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Citation for published version:
Abernethie, AJ, Gastaldello, A, Maltese, G, Morgan, RA, Mcinnes, KJ, Small, GR, Walker, BR, Livingstone, DEW, Hadoke, PWF & Andrew, R 2022, 'Comparison of mechanisms of angiostasis caused by the anti-inflammatory steroid 5-tetrahydrocorticosterone versus conventional glucocorticoids', *European Journal of Pharmacology*, pp. 175111. https://doi.org/10.1016/j.ejphar.2022.175111

Digital Object Identifier (DOI):
10.1016/j.ejphar.2022.175111

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
European Journal of Pharmacology

Comparison of mechanisms of angiostasis caused by the anti-inflammatory steroid 5α-tetrahydrocorticosterone versus conventional glucocorticoids

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Category: Endocrine Pharmacology

Highlights

- 5α-Tetrahydrocorticosterone has anti-inflammatory properties but is less angiostatic than hydrocortisone.
- 5α-Tetrahydrocorticosterone suppressed angiogenesis from mouse aortic rings *ex vivo* to a limited degree.
- 5α-Tetrahydrocorticosterone decreased *Pecam1* gene transcript associated with vascular remodelling.
- Angiostatic effects of 5α-tetrahydrocorticosterone were not blocked by glucocorticoid receptor antagonism.

Abstract

5α-Tetrahydrocorticosterone (5αTHB) is an effective topical anti-inflammatory agent in mouse, with less propensity to cause skin thinning and impede new blood vessel growth compared with corticosterone. Its anti-inflammatory effects were not prevented by RU38486, a glucocorticoid receptor antagonist, suggesting alternative mechanisms. The hypothesis that 5αTHB directly inhibits angiogenesis to a lesser extent than hydrocortisone was tested, focussing on glucocorticoid receptor mediated actions. New vessel growth from aortae from C57BL/6 male mice was monitored in culture, in the presence of 5αTHB, hydrocortisone (mixed glucocorticoid/mineralocorticoid receptor agonist) or the selective glucocorticoid receptor agonist dexamethasone. Transcript profiles were studied, as was the role of the glucocorticoid receptor, using the antagonist, RU38486. *Ex vivo*, 5αTHB suppressed vessel growth from aortic rings, but was less potent than hydrocortisone (EC50 2512 nM 5αTHB, versus 762 nM hydrocortisone). In contrast to conventional glucocorticoids, 5αTHB did not alter expression of genes related to extracellular matrix integrity or inflammatory signalling, but caused a small increase in *Per1* transcript, and decreased transcript abundance of *Pecam1* genes. RU38486 did not antagonise the residual effects of 5αTHB to suppress vessel growth or regulate gene expression, but modified effects of dexamethasone. 5αTHB did not alter expression of glucocorticoid-regulated genes *Fkbp51* and *Hsd11b1,* unlike hydrocortisone and dexamethasone. In conclusion, compared with hydrocortisone, 5αTHB exhibits limited suppression of angiogenesis, at least directly in blood vessels and probably independent of the glucocorticoid receptor. Discriminating the mechanisms employed by 5αTHB may provide the basis for the development of novel safer anti-inflammatory drugs for topical use.

Keywords: Glucocorticoids, Inflammation, 5α-Tetrahydrocorticosterone, Angiogenesis, Blood Vessel

1 Introduction

With eczema estimated to affect 230 million people worldwide, inflammatory skin diseases are highly prevalent and most commonly treated with topical glucocorticoid hormones. These drugs exert their main effects through binding to the glucocorticoid receptor (NR3C4). However, topical glucocorticoid therapy is associated with debilitating side effects, both systemically and locally on blood vessels, suppressing wound repair and inhibiting angiogenesis (Gastaldello et al., 2017). Scientists have sought to improve the therapeutic index of anti-inflammatory glucocorticoids through selective drug design and modified pharmacokinetics (Schacke et al., 2006; Schacke et al., 2004), including use of natural products (De Bosscher et al., 2005; Reeves et al., 2012). To date the ideal therapy has not been achieved, but there is much interest in selective modulation of the glucocorticoid receptor (Clark and Belvisi, 2012; De Bosscher et al., 2016; Haskell, 2003; Lonard and Smith, 2002; Meijer et al., 2018; Patel and Bihani, 2018).

The steroid, 5α-tetrahydrocorticosterone (5αTHB), is also being investigated as a safer topical anti-inflammatory treatment with fewer systemic and local side effects than current glucocorticoid therapies (Gastaldello et al., 2017; Yang et al., 2011). 5αTHB can reduce swelling and inflammatory cell infiltration in a mouse model of skin inflammation to a similar extent as corticosterone, the rodent glucocorticoid equivalent to hydrocortisone in man (Gastaldello et al., 2017). However, in contrast to corticosterone, 5αTHB did not induce systemic side effects and only invoked limited skin thinning. Accumulating evidence suggests that 5αTHB achieves its improved side effect profile by acting through different mechanisms from those of conventional glucocorticoids; its anti-inflammatory actions were not attenuated by the glucocorticoid receptor antagonist RU38486 (Gastaldello et al., 2017). Furthermore, topical 5αTHB suppressed inflammatory swelling and cell infiltration over a different timeframe to corticosterone.

One of the main use-limiting side effects of topical glucocorticoids is delayed wound repair, and crucial to wound repair is the restoration of blood flow through angiogenesis. Glucocorticoids are well known to inhibit angiogenesis, thought to be mediated in large part via the glucocorticoid receptor (Logie et al., 2010; Small et al., 2005). Whilst glucocorticoid-mediated suppression of angiogenesis occurs through diverse mechanisms, it is predominantly achieved by modifying inflammatory signalling and basement membrane/extracellular matrix degradation (McSweeney et al., 2010; Morgan et al., 2018). Suppression of inflammatory signalling is often detrimental to angiogenesis since many inflammatory cytokines are also pro-angiogenic. Likewise, degradation of the blood vessel basement membrane can inhibit endothelial cell migration and proliferation. Gastaldello *et al.* (Gastaldello et al., 2017) assessed angiogenesis *in vivo* using a sponge implantation model, in which 5αTHB-mediated prevention of new blood vessel growth was less pronounced than the response to corticosterone, potentially as a result of linked to more limited or selective actions. Corticosterone, like hydrocortisone administered clinically, is an agonist of both the glucocorticoid and mineralocorticoid (NR3C2) receptors. Since this was tested in an *in vivo* model, actions of glucocorticoids to suppress angiogenesis may have been executed directly on the blood vessels, or indirectly as consequences of their effect on other systemic processes, such as suppression of inflammation.

This investigation addressed the hypothesis that, when applied directly to blood vessels and in the absence of inflammatory cell infiltrate, 5αTHB causes more limited suppression of angiogenesis than the conventional glucocorticoid hydrocortisone, acting through a selective mechanism of action potentially independent of the glucocorticoid receptor. To address this hypothesis, the impact of 5αTHB on angiogenesis, in the absence and presence of a glucocorticoid receptor antagonist, was monitored in an *ex vivo* model and differential transcriptional responses studied.

2. Materials and Methods

2.1. Materials

Chemicals were from Sigma Aldrich (Dorset, UK) and cell culture reagents from Lonza (Berkshire, UK) unless otherwise stated. Steroids, including the glucocorticoid receptor antagonist RU38486 (PubChem CID: 55245), were from Steraloids (Newport, RI, USA). Relevant chemicals studied include 5αTHB (PubChem CID: 101790); corticosterone (PubChem CID: 5753); dexamethasone (PubChem CID: 5743).

2.2 Mice

Male mice (C57BL/6, 8-12 weeks old, between 21 and 30 g) were from Harlan Laboratories (Shardlow, UK), and were housed in groups of 4 under standard conditions of 12 h light and dark cycles at 18-22 ^{o}C for at least a week prior to experimentation, under authority of the United Kingdom Home Office. Schedule 1 procedures were performed in accordance with local guidelines.

2.3. Preparation of aortic rings

Mice were killed by asphyxiation with carbon dioxide, thoracic aortae excised and placed in Dulbecco's Modified Eagle Medium (Lonza Group Ltd, Basel, Switzerland) at 4 °C. Adherent adipose tissue was removed, and aortae divided into 1 mm rings. To stimulate angiogenesis, aortic rings were then embedded in alpha 1 type 1 collagen (200 µL, 1 mg/mL, Millipore, Hertfordshire, UK) and incubated (37 °C, 5% CO_2) in Opti-MEM (Life technologies, Paisley, UK) alone or containing a growth stimulating treatment with or without the appropriate steroid treatment. The growth stimulating treatment consisted of an initial exposure (day 0) to 1% foetal calf serum followed by 5 ng/mL Recombinant Murine Vascular Endothelial Growth Factor (PeproTech, London, UK) on days 3 and 5. Steroid treatment consisted of dexamethasone (1-1000 nM), hydrocortisone (10 nM-10 µM), or 5αTHB (10 nM-10 µM), alone or in combination with the glucocorticoid receptor antagonist, RU38486 (30 nM). Drugs were dissolved in ethanol and diluted in Opti-MEM to give a final ethanol concentration of 1-3% v/v. Medium was replaced on days 3 and 5. Experiments were performed in duplicate. New vessels were counted on days 5 and 7 using light microscopy with the investigator 'blinded' to the treatment group of each ring.

2.4. Changes in abundance of gene transcripts

Rings treated with steroid concentrations achieving between the EC50 and maximal response (30 nM dexamethasone; 1 µM hydrocortisone; 3 µM 5αTHB) were chosen to compare effects on gene expression in the aorta.

Abundance of gene transcripts was analysed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) of RNA extracted from aortic rings after 7 days of culture, which was determined by (Small et al., 2005) to be the optimal time-point to assess effects on vessel growth. Four aortic rings of the same treatment group were combined to provide sufficient RNA for analysis and were mechanically disrupted in QIAzol lysis reagent (Qiagen, Manchester, UK). Total RNA was extracted using an RNeasy Minikit according to the manufacturer's instructions. cDNA was synthesised from RNA (75 ng) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Lithuania) according to the manufacturer's instructions and RT-qPCR performed using a Light Cycler ® 480 (Roche Diagnostics, Mannheim, Germany). Primers (Table 1 or otherwise cited in (Gastaldello et al., 2017)) were designed to match intron spanning probes with the Roche Universal Probe Library using the online software Universal Probe Library Assay Design Centre (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html#assay-design-center, 2017). All samples were analysed in triplicate, and data accepted if standard deviations of their quantification cycles were lower or equal to 0.4 cycles. If greater, then replicates were checked for consistency and outliers excluded. A standard curve was generated for each transcript using a serial dilution of cDNA pooled from different samples. Standard curves were accepted if reaction efficiency was between 1.7 and 2.1. Negative controls were accepted if there was no amplification for at least 10 cycles after the most dilute part on the standard curve. Data were normalized for the mean of the transcript abundance of two reference genes, *Actb* and *Tbp*, which did not differ between treatment groups.

2.4. Data and Statistical analysis

Data were analyzed using GraphPad Prism 6 software (CA, USA), and presented as mean ± standard error of the mean following one-way Analysis of Variance and either Dunnett's or Tukey's multiple comparison test depending on whether groups were being compared to one control group or to each other, respectively. Due to differences in initial numbers of vessels between mice, steroid concentration responses in the presence or absence of RU38486 were compared by normalizing the number of vessels of each treatment group to that of stimulated controls from the same animal, and then using these normalized data to superimpose concentration-response curves in the presence and absence of RU38486 as deemed appropriate. To fit the concentration-response curves, a non-linear regression analysis was performed using the log(inhibitor) vs response (three parameters) equation and the least squares fitting method. EC50 values were calculated from the mean data. Statistical significance was taken at $p<0.05$ and trends described where $0.05<p<0.1$. Power calculations, performed using PS: Power and Sample size calculation software (by WD Dupont and WD Plummer, Jr), supported day 7 as the best time point to detect the suppression of vessel growth by dexamethasone. A sample size of 8 was required when assessing vessel growth at day 7, to achieve power of 90% with $p<0.05$ to suppress vessel growth to 28% of stimulated controls. Based on the response of *Per1*, a sample size of 4 was deemed necessary to achieve a 3.5-fold increase in gene expression with 90% power and $p<0.05$.

3. Results

3.1 Effects of steroids on vessel growth from mouse aortic rings *ex vivo*

The growth stimulating medium significantly increased the number of vessels sprouting from aortic rings after 7 days in culture (Figure 1). Dexamethasone (positive control for glucocorticoid receptor-mediated angiostasis) suppressed vessel growth in a concentration-dependent manner (Figure 1 and 2a). The EC50 concentration for suppression of vessel growth by dexamethasone at day 7 was 7.13 nM and the suppression became significant at a concentration of 10 nM. A concentration of 100 nM of dexamethasone was thus chosen as a robust positive control during the subsequent experiments testing the concentration-dependent effects of hydrocortisone and 5αTHB on vessel sprouting (described below).

Hydrocortisone (Figure 1 and 2b) and 5αTHB (Figure 1 and 2c) both caused concentration-dependent suppression of vessel outgrowth from mouse aortic rings, to levels equivalent to unstimulated controls. Hydrocortisone (EC50 762 nM) achieved significant suppression with concentration 1 μM and above. 5αTHB was less potent than hydrocortisone at suppressing vessel growth. It had a higher EC50 (2512 nM) and required a higher concentration (3 μM) for its suppression of vessel growth to reach significance.

3.2. Effects of steroids on gene expression in mouse aortic rings during angiogenesis

Comparisons were made using steroid concentrations between the EC50 and the concentration that produced the maximal response, achieving as close as possible to 30% of stimulated vessel growth. This was achieved with 30 nM dexamethasone, 1 μM hydrocortisone, and 3 μM 5αTHB, which suppressed vessel growth to 30±7%, 35±11% and 26±14% of stimulated controls, respectively. In relation to genes involved in inflammation and signalling, exposure to dexamethasone or hydrocortisone decreased the abundance of transcripts for *Cxcl5* (Figure 3a) and increased those of *Dusp1* (dexamethasone achieving a strong trend ($p=0.053$)) (Figure 3b). 5αTHB did not affect transcript abundance of these genes but *Mcp1* transcripts were significantly higher ($p<0.01$) in aortic rings exposed to 5αTHB compared to dexamethasone or hydrocortisone, neither of which had any effect (Figure 3c). In relation to genes involved in remodelling of the extracellular matrix, the transcript abundance of *Col4a1* was increased by hydrocortisone, with a strong trend (p=0.0501) for an increase by dexamethasone (Figure 3d). Both dexamethasone and hydrocortisone decreased abundance of *Mmp9* transcripts (Figure 3e). 5αTHB did not alter abundance of any of these gene transcripts. In relation to genes involved in vasculature remodelling, only 5αTHB suppressed *Pecam1* transcripts in comparison to vehicle treatment (Figure 3f). Finally, genes known to be directly regulated by the glucocorticoid receptor were assessed and, as anticipated, dexamethasone and hydrocortisone increased the abundance of *Per1, Hsd11b1,* and *Fkbp51* transcripts (Figure 3g,h, and i). In contrast, 5αTHB only increased the abundance of *Per1* transcripts and to a lesser extent than hydrocortisone or dexamethasone (Figure 3g).

3.3. Glucocorticoid receptor-dependency of the effects of 5αTHB in the mouse aorta during angiogenesis

The glucocorticoid receptor antagonist RU38486 was used to determine whether the changes observed in the mouse aorta in response to 5αTHB were mediated through the glucocorticoid receptor. Dexamethasone was used for this comparison as it is a glucocorticoid receptor selective agonist. In the presence of RU38486, a rightward shift was induced in the concentration-response curve for the suppression of vessel growth by dexamethasone, causing an increase in the EC50 from 7.13 nM to 288 nM (Figure 4a). In contrast, RU38486 did not alter the concentration-response curve for the suppression of vessel growth by 5αTHB (Figure 4b).

Finally, the contribution of the glucocorticoid receptor to transcript regulation by dexamethasone and 5αTHB was assessed by investigating the effect of RU38486 to antagonise the changes in gene expression by steroid treatment (either dexamethasone or 5αTHB) of aortic rings in Figure 5: Only genes, whose transcript levels were significantly altered by dexamethasone in comparison to vehicle treated aortic rings (Figure 5: *Cxcl5*, *Mmp9*, *Fkbp51*, *Hsd11b1* and *Per1*) and by 5αTHB (Figure 5:

Pecam1 and *Per1)* were pursued to assess the role of the glucocorticoid receptor in regulation of steroid-induced transcript. In the presence of RU38486, changes in the expression of *Cxcl5*, *Mmp9*, and *Hsd11b1* mRNA induced by dexamethasone were no longer evident (Figures 5a, b, and d). RU38486 also tended to antagonise dexamethasone-induced up-regulation of *Per1* (p=0.06) and *Fkbp51* (P=0.08) (Figures 5e and c). Interestingly, RU38486 down-regulated *Mmp9* when administered alone (Figure 3b). In contrast, RU38486 did not antagonise the effect of 5αTHB on *Pecam1* and *Per1* transcripts (Figures 5f,g). RU38486 alone did decrease *Pecam1* transcript abundance but did not alter that of *Per1* (Figure 5f,g).

4. Discussion

Previous work suggested 5αTHB as an effective anti-inflammatory steroid (Gastaldello et al., 2017). Due to its pharmacokinetic properties, 5αTHB is better suited to topical application, and studies of irritant dermatitis in mice suggested 5αTHB had less local side-effects to reduce wound repair than current glucocorticoid treatments, suppressing angiogenesis less than corticosterone (Gastaldello et al., 2017). However, indirect effects of steroids could influence angiogenesis *in vivo*, so direct effects of 5αTHB on vessel growth are of interest. Furthermore, glucocorticoid receptor-dependency of 5αTHB's effects on blood vessels was assessed, given that 5αTHB suppresses inflammation independently of glucocorticoid receptors *in vivo* (Gastaldello et al., 2017) and glucocorticoid suppression of angiogenesis is believed largely mediated via glucocorticoid receptors. Consistent with *in vivo* studies, 5αTHB suppresses angiogenesis more weakly than hydrocortisone, and its residual effects on blood vessels were independent of glucocorticoid receptors, supporting its potential as a safer topical anti-inflammatory drug.

Gastaldello *et al.* (Gastaldello et al., 2017) previously demonstrated that 5αTHB inhibited angiogenesis less than corticosterone (rodent equivalent of hydrocortisone) in *in vivo* sponge implants. While systemic models replicate complex *in vivo* scenarios, mechanistic interpretation is confounded since inflammatory cell recruitment precedes angiogenesis during wound repair. Therefore, effects of glucocorticoids could occur indirectly through suppressed systemic inflammation (Yang et al., 2011) or directly on vessels. Here, direct vascular responses to glucocorticoids were assessed in *ex-vivo* aortic rings, with several cell types represented. Suppression of angiogenesis by both corticosterone (Small et al., 2005) and hydrocortisone (Morgan et al., 2018) has been studied previously in this model, and blocked by antagonists of glucocorticoid but not mineralocorticoid receptors.

Ex vivo, 5αTHB possessed suppressed angiogenesis less potently than hydrocortisone, suggesting direct effects on blood vessels do indeed play a role *in vivo* and reinforcing that at equipotent anti-inflammatory doses 5αTHB is less detrimental to angiogenesis (and hence wound repair) than conventional glucocorticoids. Concentration-dependent suppression of vessel growth occurred in order of potency: dexamethasone>hydrocortisone>5αTHB. There is considerable bioassay variability, even between rings from one mouse, underlying derivation of EC50s from mean data. Inherent variability arises from ring size, handling, and the exact aortic location (Baker et al., 2012). The 7 days time point was chosen to minimize variability.

Although glucocorticoids suppress angiogenesis largely through glucocorticoid receptors (Small et al., 2005), downstream mechanisms whereby glucocorticoids affect vascular function are diverse and poorly understood (Logie et al., 2010; Morgan et al., 2018; Small et al., 2005). Glucocorticoids alter angiogenic cytokine release from inflammatory cells recruited to the vasculature (Gelati et al., 2008), and *ex vivo* assays are needed to study vascular responses to glucocorticoids without influences from circulating immune cells. Indeed RNA-Sequencing showed 13 KEGG pathways were down-regulated in hydrocortisone-treated aortae, 9 associated with inflammation, but also 4 with extracellular matrix or cytoskeletal function (Morgan et al., 2018). Glucocorticoids degrade extracellular matrix components of vessel basement membranes, impairing remodelling that allows endothelial cell migration and proliferation required to form new vessels (Drebert et al., 2017; Morgan et al., 2018). Glucocorticoids also alter ability of endothelial cells to form cell-cell connections (Logie et al., 2010). Current findings are consistent with published work, whereby hydrocortisone and dexamethasone decreased *Cxcl5 and Mmp9* expression and increased *Dusp1* and *Col4a1* expression (Morgan et al., 2018). Whilst some gene expression changes were subtle, previous work suggests they are biologically meaningful; changes of similar magnitudes are accompanied by changes in protein expression or functional effects (Guo et al., 2018; Koyanagi et al., 2006; Mylonas et al., 2017; Neubauer et al., 2008; Sakuma-Zenke et al., 2005). Since CXCL5 and DUSP1 have anti-inflammatory functions (Abraham and Clark, 2006; Frangogiannis, 2012; Kobayashi, 2008; Lang et al., 2006), changes in response to steroids indicate that hydrocortisone and dexamethasone suppress inflammatory signalling. Indeed glucocorticoids suppress activity of both neutrophils and macrophages (Gastaldello et al., 2017; Yang et al., 2011), and immune cells promote angiogenesis through synthesis of proangiogenic mediators involved in endothelial cell proliferation, migration and activation (Ribatti and Crivellato, 2009). Decreases in *Mmp9* and increases in *Col4a1* are consistent with extracellular matrix remodelling, since *Col4a1* encodes the α1 chain of collagen IV, the main collagen present in basement membrane surrounding endothelial and vascular smooth muscle cells (Vahedi and Alamowitch, 2011). Matrix metallopeptidase 9 degrades collagen and gelatin in basement membranes, allowing endothelial cells to migrate and proliferate outwards into new tubes (Chen et al., 2013). These data, therefore, support hydrocortisone suppressing angiogenesis largely through effects on vessel basement membrane extracellular matrix and inflammatory signalling, but crucially reveal that 5αTHB does not impair angiogenesis through these mechanisms. Mechanisms studied were guided by Gastaldello et al. (Gastaldello et al., 2017) and are not exhaustive, with other potential transcriptional changes induced by hydrocortisone suitable for further study e.g. heme oxygenase-1 mediate VEGF stimulation of angiogenesis (Chen et al., 2016).

Unlike the topical glucocorticoid hydrocortisone and selective glucocorticoid receptor agonist dexamethasone, 5αTHB did not affect expression of *Cxcl5*, *Dusp1*, *Col4a1* or *Dusp1* in mouse aortae. This mirrors findings from sponge implantation studies (Gastaldello et al., 2017) where 5αTHB had more limited effects than corticosterone on gene transcripts involved in extracellular matrix homeostasis. However, in both settings (Gastaldello et al., 2017), 5αTHB decreased transcript levels of the endothelial adhesion protein *Pecam1*. *Pecam1* encodes Platelet endothelial cell adhesion molecule (CD31), a cell surface glycoprotein expressed by all vascular cells but particularly abundant at endothelial cell-cell junctions where it may modify permeability and transmigration (Ilan and Madri, 2003; Lertkiatmongkol et al., 2016; Solowiej et al., 2003; Woodfin et al., 2007). Residual angiostatic effects of 5αTHB may be mediated by interfering with formation of new endothelial cell-cell contacts. Alternatively 5αTHB may induce endothelial-to-mesenchymal transition, also associated with loss of *Pecam1* and decreased angiogenesis (Miscianinov et al., 2018). Limited numbers of aortic rings per mouse prevented complementary analysis of associated proteins. Indeed, transcript analysis required pooled rings. Of note doses of 5αTHB required to attenuate angiogenesis were greater than those of hydrocortisone and dexamethasone, yet caused fewest changes in gene expression, reinforcing conclusions that 5αTHB may be a safer topical anti-inflammatory steroid. Questions remain as to which vascular cell types respond to 5αTHB but could include endothelial or resident immune cells.

Differences in potency and profile of 5αTHB to suppress angiogenesis, despite equivalent topical anti-inflammatory properties, prompts questions over its mechanism of action. Previous work (Gastaldello et al., 2017) raised doubt over whether anti-inflammatory effects of 5αTHB were mediated through glucocorticoid receptors. Although glucocorticoids act largely via glucocorticoid receptors to suppress angiogenesis (Small et al., 2005), there are also reports of non-glucocorticoid receptor mediated angiostasis. Epi-cortisol (Folkman and Ingber, 1987) which lacks both glucocorticoid and mineralocorticoid receptor agonist activity inhibited angiogenesis *ex vivo*. Notably both dexamethasone and hydrocortisone increased expression of glucocorticoid receptor responsive genes (*Per1*, *Hsd11b1*, *Fkbp51*), whereas 5αTHB only marginally increased *Per1* expression, again suggesting that 5αTHB does not strongly activate glucocorticoid receptors. The glucocorticoid receptor antagonist RU38486 caused rightward shifts in the concentration-response curve to inhibit vessel growth of the glucocorticoid receptor agonist dexamethasone, but not 5αTHB, suggesting again suppression of angiogenesis by 5αTHB is largely independent of glucocorticoid receptors. Effects of 5αTHB on *Pecam1* and *Mcp1* gene expression were also not blocked by RU38486, again contrasting with dexamethasone, and implicating a different receptor.

RU38486 competitive antagonizes the glucocorticoid receptor (Castinetti et al., 2012; Fleseriu et al., 2012; Nguyen and Mizne, 2017) but has limitations. It is not completely selective for glucocorticoid receptors, also antagonizing progesterone receptors (NR3C3) (Castinetti et al., 2012; Sun et al., 2014). However, research *in vivo* supports a pro-angiogenic role for progesterone receptors (Karas et al., 2001; Nakamura et al., 2005; Walter et al., 2005; Yu et al., 2017). Furthermore, RU38486 has mixed agonist/antagonist properties (Beck et al., 1993a; Beck et al., 1993b; Chien et al., 2009; Zhang et al., 2007), evident in our data. Low concentrations of RU38486 were used to minimize agonist activity on vessel growth, but this was not fully realised and some independent effects of RU38486 on gene transcript abundance were also observed. Furthermore concentrations were finely tuned to avoid excessive independent effects of RU38486 on vessel growth. With further knowledge of cell-type involvement, selective targeting of glucocorticoid receptors *in vivo* in mice may be employed.

The involvement of mineralocorticoid receptors or mineralocorticoid-glucocorticoid receptor heterodimers may be future avenues to explore, although activation of mineralocorticoid receptors promote, rather than suppress, inflammatory responses in macrophages (Bene et al., 2014). Mineralocorticoid receptor antagonists do not attenuate glucocorticoid-induced angiostasis (Small et al., 2005) and, moreover, dexamethasone and hydrocortisone induced similar changes in gene transcription. Hence additional involvement of mineralocorticoid receptors was not apparent. The possibility remains that 5αTHB may bind at an allosteric glucocorticoid receptor site and trigger non-genomic signalling, or may act through another glucocorticoid-binding receptor, such as membrane glucocorticoid receptor or other low affinity binding proteins (Falkenstein et al., 2000; Strehl and Buttgereit, 2013). Indeed membrane glucocorticoid receptors have different ligand binding specificity from cytosolic glucocorticoid receptors (Mitre-Aguilar et al., 2015) and are present in immune cells (Buttgereit et al., 2004; Stahn et al., 2007). Non-genomic cardioprotective effects of glucocorticoids have been demonstrated (Haller et al., 2008; Tasker et al., 2006) having rapid therapeutic effects after stroke or myocardial infarction (Song and Buttgereit, 2006). In fact fewer adverse cardiovascular effects could be achieved through making use of non-genomic mechanisms of glucocorticoids; for example to decrease/eliminate genomic effects on wound repair and cardiac cell remodelling (Lee et al., 2012). Some non-genomic signalling cascades may indirectly modify gene expression, consistent with slower suppression of topical inflammation by 5αTHB, whereas corticosterone-induced inhibition was evident in 6 hours.

These data add to our understanding of the actions of 5αTHB, strengthening *in vivo* findings. There were limitations in that limited pools of vessels and inherent bioassay variability made it ethically challenging to conduct a well powered study of protein expression. RU38486 is an imperfect inhibitor confounding dose response fitting. Lastly human translation is still required. Further dissection of cell types could also be achieved using endothelial cell lines and studying features such as tube formation (Logie et al., 2011).

In summary, 5αTHB was less angiostatic than hydrocortisone and acts through selective mechanisms which differ from classical glucocorticoids. 5αTHB inhibits angiogenesis without altering inflammatory signalling or basement membrane composition and likely acts independent of glucocorticoid receptors. Angiogenesis is just one stage during wound repair, and future work should determine ultimate effects of 5αTHB on wound closure *in vivo*. Wound closure models are also excellent for studying impact of endothelial to mesenchymal transition on vessel growth during wound healing (Miscianinov et al., 2018). 5αTHB is therefore a promising candidate for a safer topical anti-inflammatory therapy. Identification of mechanisms through which 5αTHB signals may lead to development of new prototypes of anti-inflammatory drugs which could also be used systemically with a reduced side effect profile.

Acknowledgements and Funding Sources

The authors thank Dr Junxi Wu for technical help. This work was support the British Heart Foundation (and its Centre for Research Excellence; RG-05-008, BHF RE/08/001) and the Society for Endocrinology. Brian R Walker is a Wellcome Trust Senior Investigator. For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission

Declarations of Competing Interests: None

Figure Legends

Figure 1: Light microscopy images of angiogenesis from aortic rings showing suppression of growth following treatment with steroids

5α-Tetrahydrocorticosterone (5αTHB) induced a concentration-dependent suppression of angiogenesis in mouse aortic rings *ex vivo* but was less potent than hydrocortisone or dexamethasone. Aortic rings were maintained in medium either containing vehicle (control), or angiogenic medium (Stimulus) with or without dexamethasone (100 nM; positive control). Concentration-responses were assessed to dexamethasone (1, 3, 10, 30, 100, 300, or 1000 nM), hydrocortisone (HC: 10, 30, 100, 300, 1000, 3000, 10000 nM), or 5αTHB (10, 30, 100, 300, 1000, 3000, 10000 nM). Vessel outgrowths from aortic rings were counted 7 days after plating. All steroids induced a concentration-dependent suppression of vessel outgrowth, with the effect more apparent in lower concentrations of dexamethasone and hydrocortisone than 5αTHB with representative images shown

Figure 2:

5α-Tetrahydrocorticosterone (5αTHB) induced a concentration-dependent suppression of angiogenesis in mouse aortic rings *ex vivo* but was less potent than hydrocortisone or dexamethasone. Aortic rings were maintained in medium either containing vehicle (control), or angiogenic medium with or without dexamethasone (100 nM Dex; positive control). Concentration-response curves were obtained to (a) dexamethasone (1, 3, 10, 30, 100, 300, or 1000 nM, left to right on graph), (b) hydrocortisone (HC: 10, 30, 100, 300, 1000, 3000, 10000 nM, left to right on graph), or (c) 5αTHB (10, 30, 100, 300, 1000, 3000, 10000 nM, left to right on graph). Vessel outgrowths from aortic rings were counted 7 days after plating. All steroids induced a concentration-dependent suppression of vessel outgrowth, with the effect becoming significant at 10 nM Dex, 1 μM HC, and 3 μM 5αTHB. Concentration-response curves (R^2 = 0.3403, 0.2398, and 0.2763, respectively) were plotted and from these the steroid concentrations required to inhibit vessel growth by half (EC50) were determined as 7.13 nM Dex, 762 nM HC, and 2512 nM 5αTHB. The EC50s were calculated from mean data due to inherent variability in the aortic ring assay. Graphs show mean ± standard error of the mean of n=9 for dexamethasone and hydrocortisone and n=7 for 5αTHB. **=p<0.01, ***=p<0.001 vs control, #=p<0.05, ##=p<0.01, ###=p<0.001 vs stimulus. Controls were analysed by Student's unpaired t-test, and steroid doses were analysed by one-way Analysis of variance, followed by Dunnett's multiple comparisons test versus stimulus. Individual data points are shown in Supplementary Figure 1.

Figure 3:

5α-Tetrahydrocorticosterone regulates a different profile of genes from dexamethasone and hydrocortisone in the mouse aorta during angiogenesis. RNA was extracted from stimulated mouse aortic ring sections treated with either vehicle (stimulus only, S), dexamethasone (D; 30 nM), hydrocortisone (HC; 1 µM), or 5α-tetrahydrocorticosterone (T; 3 µM) and analysed by real-time qPCR for abundance of transcripts of genes involved in (a, b, c) inflammatory signalling (*Cxcl5, Dusp1, Mcp1*), (d, e) extracellular matrix remodelling (*Col4a1, Mmp9*), (f) vasculature remodelling (*Pecam1*), and (g, h, i) glucocorticoid-related signalling (*Per1, Hsd11b1, Fkbp51*). The number of RNA samples produced varied according to the number of aortic rings available and were as follows: n=11 for stimulus-only treated group, n=8 for dexamethasone, n=5 for hydrocortisone, and n=9 for 5α-Tetrahydrocorticosterone. Data (mean ± standard error of the mean) were analysed by one-way Analysis of variance followed by Tukey's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001 vs stimulus; #=p<0.05, ##=p<0.01 vs hycrocortisone; $=p<0.05, $$=p<0.01, $$$=p<0.001 vs dexamethasone.

Figure 4:

RU38486 did not antagonise 5α-tetrahydrocorticosterone-mediated suppression of angiogenesis. Steroid-induced suppression of vessel growth from mouse aortic rings was compared in the presence and absence of RU38486 (30 nM). Murine aortic rings were cultured in medium with a stimulus for vessel growth in the presence of dexamethasone (1-1000 nM) or 5α-tetrahydrocorticosterone (5αTHB; 10-10000 nM) alone or in the presence of RU38486 (30 nM). After

7 days the vessels which had grown from the rings were counted and normalized to stimulated controls from the same animal in the presence and absence of RU38486 for (a) dexamethasone, and (b) 5αTHB. Whereas RU38486 antagonized the effect of low concentrations of dexamethasone, it did not antagonise the effect of 5αTHB. Graphs show mean ± SEM of n=8, expect 5αTHB which is n=7. Individual data points are shown in Supplementary Figure 2. * $p<0.05$ vs steroid alone assessed by Student's t-test.

Figure 5:

Transcriptional changes induced by the glucocorticoid receptor agonist dexamethasone, but not those in response to 5α-tetrahydrocorticosterone were blocked by glucocorticoid receptor antagonism with RU38486. RNA was extracted from cultured mouse aortic ring sections treated with vehicle (stimulus only, S) alone, or in combination with either dexamethasone (D; 30 nM), 5α-tetrahydrocorticosterone (T; 3 μM), the glucocorticoid receptor antagonist RU38486 (RU, 30 nM), or a combination of RU38486 and dexamethasone (RU+ D), or RU38486 and 5αTHB (RU + T). RNA was reverse transcribed into cDNA, and real-time qPCR was used to assess the ability of RU38486 to antagonise dexamethasone-mediated changes in transcription abundance of (a) *Cxcl5*, (b) *Mmp9,* (c) *Fkbp51*, (d) *Hsd11b1*, and (e) *Per1*; and 5αTHB-mediated transcriptional changes of (f) *Pecam1* and (g) *Per1*. RU38486 tended ($0.05<p<0.1$) to antagonise the effect of dexamethasone on transcript abundance of *Mmp9* (P=0.089), *Per1* (P=0.058), *Hsd11b1* (P=0.063) *and Fkbp51* (P=0.081). In contrast, in the presence of RU38486 the effects of 5αTHB were unchanged. n=11 for stimulus-only treated group, n=8 for dexamethasone, n=9 for 5αTHB, n=6 for RU, n=5 for RU+D, and n=5 for RU+T. Graphs (mean ± standard error of the mean) were analysed by one-way Analysis of variance followed by Tukey's multiple comparisons test, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$ vs stimulus, ##=$p<0.01$, ####=$p<0.0001$ vs dexamethasone, \$=$p<0.05$ vs RU38486.

Figure 1:

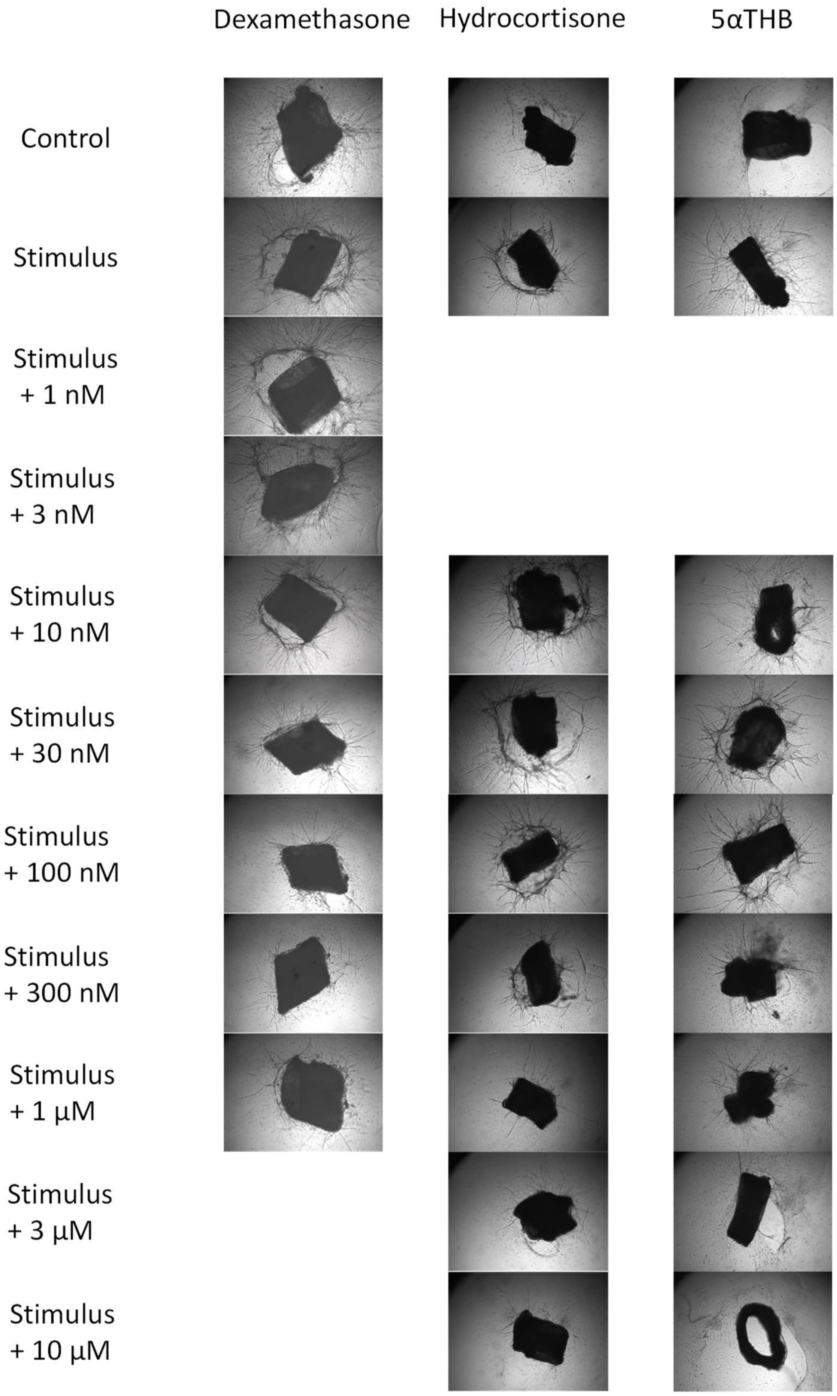

Dexamethasone
Hydrocortisone
5αTHB
Control
Stimulus
Stimulus + 1 nM
Stimulus + 3 nM
Stimulus + 10 nM
Stimulus + 30 nM
Stimulus + 100 nM
Stimulus + 300 nM
Stimulus + 1 µM
Stimulus + 3 µM
Stimulus + 10 µM

Figure 2:

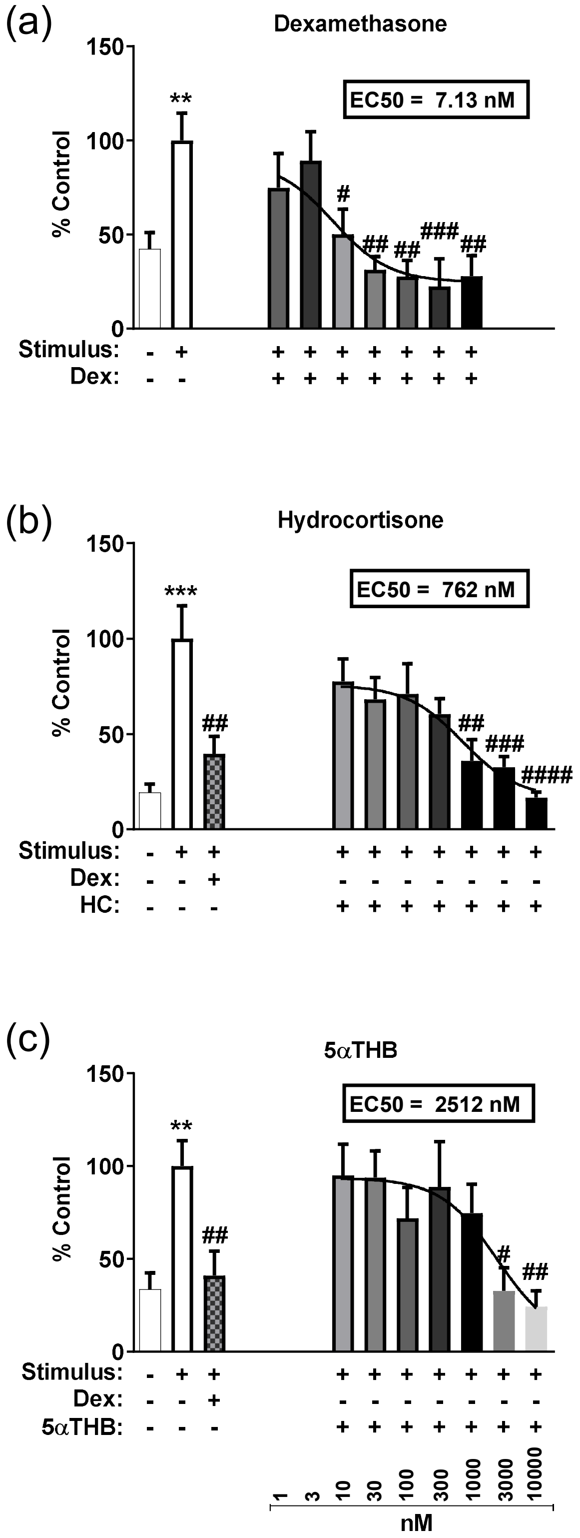

(a)
Dexamethasone
EC50 = 7.13 nM
% Control
150
100
50
0
**
#
##
##
###
##
Stimulus: - + + + + + + + +
Dex: - - + + + + + + +
(b)
Hydrocortisone
EC50 = 762 nM
% Control
150
100
50
0
***
##
##
###
####
Stimulus: - + + + + + + + + + +
Dex: - - + - - - - - - -
HC: - - - + + + + + + +
(c)
5αTHB
EC50 = 2512 nM
% Control
150
100
50
0
**
##
#
##
Stimulus: - + + + + + + + + + +
Dex: - - + - - - - - - -
5αTHB: - - - + + + + + + +
1
3
10
30
100
300
1000
3000
10000
nM

Figure 3:

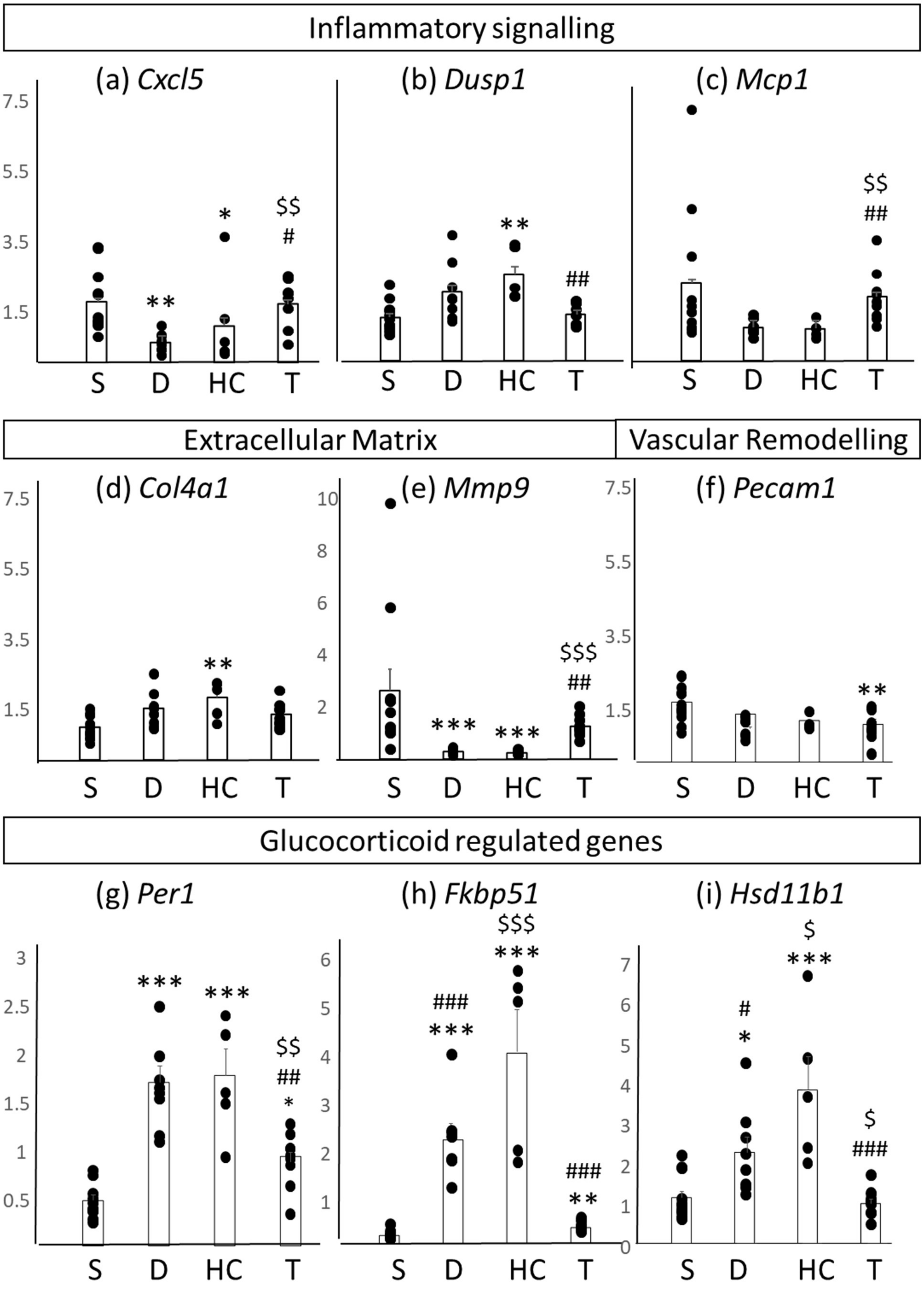
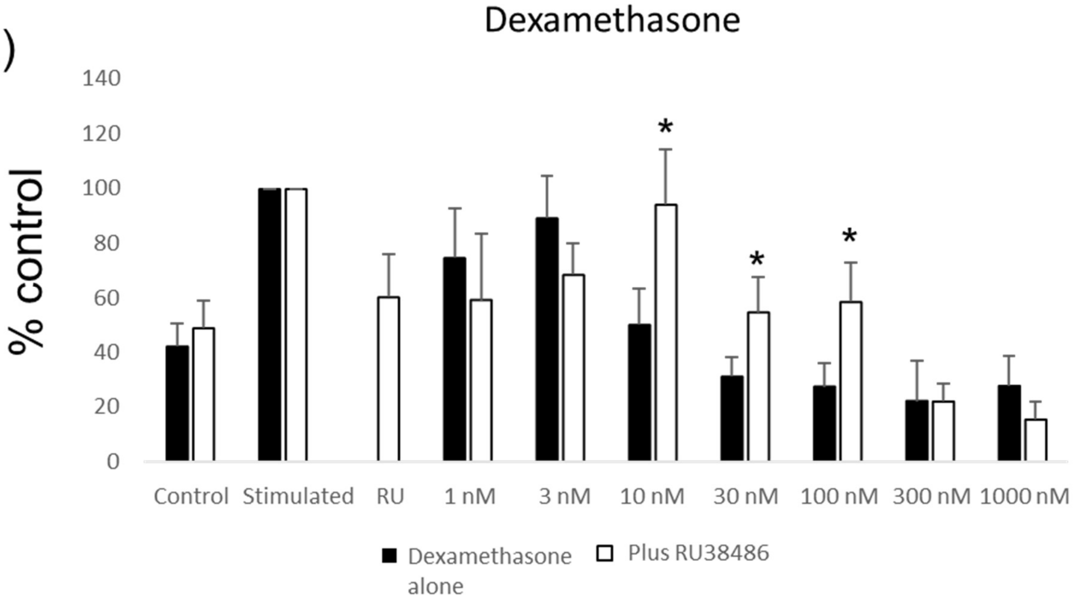


Figure 4

a)


Dexamethasone
% control
0
20
40
60
80
100
120
140
Control
Stimulated
RU
1 nM
3 nM
10 nM
30 nM
100 nM
300 nM
1000 nM
*
*
*
■ Dexamethasone alone
□ Plus RU38486

b)

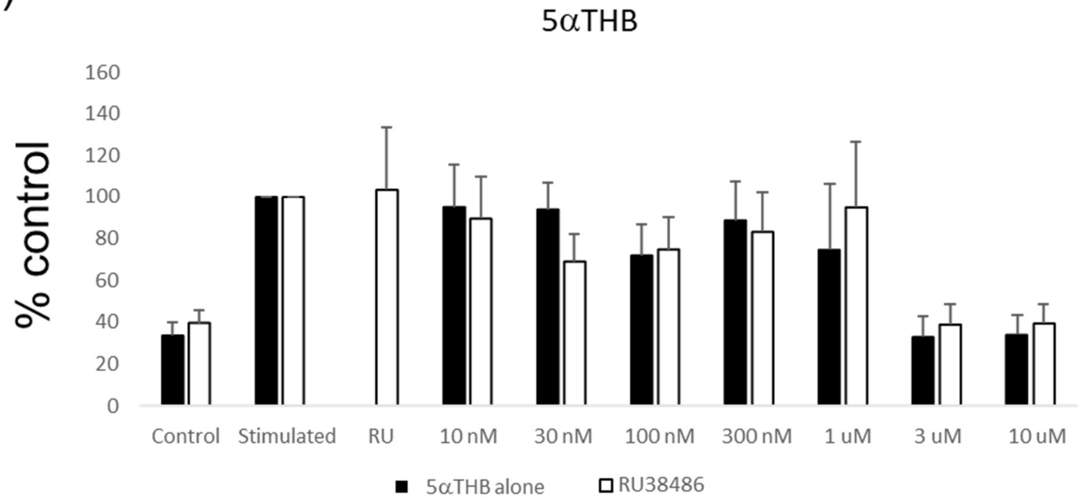

5αTHB
% control
0
20
40
60
80
100
120
140
160
Control
Stimulated
RU
10 nM
30 nM
100 nM
300 nM
1 uM
3 uM
10 uM
■ 5αTHB alone
□ RU38486

Figure 5:

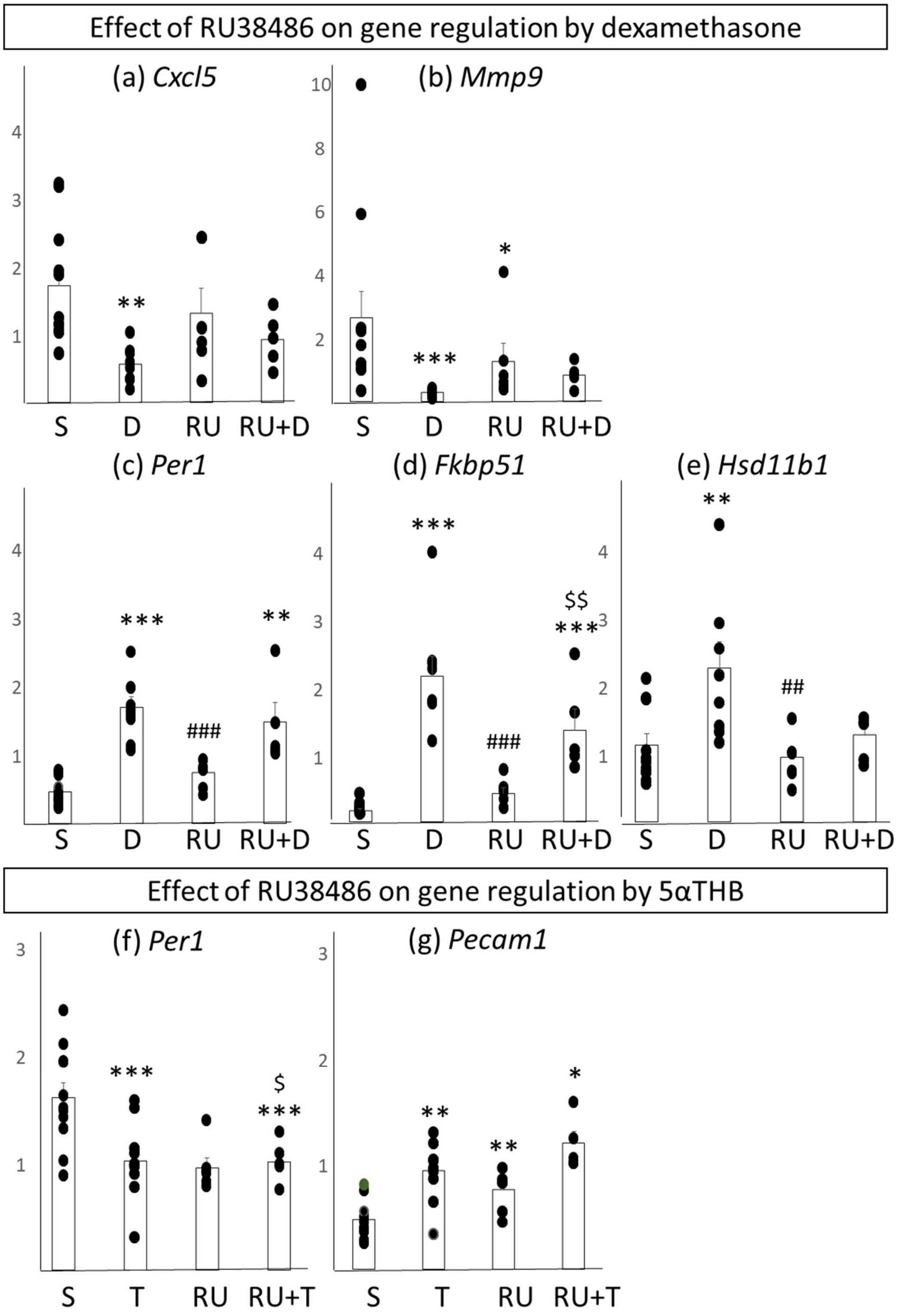

Effect of RU38486 on gene regulation by dexamethasone
(a) *Cxcl5*
4
3
2
1
**
S
D
RU
RU+D
(b) *Mmp9*
10
8
6
4
2
*
***
S
D
RU
RU+D
(c) *Per1*
4
3
2
1
***
**
###
S
D
RU
RU+D
(d) *Fkbp51*
4
3
2
1
***
###
$$
***
S
D
RU
RU+D
(e) *Hsd11b1*
4
3
2
1
**
##
S
D
RU
RU+D
Effect of RU38486 on gene regulation by 5αTHB
(f) *Per1*
3
2
1
***
$
***
S
T
RU
RU+T
(g) *Pecam1*
3
2
1
**
**
*
S
T
RU
RU+T

Table 1:

Details of primers and probes for real-time PCR analysis of mouse aortic ring samples. Any primers used but not listed are reported by (Gastaldello et al., 2017). UPL denotes Universal Probe Library fluorescent probe number (Roche Diagnostics Ltd, Burgess Hill, UK). Gene names: *Cxcl5* = C-X-C motif chemokine 5; *Per1* = Period circadian protein homolog 1; *Hsd11b1* = Hydroxysteroid 11-beta dehydrogenase 1; *Fkbp51* = FK506- binding protein 51; *Actb* =Beta-actin. Reference genes were Actb and TATA-binding protein (*Tbp)*. Primers were designed to match the given intron-spanning probes with the Roche Universal Probe Library (UPL) using the online software Universal Probe Library Assay Design Center which confirms specificity by blasting against the mouse genome (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html#assay-design-center, 2017). Reaction conditions were standardised for all PCR assays, with an annealing temperature of 60°C.

Gene Symbol	**Forward Primer**	**Reverse Primer**	**UPL**	**Accession Number**	**Amplicon size (base pairs)**
Cxcl5	cagtgggtttgagaacaccata	ctggaggctcattgtggac	25	NM_009141	114
Per1	gcttcgtggacttgacacct	tgctttagatcggcagtggt	71	NM_011065	100
Hsd11b1	tctacaaatgaagagttcagaccag	gccccagtgacaatcacttt	1	NM_008288	62
Fkbp51	tgttcaagaagttcgcagagc	ccttcttgctcccagcttt	69	U16959	63
Actb	ctaaggccaaccgtgaaaag	accagaggcatacagggaca	64	NM_007393	114

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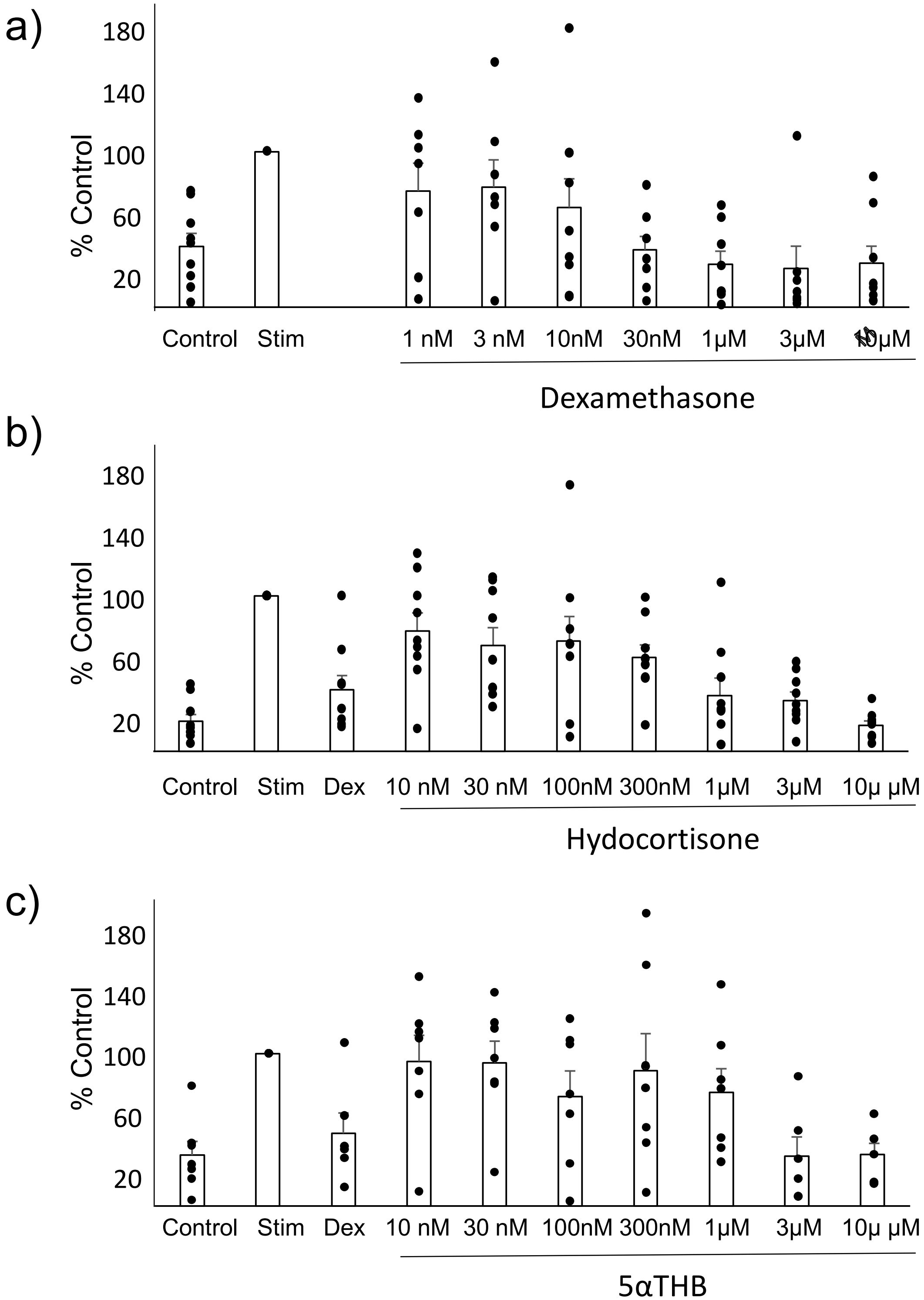
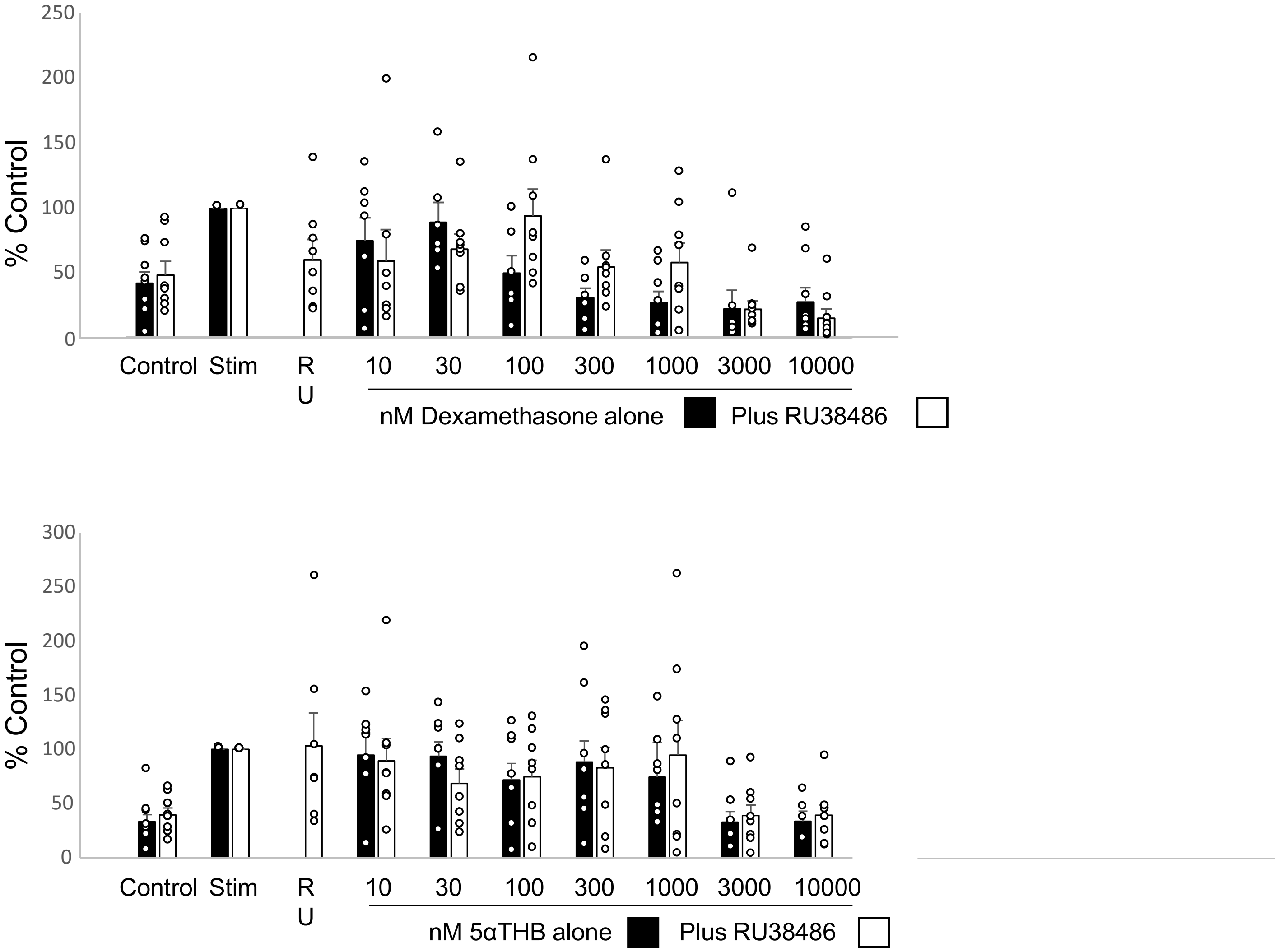
Abernethie et al Supplement Figure 1



5αTHB induced a concentration-dependent suppression of angiogenesis in mouse aortic rings *ex vivo* but was less potent than hydrocortisone or dexamethasone. Aortic rings were maintained in medium either containing vehicle (control), or in medium with a stimulus for vessel growth (Stim) with or without dexamethasone (100 nM Dex; positive control). Concentration-responses were obtained to (a) Dexamethasone (1, 3, 10, 30, 100, 300, or 1000 nM, left to right on graph), (b) hydrocortisone (HC: 10, 30, 100, 300, 1000, 3000, 10000 nM, left to right on graph), or (c) 5αTHB (10, 30, 100, 300, 1000, 3000, 10000 nM, left to right on graph). Vessel outgrowths from aortic rings were counted 7 days after plating. All steroids induced a concentration-dependent suppression of vessel outgrowth. Graphs show individual data points and mean ± SEM of n=8 experiments.

Abernethie et al Supplement Figure 2



RU38486 did not antagonise 5α-tetrahydrocorticosterone-mediated suppression of angiogenesis. Steroid-induced suppression of vessel growth from mouse aortic rings was compared in the presence or absence of RU38486 (30 nM). Murine aortic rings were cultured in medium with a stimulus (Stim) for vessel growth in the presence of dexamethasone (1-1000 nM) or 5α-tetrahydrocorticosterone (5αTHB; 10-10000 nM) alone or in the presence or absence of RU38486 (30 nM). After 7 days the vessels which had grown from the rings were counted and normalized to stimulated controls from the same animal in the presence or absence of RU38486 for (a) dexamethasone, and (b) 5αTHB. Whereas RU38486 antagonized the effect of low concentrations of dexamethasone, it did not antagonise the effect of 5αTHB. Graphs show individual data points and mean ± SEM of n=8, except 5αTHB which is n=7.