GPCR and a membrane calcium channel (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

The above pharmacological arguments appear strong enough to consider that the cell membrane is (maybe not exclusively) a site of action of 20E in mammalian cells. The present experiments have been undertaken in an attempt to identify the/one GPCR involved in 20E effects and to understand its estrogen-like effects using gene silencing or different pharmacological approaches.

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Materials and methods

Chemicals

Except where otherwise mentioned, all the reagents and chemicals were from Sigma. Peptides such as angiotensin(1–7) and A779 (Asp-Arg-Val-Tyr-Ile-His-D-Ala) were custom-prepared by the IBPS Peptide Synthesis Platform (Sorbonne University, Paris, France). 20-Hydroxyecdysone (20E) was obtained from Chemieliva Pharmaceutical (Chongqing, China) or from Patheon (Regensburg, Germany) and had a purity of 96.5–97%.

Preparation of HSA-conjugated 20-hydroxyecdysone

22-Succinyl-20E was prepared according to Dinan *et al.* (2003). Coupling the 20E derivative to human serum albumin (HSA) was performed either with the Pierce Protein Coupling Kit (Product 77672, Thermo Scientific, using 1 mg 22-succinyl-20E and 10 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as coupling reagent per milligram HSA with a reaction time of 2 h at RT; batch 1) or according to a method provided by Dr J-P Delbecque (J-P Delbecque and M de Reggi, personal communication; batch 2) using ethylchloroformate as the coupling reagent. The 20E-HSA conjugate was repeatedly washed with H_2O/DMSO (9:1, v/v) in an Amicon filter (Millipore; 30K cut-off) to remove non-covalently bound steroid, lyophilized and then analyzed by mass spectrometry to determine the number of 20E molecules coupled to each HSA molecule. Analyses were performed in a 4700 MALDI TOF/TOF proteomics analyzer (Applied Biosystems). The 20E conjugate was studied in linear mode, positive-ion mode. Laser acceleration was set at 20 kV and the default laser fluency was set at 2000 and modified according to the signal-to-noise quality. The matrix used was α-cyano-4-hydroxycinnamic acid (HCCA). Samples were prepared following the dried-droplet method, 1 µL of a mixture of 1 µL of matrix (10 mg/mL) and 1 µL of sample were spotted and dried with gaseous nitrogen. The mass shift, relative to HSA, after coupling indicates that 11 molecules of 20E derivative are coupled to each albumin molecule for batch 1 and 9 for batch 2.

Cell culture

The C2C12 mouse myoblast cell line (Yaffe & Saxel 1977) was purchased from ATCC (CRL-1772). Except where otherwise mentioned, culture media, serum, antibiotics and supplements were from Life Technologies. All cultures contained 100 U/mL of penicillin and 100 µg/mL of streptomycin and were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C. For C2C12 proliferation, cells were maintained in DMEM medium containing 4.5 g/L glucose, supplemented with 10% FBS. C2C12 cells were maintained at low passage (3–20 passages) for all experiments to maintain the differentiation potential of the cultures. Cell confluency was always kept below or equal to ~80%. For all experiments, cells were first seeded at 30,000 cells per well in 24-well plates. To induce differentiation, C2C12 cells at ~80% confluency in proliferation medium were shifted to DMEM medium supplemented with either 2% FBS or 2% horse serum.

Protein synthesis (^{3}H-leucine incorporation)

C2C12 cells were grown in 24-well plates at a density of 30,000 cells/well in 0.5 mL of growth medium (DMEM + 4.5 g/L glucose supplemented with 10% FBS). Twenty-four hours after plating, the differentiation into multinucleated myotubes was induced in DMEM + 4.5 g/L glucose containing 2% FBS. After 5 days, cells were pre-incubated in Krebs medium for 1 h at 37°C before being incubated in DMEM media without serum for 2.5 h in the presence of radiolabeled leucine (5 µCi/mL) and DMSO (control condition) or insulin growth factor (IGF-1, 100 ng/mL) or 20E (0.1, 0.5, 1, 5 or 10 µM). At the end of the incubation, supernatants were discarded and cells were lysed in 0.1 N NaOH for 30 min. The radioactivity associated with the cell-soluble fraction was then counted using a Wallac Microbeta 1450-021 TriLux Luminometer Liquid Scintillation Counter (Wallac EG&G, Gaithersburg, MD, USA) and protein quantification was performed using the colorimetric Lowry method.

Myostatin and MAS gene expression assays

Cells were plated at a density of 30,000 cells per well in 24-well plates and were grown overnight in 5% CO_2 at 37°C. On day 5 of differentiation, treatments were carried out for 6 h. At the end of the incubation, RNAs were extracted and purified using RNAzol (Eurobio, Les Ulis, France). RNAs were converted into cDNAs using a high-capacity cDNA RT Kit (Applied Biosystems, ThermoFisher) before performing quantitative PCR using iTaq SybrGreen (Biorad). Q-RT-PCRs were then performed using a 7900HT Fast Real-Time PCR detection system (Applied Biosystems) and a standard qPCR program (1 cycle at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min). QRT-PCR master mix contained the 100 ng cDNA samples and a set of

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primers at final concentration of 200 nM designed for two different exons and described below. The quality of RNA was checked using NanoDrop™ technology (ThermoFisher) when necessary.

The relative differences in gene expression levels between treatments were expressed as increases or decreases in cycle time (Ct) numbers compared to the control group where the Ct value of each gene was normalized to the beta-actin gene or hypoxanthine guanine phosphoribosyl transferase (HPRT) gene. Results of gene expression were expressed as $2^{-\Delta\Delta CT}$ after normalization with house-keeping genes. Primer sequences used are described in Table 1.

siRNA MAS assay

Cells were plated at a density of 10,000 cells per well in 24-well plates. After 3 days of differentiation, cells were transfected either with scramble siRNA (10 nM) or Mas1 siRNA (10 nM) according to the manufacturer's instructions (Origene Technologies, Rockville, MD, USA). Two days after transfection, myotubes were treated with either DMSO or IGF-1 or 20E or angiotensin(1–7) for 6 h. At the end of incubation, RNA was extracted and analyzed by QRT-PCR as described above.

Binding studies

The relative affinities of 20E for human nuclear steroid receptors such as androgen receptor (AR), estrogen receptors alpha and beta (ERα, ERβ) and glucocorticoid receptor (GR) were determined by radioligand binding assays (CEREP/Eurofins). The selective ligands [^{3}H]-methyltrienolone, [^{3}H]-estradiol and [^{3}H]-dexamethasone were employed with cells expressing either human endogenous or recombinant AR, ERα or β, or GR, respectively. 20E was used at concentrations up to 100 µM as a potential competitor. Inhibition of control specific binding was determined, and IC_{50} and K_i were calculated when possible. Additionally, a receptor screen was carried out on 45 GPCR and 5 nuclear receptors at a fixed concentration of 20E (10 µM). Radioligand binding assays were performed according to the manufacturer's instructions employing ^{3}H- or ^{125}I-labeled specific ligands of each receptor (SafetyScreen87 Panel, Panlabs, Taipei, Taiwan).

Table 1 Primers used for mRNA quantification by RT-QPCR.

Gene	Sequence
Mstn	F 5′-GAGTCTGACTTTCTAATGCAAG-3′ R 5′-TGTTGTAGGAGTCTTGACGG-3′
Mas1	F 5′-TGCCTTGGTGACCACCATGG-3′ R 5′-ACCAAGATGGTGCTGGACAC-3′
Actb	F 5′-CTCTAGACTTCGAGCAGGAG-3′ R 5′-GGTACCACCAGACAGCACT-3′
Hprt	F 5′-TCCTCATGGACTGATTATGGA-3′ R 5′-TCCAGCAGGTCAGCAAAGAA-3′

Statistical analyses

Statistical analysis was performed using Graph Pad Prism® Software. ANOVA followed by a Dunnett *t*-test or a Kruskal–Wallis test followed by a Dunn's test (when the variances significantly differed) have been performed. To evaluate the significance of differences between two groups, the choice of parametric Student's *t*-test or non-parametric Mann–Whitney test was based on the normality or non-normality of data distribution, respectively (D'Agostino–Pearson test). The results are considered significant at P-value < 0.05 (*), <0.01(**), <0.001 (***).

Results

20-Hydroxyecdysone stimulates muscle anabolism

20-Hydroxyecdysone (20E) effects were investigated on pre-established murine myotubes (following 6 days of differentiation). An anabolic effect was investigated by means of a *de novo* protein synthesis assay. A dose-dependent increase in protein synthesis was observed in response to 20E treatment of C2C12 myotubes vs untreated conditions (Fig. 1A). IGF-1 (100 ng/mL), employed as positive control (Semsarian *et al.* 1999), displayed, as expected, an improvement in [^{3}H] Leu incorporation (+18 %, P < 0.01). The 20E effect was significant from 1 to 5 µM. The maximal effect (+22 %, P < 0.001) was measured with 5 µM of 20E, while treatment with a higher concentration of 20E (10 µM) appeared to be notably less efficient (+14%, ns) than the previous concentration tested.

Myostatin is a major autocrine regulator that inhibits muscle growth in mammals. The myostatin transcript bioassay was developed and standardized in order to assess ecdysteroid activity (Zubeldia *et al.* 2012). IGF-1 (100 ng/mL), used as a positive control, demonstrated a significant inhibition of myostatin gene expression (60% of untreated control cells, P < 0.001, Fig. 1B). A dose-dependent and partial inhibition of myostatin gene expression was observed in response to 20E treatment at concentrations between 0.05 and 10 µM. This inhibition was significant from 1 µM 20E (Fig. 1B). The inhibition of myostatin gene expression was then employed as a readout for 20E activity.

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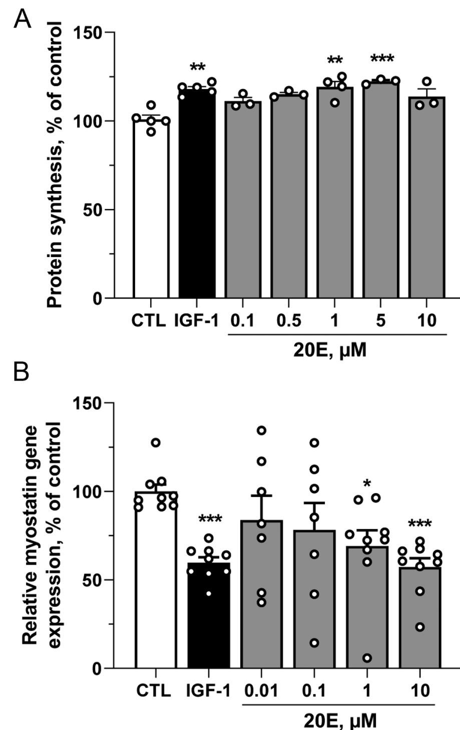

Figure 1
Effects of 20E on protein synthesis and myostatin gene expression in C2C12 cells. (A) Representative results from at least three distinct experiments displaying 20E effects on protein synthesis in differentiated myotubes detected by [^{3}H]-leucine incorporation. (B) C2C12 mouse myoblasts were differentiated for 6 days into myotubes. They were then treated for 6 h with concentrations of 20E ranging from 0.001 to 10 µM. Myostatin gene expression was detected by qRT-PCR. Results are shown as means ± s.e.m. of at least seven independent experiments with ***P < 0.001, **P < 0.01, *P < 0.05 vs untreated control (Kruskal–Wallis one-way ANOVA followed by uncorrected Dunn's test).

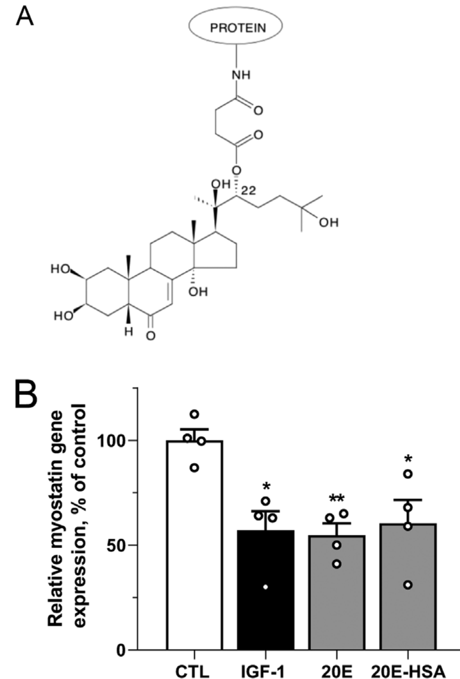

Figure 2
20E acts on C2C12 myotubes from outside of the cell. (A) Structure of 20E 22-succinate coupled with human serum albumin. (B) C2C12 mouse myoblasts were differentiated for 6 days into myotubes. They were then treated for 6 h with IGF-1 (100 nM), 20E (10 µM) or 20E-HSA (10 µM 20E-equivalent). Myostatin gene expression was detected by qRT-PCR. Results are shown as means ± s.e.m. of four independent experiments with *P < 0.05, **P < 0.01 vs untreated control (Kruskal–Wallis one-way ANOVA followed by uncorrected Dunn's test).

20-Hydroxyecdysone acts on cell membranes

In order to confirm that 20E acts primarily at the cell membrane, or if it needs to penetrate into the cell to exert its effects, a membrane-impermeable derivative of 20E was produced (Fig. 2A). We compared the effects of free 20E and its 22S-HSA conjugate (batch 1) on myostatin gene expression at the concentration of 10 µM 20E-equivalents (Fig. 2B). We observed that the membrane-impermeable 20E-derivative retained an activity similar to that of free 20E. Similar results were obtained with another batch of 20E-22S-HSA conjugate prepared with another coupling method (batch 2) and also when using 20E-6-CMO coupled to albumin (data not shown). This result demonstrates that covalent attachment to a bulky protein does not prevent 20E activity and is an additional argument for the interaction of 20E with a membrane receptor, as proposed by Gorelick-Feldman *et al.* (2010).

20-Hydroxyecdysone acts via a GPCR-type receptor

The GPCR hypothesis is based on the inhibition of 20E effects by pertussis toxin, but there are still numerous possible GPCR candidates. We selected a set of GPCR receptors based on available literature and using different criteria corresponding to well-established effects of 20E: (i) involvement in the control of muscle cells activity and glycemia/insulin sensitivity and (ii) ability to reduce fat mass gain in high-fat fed animals (Kizelsztein *et al.* 2009, Foucault *et al.* 2012). A set of GPCRs was thus selected including TGR5 (bile acid receptor: Thomas *et al.* 2009), GPER/GPR30 (estradiol, aldosterone receptors: Prossnitz *et al.* 2008, Evans *et al.* 2014), LPA1 (lysophosphatidic acids receptor: Jean-Baptiste *et al.* 2005), APJ (apelin receptor: Bertrand *et al.* 2013), OXTR (oxytocin receptor: Breton *et al.* 2002), AVPR1 (vasopressin receptor: Nervi *et al.* 1995), MrgD (alamandine receptor: Lautner *et al.* 2013), MARRS (vitamin D3 receptor: Khanal & Nemere 2007) and MAS (angiotensin(1–7) receptor: Santos *et al.* 2003, Muñoz *et al.* 2010). The possible interaction of 20E with those receptors was assayed using different approaches according

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to available methodologies: (1) *in silico* binding when 3D structures were available (Lapenna *et al.* 2015), (2) *in vitro* direct binding studies by competition with a radioactive ligand (e.g. Supplementary Table 1), (3) comparison of the effects of known receptor agonists with those of 20E on C2C12 cells or (4) effect of known antagonists of those receptors on the response of C2C12 cells to 20E. Using this approach, the only receptor which gave positive data was MAS, the receptor of angiotensin(1–7).

20-Hydroxyecdysone and angiotensin(1–7) act via MAS receptor activation

Using the myostatin gene expression assay, a pharmacological approach was employed to compare the effects of the endogenous MAS receptor agonist (angiotensin(1–7)) with those of 20E on C2C12 cells in the presence or absence of a known antagonist.

Angiotensin(1–7) (Fig. 3A), as well as 20E (Fig. 1B), partially inhibits myostatin gene expression in a dose-dependent manner. As expected, this inhibition by angiotensin(1–7) (10 µM) was totally abolished by specific angiotensin(1–7) antagonist (A779; 10 µM) (Fig. 3B). Interestingly, 20E inhibitory effects on myostatin gene expression were also fully reversed by the same antagonist (Fig. 3B), suggesting that inhibition of myostatin gene expression by 20E (or angiotensin(1–7)) is mediated by the receptor of angiotensin(1–7). In contrast, the effect of IGF-1, which acts through its own receptor (insulin-like growth factor 1 receptor; IGF1R) remains unchanged in the presence or in the absence of angiotensin(1–7) antagonist (not shown).

In order to further assess the involvement of MAS receptor in 20E activity on myotubes, we designed a gene interference experiment using silencing RNA directed against MAS receptor (siRNA Mas1). The efficiency of MAS receptor downregulation by siRNA was tested first. A significant decrease of MAS gene expression by a factor 2 in all transfected groups by directed siRNA vs scramble siRNA was observed (data not shown). In a similar way to what was observed with antagonists (Fig. 3B), downregulation of MAS receptor reversed 20E or angiotensin(1–7) effects on myostatin gene expression (Fig. 3C). As expected, and consistent with the pharmacological approach, downregulation of MAS receptor had no impact on the effect of IGF-1 on myostatin gene expression (data not shown). Finally, we employed MCF-7 human breast cancer cells which highly and constitutively express MAS receptor (Luo *et al.* 2015; Supplementary Fig. 2A) to assess NO production in response to MAS receptor activation by 20E. As shown in Supplementary Fig. 2B, the DAF-FM relative fluorescence of 20E-treated MCF7 cells was significantly enhanced at all concentrations used when compared to untreated control, indicating that 20E enhanced NO production. Similar levels of NO production in the presence of 10 µM 20E or angiotensin(1–7) were observed (+38% ($P < 0.05$) and +34% ($P < 0.01$), respectively). NO production induced by 20E and angiotensin(1–7) was inhibited by A779 treatment (Supplementary Fig. 2B). These results are consistent with our data obtained on myostatin gene expression (Fig. 3) and further demonstrate the role of MAS

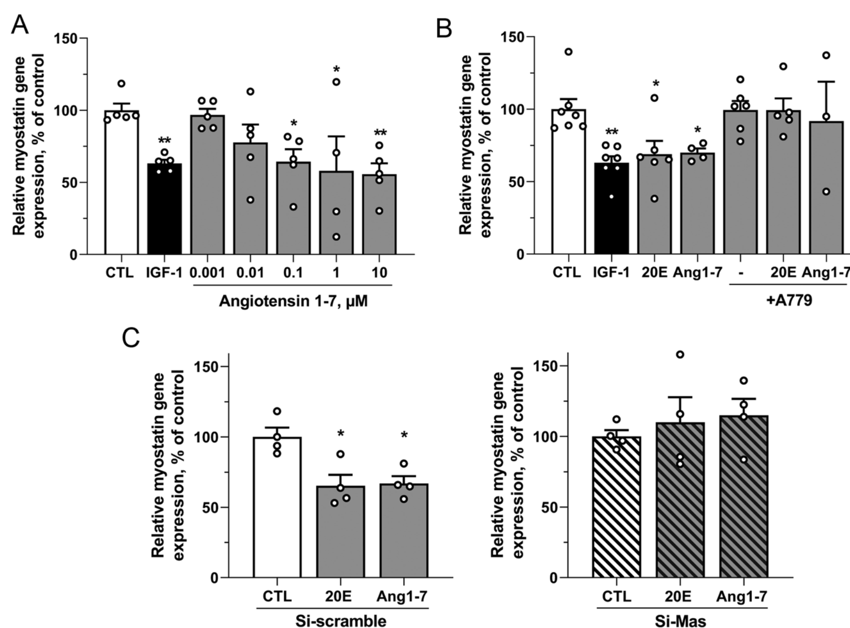

Figure 3
20-Hydroxyecdysone effects are mediated through Mas receptor activation. C2C12 mouse myoblasts were differentiated for 6 days into myotubes. (A) They were then treated for 6 h with concentrations of angiotensin(1–7) ranging from 0.001 to 10 µM. Myostatin gene expression was determined using qRT-PCR. (B) Effect of MAS antagonist (A779, 10 µM) on 20E- and angiotensin(1–7)-induced myostatin gene expression inhibition. (C) Effect of MAS siRNA on 20E- and angiotensin(1–7)-induced myostatin gene expression inhibition. Results are shown as means ± s.e.m. of at least three independent experiments with *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Kruskal–Wallis one-way ANOVA followed by uncorrected Dunn's test compared to untreated control.

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receptor activation in 20E-mediated effects. These data are in concordance with previously published studies by Heitsch *et al.* (2001) and Sampaio *et al.* (2007).

20-Hydroxyecdysone does not bind to a set of nuclear receptors

Parr *et al.* (2014, 2015) have proposed that 20E effects are explained by its binding to estrogen receptor ERβ. It is however difficult to consider that this receptor corresponds to a canonical nuclear receptor of estradiol, given that several previous binding studies of 20E to nuclear ERs were unsuccessful (Báthori *et al*. 2008, Gorelick-Feldman *et al*. 2010, Seidlova-Wuttke *et al*. 2010). We too performed competitive binding tests of 20E for AR, ERα and ERβ which were all negative at up to 100 µM (data not shown). Similarly, an off-target safety screen for 87 receptors was equally negative for ERα and AR (Supplementary Table 1). Nevertheless, Parr's results are not unique, since Gao *et al.* (2008) showed that 20E can activate several ERβ target genes, and indeed there are multiple similarities between the effects of 20E and 17β-estradiol (E2) on muscles (Velders *et al*. 2012) or skin cells (Ehrhardt *et al*. 2011). Thus, while the above binding studies seem to exclude canonical nuclear forms of ERs, the question remains open for the membrane ones.

Estradiol effects on C2C12 myotubes

In an attempt to explain this discrepancy, we engaged in a set of experiments to characterize estradiol effects on C2C12 cells and to identify which type of E2 receptor could be involved in 20E effects. We first checked if, like 20E, E2 was able to impact myostatin gene expression in C2C12 cells. Myotubes were treated with increasing doses of E2 during 6 h and myostatin expression was then determined at transcriptional level. E2 significantly inhibits myostatin gene expression from 0. 1 to 1 µM (Fig. 4A). To determine if E2 activity relies on an interaction with a plasma membrane receptor, we employed the same strategy as the one presented above for 20E (Fig. 2). We used a membrane-impermeable conjugate of E2 made of 6-hydroxy-E2 attached via a 6-carboxymethyloxime (CMO) linker to BSA. E2-6-CMO itself significantly inhibited myostatin gene expression (−34%, $P < 0.01$) with the same trend as IGF-1 positive control (−44%, $P < 0.01$). By contrast, the E2-6-CMO-BSA conjugate was inactive (Fig. 4B) and did not significantly decrease myostatin expression (−13%, ns). This experiment allows the exclusion of an interaction of E2 with a transmembrane receptor but rather could

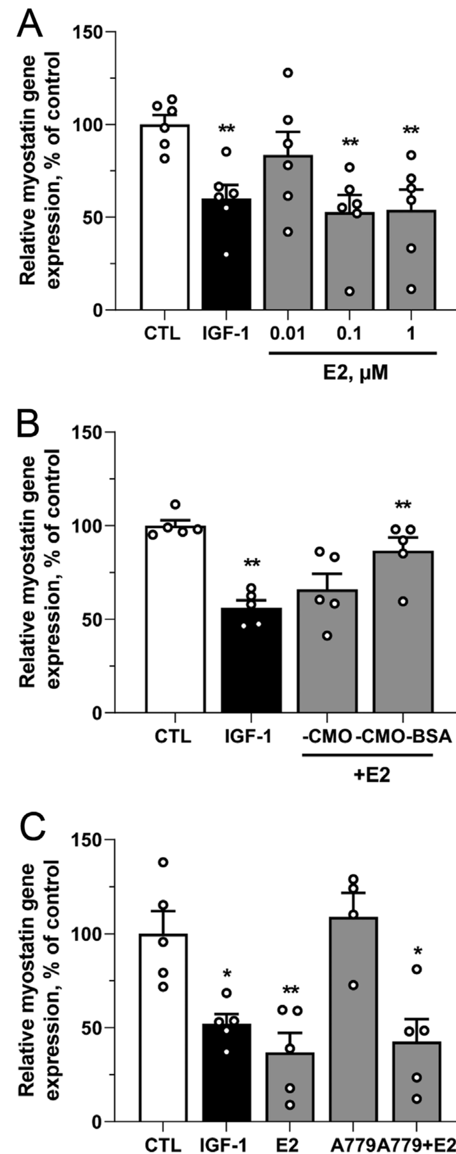

Figure 4
17β-Estradiol-mediated myostatin inhibition is not linked with a transmembrane receptor and is not blunted by a MAS antagonist. (A) Differentiated C2C12 cells were treated with IGF-1 (100 ng/mL) or 17β-estradiol (E2; 0.01, 0.1 and 1 µM) for 6 h. Myostatin gene expression was detected by qRT-PCR. (B) Effect of E2-CMO (0.1 µM) and E2-CMO-BSA (0.1 µM) on myostatin gene expression (qRT-PCR). (C) Effect of E2 (0.02 µM) in combination with Mas antagonist A779 (10 µM) on myostatin gene expression. Results are shown as means ± s.e.m. of at least five independent experiments with $*P < 0.05$, $**P < 0.01$ (Kruskal–Wallis one-way ANOVA followed by uncorrected Dunn's test) compared to untreated control.

possibly fit with a receptor bound to the cytoplasmic side of the cell membrane by a lipid anchor.

Sobrino *et al.* (2017) showed that the vasodilatory effect of E2 was blunted by a MAS antagonist. This encouraged us to check if a MAS antagonist would inhibit the effect of E2 on myostatin gene expression by C2C12 cells. E2's effect on

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myostatin gene expression was tested in the presence of a MAS antagonist (A779), but, unexpectedly, the effect of E2 was not inhibited by A779 (Fig. 4C).

17α-E2 is an epimer of estradiol which does not bind nuclear ERα or ERβ with high affinity and is known to bind only membrane forms of ER (Dykens *et al*. 2005). This compound proved active for the inhibition of myostatin gene activity (Supplementary Fig. 3). This result provides an additional argument for the involvement of a non-nuclear ER.

Discussion

Our data combined with those previously available from the literature allow us to conclude that the effects of 20E on C2C12 cells involve both Mas receptor and a non-nuclear estradiol receptor. Although these results do not allow the unambiguous identification of its primary target, they allow a reconciliation of the findings of Gorelick-Feldman *et al.* (2010) and Parr *et al.* (2014) by invoking a mixed mechanism of action.

Indeed, of paramount importance is our finding that a membrane-impermeable form of E2 is inactive, whereas a membrane-impermeable form of 20E still remains active. This allows us to conclude that 20E does not bind to the concerned ER receptor and that the activation of this estrogen receptor by 20E is secondary to MAS activation.

Five different E2 receptors (not including splice variants) have been described including two GPCRs (Micevych & Christensen 2012), GPR30 (Filardo *et al*. 2000) and Gq-mER (Lagrange *et al*. 1997), plus possibly another plasma membrane-associated receptor, ER-X (Toran-Allerand 2005). Our experiments with GPR30 agonists and antagonists exclude GPR30 from the candidates. In addition, nuclear canonical receptors being excluded, the question remains open regarding which membrane E2 receptor form is involved in the effects of E2 and 20E on muscle cells.

Different membrane forms of the nuclear estrogen receptors (ERα and ERβ), produced by alternative splicing, have been described. These may correspond to either palmitoylated full forms or truncated forms unmasking a potential transmembrane alpha-helix sequence (Meitzen *et al*. 2013, Maneix *et al*. 2015, Schreihofer *et al*. 2018). Non-truncated ERs may reversibly bind to the cytoplasmic face of the membrane by a S-palmitoyl anchor (Marino & Ascenzi 2006).

In this respect, experiments on C2C12 cells treated with diarylheptanoid compounds (HPPH) provide very important information (Tipbunjong *et al*. 2017, 2019). These molecules display growth- and differentiation-promoting effects on C2C12 cells that involve a membrane form of ERα receptor bound by a palmitoyl anchor, and they were abolished by 2-bromohexadecanoic acid, an inhibitor of palmitoylation (Tipbunjong *et al*. 2019).

Interestingly, Garratt *et al.* (2019) have shown that 17αE2, an E2 epimer that does not bind nuclear forms of estrogen receptors, has beneficial effect on muscles, notably during sarcopenia. These results demonstrate that nuclear and membrane forms of E2 receptors display different ligand specificities. Therefore, the use of 'selective' inhibitors based on their effects on nuclear ERs does not allow unambiguous conclusions about whether ERα or ERβ membrane forms are targeted, and specific silencing experiments have to be preferred.

Membrane forms of ERs can associate with a GPCR. For example, in neuronal membranes, ER is associated with mGLUR1a (a glutamate receptor) and in this system, E2 effects are blunted by a mGLUR1a antagonist (Pastore *et al.* 2019).

A functional interaction between ER and MAS receptor has already been observed by Sobrino *et al.* (2017) in HUVEC cells. These authors observed that an E2 effect (increased NO synthesis) was abolished by an antagonist of MAS receptor. An association between MAS and a non-nuclear form of ER seemed therefore highly probable.

Taken together, our data best fit with the presence of a complex associating MAS and a estrogen membrane receptor (Fig. 5), likely together with a caveolin

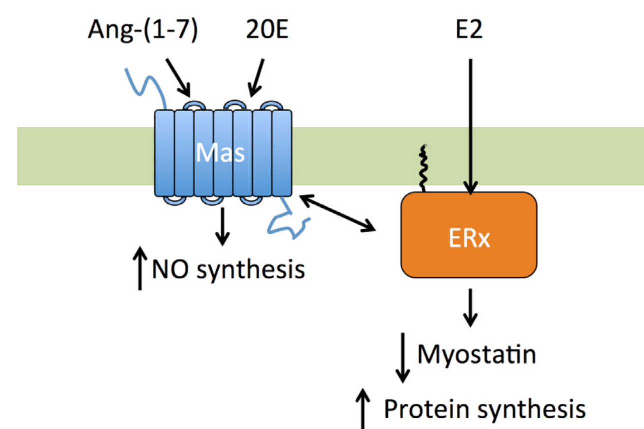

Figure 5
Proposed mechanism of myostatin gene control by angiotensin(1–7), 20E and E2. According with this scheme, myostatin and protein synthesis would be controlled directly by ER and indirectly by MAS, while NO synthesis would be controlled directly by angiotensin(1–7) (Tirupula *et al.* 2015) or 20E (Omanakuttan *et al.* 2016), and indirectly by E2 (Sobrino *et al.* 2017).

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and/or striatin, as well as additional transduction effectors (e.g. Gq, NOS). The demonstration of such a functional association will require membrane fractionation combined with various immunoprecipitation techniques. Whether a symmetrical interaction of this ER and IGFR exists is an attractive possibility, which is presently under investigation.

The case of ER is not unique. Ruhs *et al.* (2017) described an association between aldosterone receptor (MR) and GPER/GPR30. Accordingly, some effects of aldosterone are inhibited by G15, a GPER inhibitor (Feldman *et al.* 2016) and, most interestingly, they also showed an association between MR and the angiotensin II receptor AT1. Interestingly, there would thus be the presence of both a complex between aldosterone and angiotensin II AT1 receptors and, symmetrically, of a complex between estradiol and angiotensin(1–7) MAS receptors, displaying opposite and competing physiological effects.

It is worth mentioning that 20E will only activate a particular membrane form of ER, whereas E2 would in addition bind nuclear receptor(s). Thus 20E is devoid of any feminizing activity (Prabhu & Nayar 1974, Seidlova-Wuttke *et al*. 2010) and is probably inactive on estrogen-dependent cancer cells.

Conclusion

It is noteworthy that angiotensin(1–7), E2 and 20E display similar pleiotropic effects on many different organs/functions (Supplementary Table 2). During recent years, the number of identified beneficial effects of the protective arm of the renin–angiotensin–aldosterone system has been continuously increasing, and from the available data, it is expected that 20E will provide similar beneficial effects on several types of diseases (sarcopenia, diabetes, metabolic syndrome, etc.).

Based on the above findings, a pharmaceutical grade preparation of 20E (BIO101) has been developed and has been assayed in a phase 2 clinical trial for treating sarcopenia (a double-blind, placebo controlled, randomized interventional clinical trial (SARA-INT), ClinicalTrials #NCT03452488). We are confident that the beneficial effects of 20E/BIO101 will also be further established on, for example, lungs, kidneys and cardiovascular pathologies and could offer new therapeutic strategies.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JME-21-0033.

Declaration of interest

R L, M S, B D B, S R, L G, L D, S V, M L, W D and P J D are, or were, employees of Biophytis. They declare, however, that the company's potential commercial interests had no impact on the conduct of this research work.

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Author contribution statement

R L and P J D performed formal analysis, conceptualization, supervision, validation, writing review and editing and provided methodology. W D performed formal analysis, conceptualization, supervision and provided methodology; L D performed conceptualization, writing review and editing; M L performed formal analysis, conceptualization, writing review and editing and provided methodology; M S performed investigation, formal analysis, writing review and editing and provided methodology. S R and B D B performed investigation, formal analysis and provided methodology; L G performed investigation; S V performed funding acquisition, validation and provided resources.

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