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

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Highlights

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

Abstract

5 α -Tetrahydrocorticosterone (5 α THB) 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 5 α THB 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 5 α THB, 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*, 5 α THB suppressed vessel growth from aortic rings, but was less potent than hydrocortisone (EC₅₀ 2512 nM 5 α THB, versus 762 nM hydrocortisone). In contrast to conventional glucocorticoids, 5 α THB 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 5 α THB to suppress vessel growth or regulate gene expression, but modified effects of dexamethasone. 5 α THB did not alter expression of glucocorticoid-regulated genes *Fkbp51* and *Hsd11b1*, unlike hydrocortisone and dexamethasone. In conclusion, compared with hydrocortisone, 5 α THB exhibits limited suppression of angiogenesis, at least directly in blood vessels and probably independent of the glucocorticoid receptor. Discriminating the mechanisms employed by 5 α THB may provide the basis for the development of novel safer anti-inflammatory drugs for topical use.

Keywords: Glucocorticoids, Inflammation, 5 α -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, 5 α -tetrahydrocorticosterone (5 α THB), 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). 5 α THB 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, 5 α THB did not induce systemic side effects and only invoked limited skin thinning. Accumulating evidence suggests that 5 α THB 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 5 α THB 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 5 α THB-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, 5 α THB 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 5 α THB 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 5 α THB (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 °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 μ L, 1 mg/mL, Millipore, Hertfordshire, UK) and incubated (37 °C, 5% CO₂) 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 μ M), or 5 α THB (10 nM-10 μ M), 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 μ M hydrocortisone; 3 μ M 5 α THB) 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 $\text{\textcircled{R}}$ 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 \pm 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 5 α THB on vessel sprouting (described below).

Hydrocortisone (Figure 1 and 2b) and 5 α THB (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 μ M and above. 5 α THB was less potent than hydrocortisone at suppressing vessel growth. It had a higher EC₅₀ (2512 nM) and required a higher concentration (3 μ M) 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 μ M hydrocortisone, and 3 μ M 5 α THB, which suppressed vessel growth to 30 \pm 7%, 35 \pm 11% and 26 \pm 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). 5 α THB did not affect transcript abundance of these genes but *Mcp1* transcripts were significantly higher ($p<0.01$) in aortic

rings exposed to 5 α THB 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). 5 α THB did not alter abundance of any of these gene transcripts. In relation to genes involved in vasculature remodelling, only 5 α THB 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, 5 α THB 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 5 α THB 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 5 α THB 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 5 α THB (Figure 4b).

Finally, the contribution of the glucocorticoid receptor to transcript regulation by dexamethasone and 5 α THB was assessed by investigating the effect of RU38486 to antagonise the changes in gene expression by steroid treatment (either dexamethasone or 5 α THB) 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 5 α THB (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 5 α THB 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 5 α THB as an effective anti-inflammatory steroid (Gastaldello et al., 2017). Due to its pharmacokinetic properties, 5 α THB is better suited to topical application, and studies of irritant dermatitis in mice suggested 5 α THB 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 5 α THB on vessel growth are of interest. Furthermore, glucocorticoid receptor-dependency of 5 α THB's effects on blood vessels was assessed, given that 5 α THB 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, 5 α THB 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 5 α THB 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, 5 α THB 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 5 α THB 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>5 α THB. 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 $\alpha 1$ chain of collagen IV, the main collagen present in basement membrane surrounding endothelial and vascular smooth muscle cells (Vahedi and Alamowitch, 2011). Matrix metalloproteinase 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 5 α THB 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, 5 α THB 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 5 α THB had more limited effects than corticosterone on gene transcripts involved in extracellular matrix homeostasis. However, in both settings (Gastaldello et al., 2017), 5 α THB 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 5 α THB may be mediated by interfering with formation of new endothelial cell-cell contacts. Alternatively 5 α THB 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 5 α THB required to attenuate angiogenesis were greater than those of hydrocortisone and dexamethasone, yet caused fewest changes in gene expression, reinforcing conclusions that 5 α THB may be a safer topical anti-inflammatory steroid. Questions remain as to which vascular cell types respond to 5 α THB but could include endothelial or resident immune cells.

Differences in potency and profile of 5 α THB 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 5 α THB 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 5 α THB only marginally increased *Per1* expression, again suggesting that 5 α THB 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 5 α THB, suggesting again suppression of angiogenesis by 5 α THB is largely independent of glucocorticoid receptors. Effects of 5 α THB 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 5 α THB 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 Buttgerit, 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 (Buttgerit 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 Buttgerit, 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 5 α THB, whereas corticosterone-induced inhibition was evident in 6 hours.

These data add to our understanding of the actions of 5 α THB, 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, 5 α THB was less angiostatic than hydrocortisone and acts through selective mechanisms which differ from classical glucocorticoids. 5 α THB 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 5 α THB 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). 5 α THB is therefore a promising candidate for a safer topical anti-inflammatory therapy. Identification of mechanisms through which 5 α THB signals may lead to development of new prototypes of anti-inflammatory drugs which could also be used systemically with a reduced side effect profile.

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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

5 α -Tetrahydrocorticosterone (5 α THB) 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 5 α THB (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 5 α THB with representative images shown

Figure 2:

5 α -Tetrahydrocorticosterone (5 α THB) 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) 5 α THB (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 μ M HC, and 3 μ M 5 α THB. Concentration-response curves ($R^2 = 0.3403, 0.2398, \text{ and } 0.2763$, 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 5 α THB. The EC₅₀s

were calculated from mean data due to inherent variability in the aortic ring assay. Graphs show mean \pm standard error of the mean of n=9 for dexamethasone and hydrocortisone and n=7 for 5 α THB. **=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:

5 α -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 μ M), or 5 α -tetrahydrocorticosterone (T; 3 μ M) 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 5 α -Tetrahydrocorticosterone. Data (mean \pm 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 hydrocortisone; \$=p<0.05, \$\$=p<0.01, \$\$\$=p<0.001 vs dexamethasone.

Figure 4:

RU38486 did not antagonise 5 α -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 5 α -tetrahydrocorticosterone (5 α THB; 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) 5 α THB. Whereas RU38486 antagonized the effect of low concentrations of dexamethasone, it did not antagonise the effect of 5 α THB. Graphs show mean \pm SEM of n=8, except 5 α THB 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 5 α -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), 5 α -tetrahydrocorticosterone (T; 3 μ M), the glucocorticoid receptor antagonist RU38486 (RU, 30 nM), or a combination of RU38486 and dexamethasone (RU+ D), or RU38486 and 5 α THB (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 5 α THB-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 5 α THB were unchanged. n=11 for stimulus-only treated group, n=8 for dexamethasone, n=9 for 5 α THB, n=6 for RU, n=5 for RU+D, and n=5 for RU+T. Graphs (mean \pm 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:

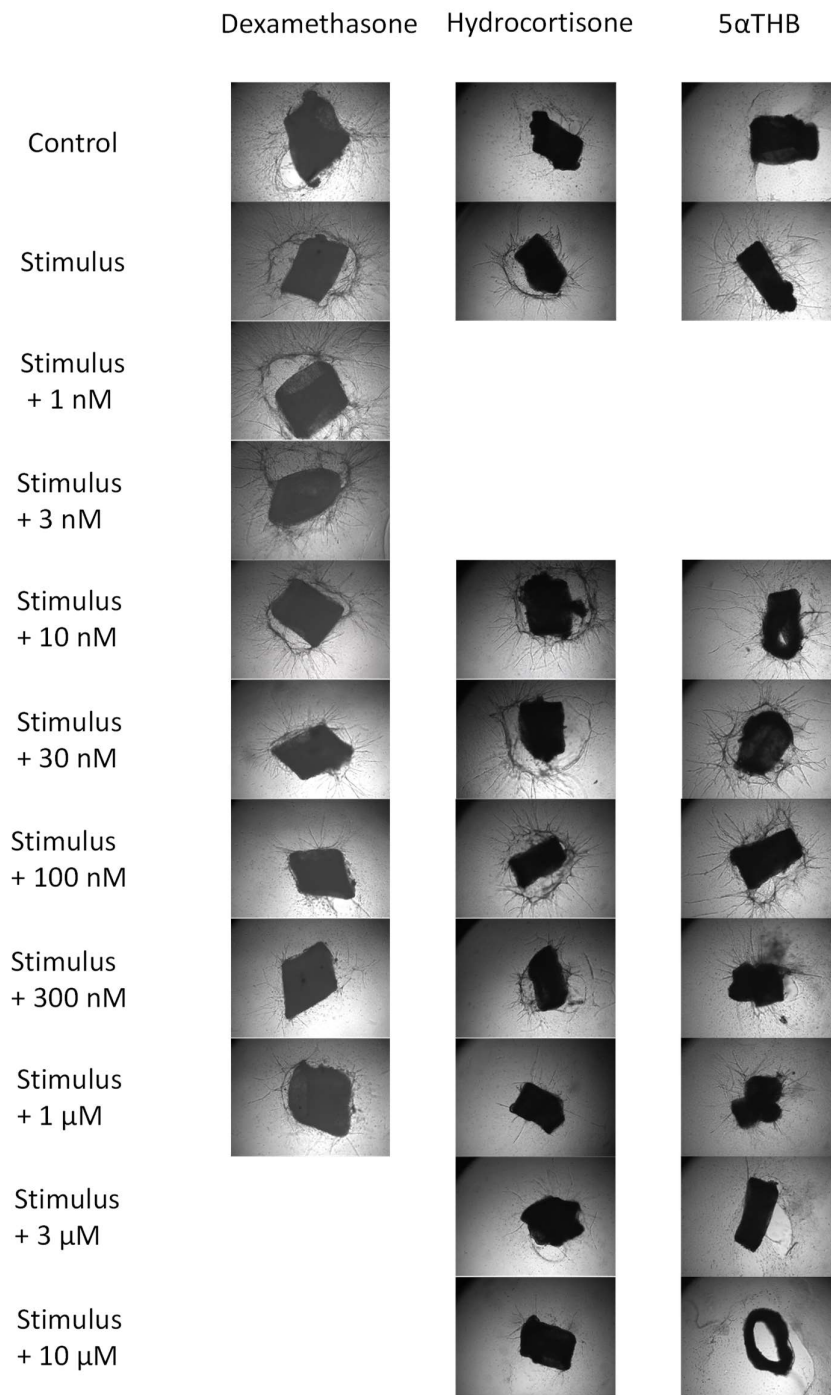


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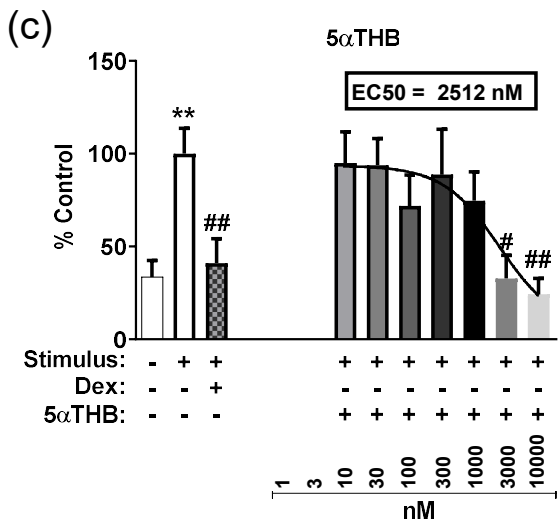
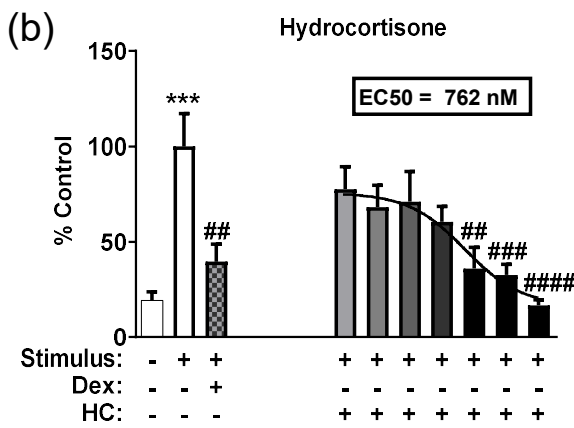
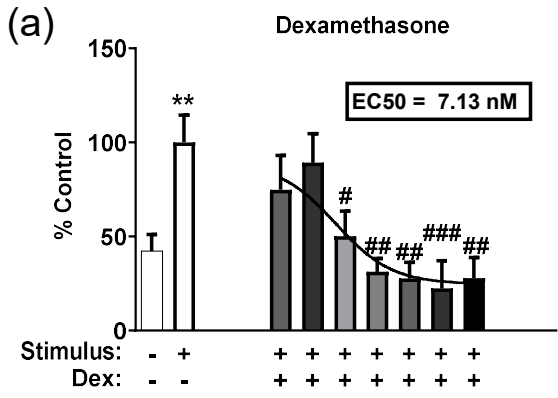


Figure 3:

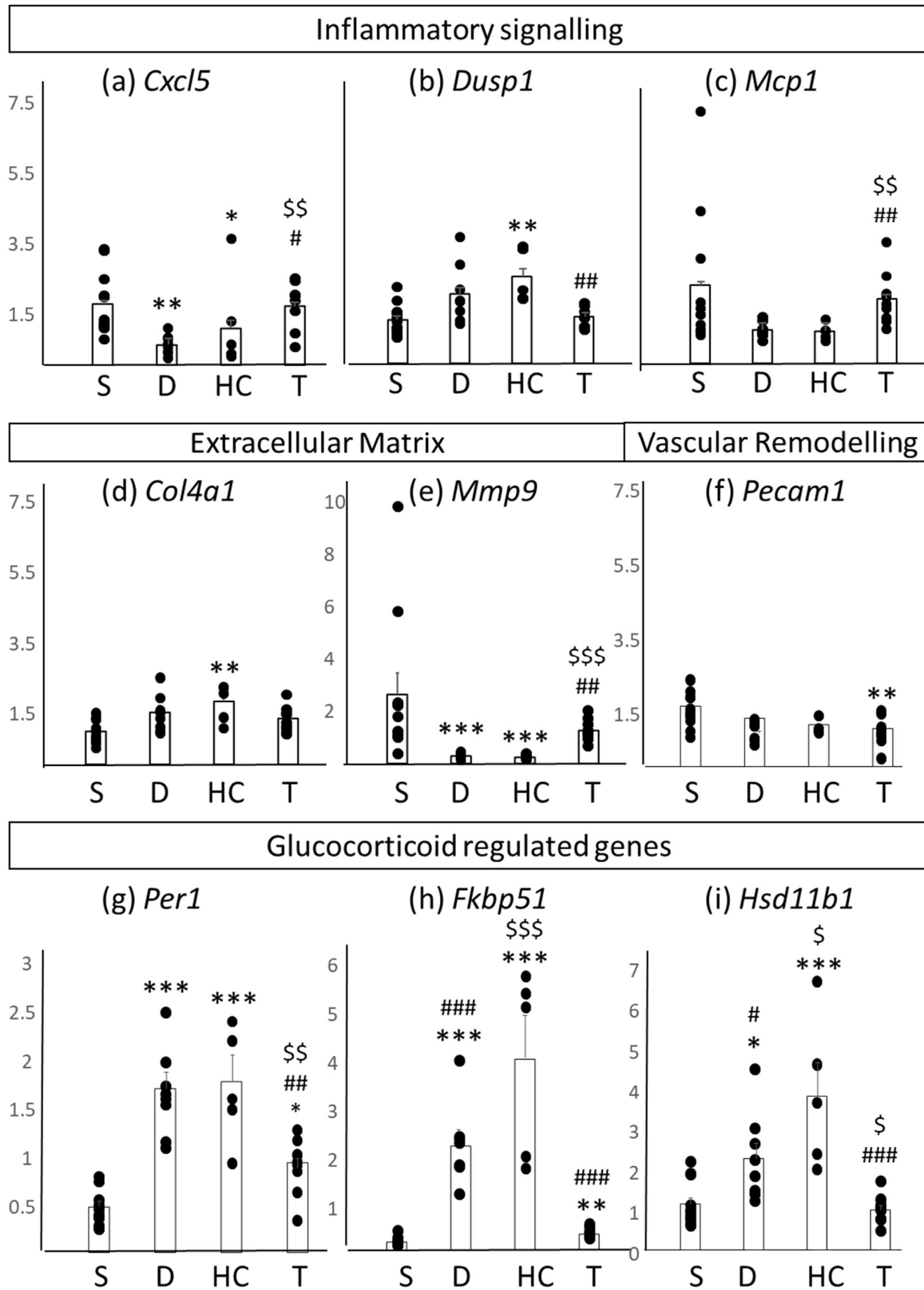


Figure 4

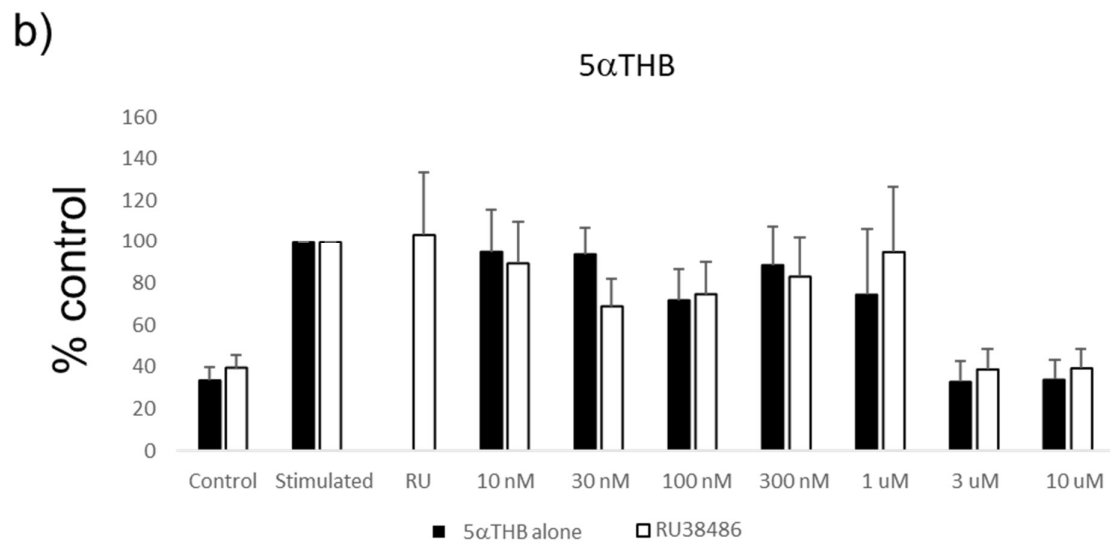
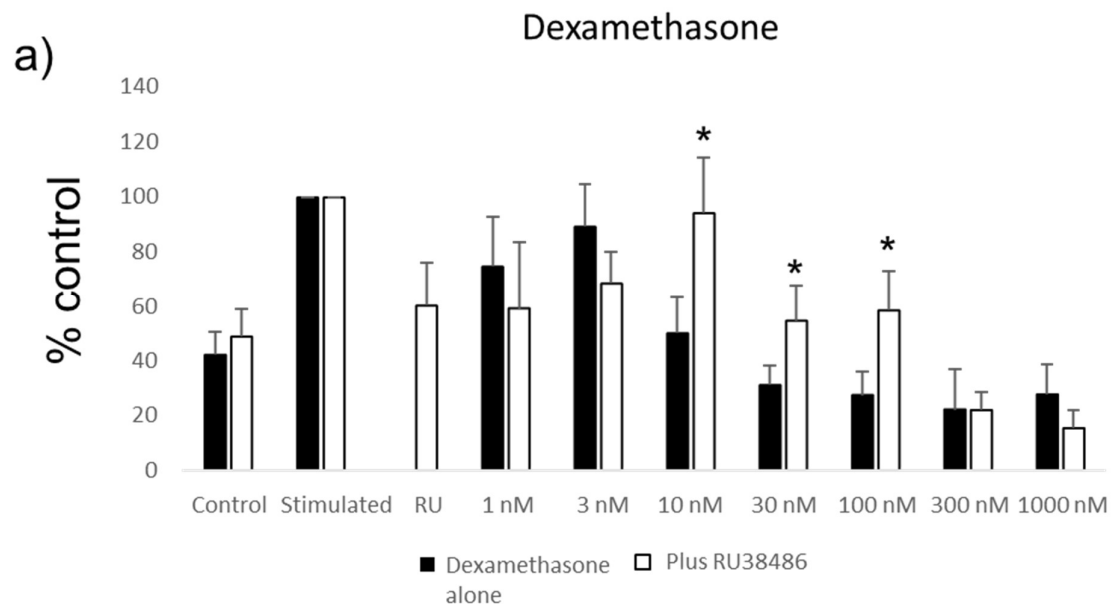
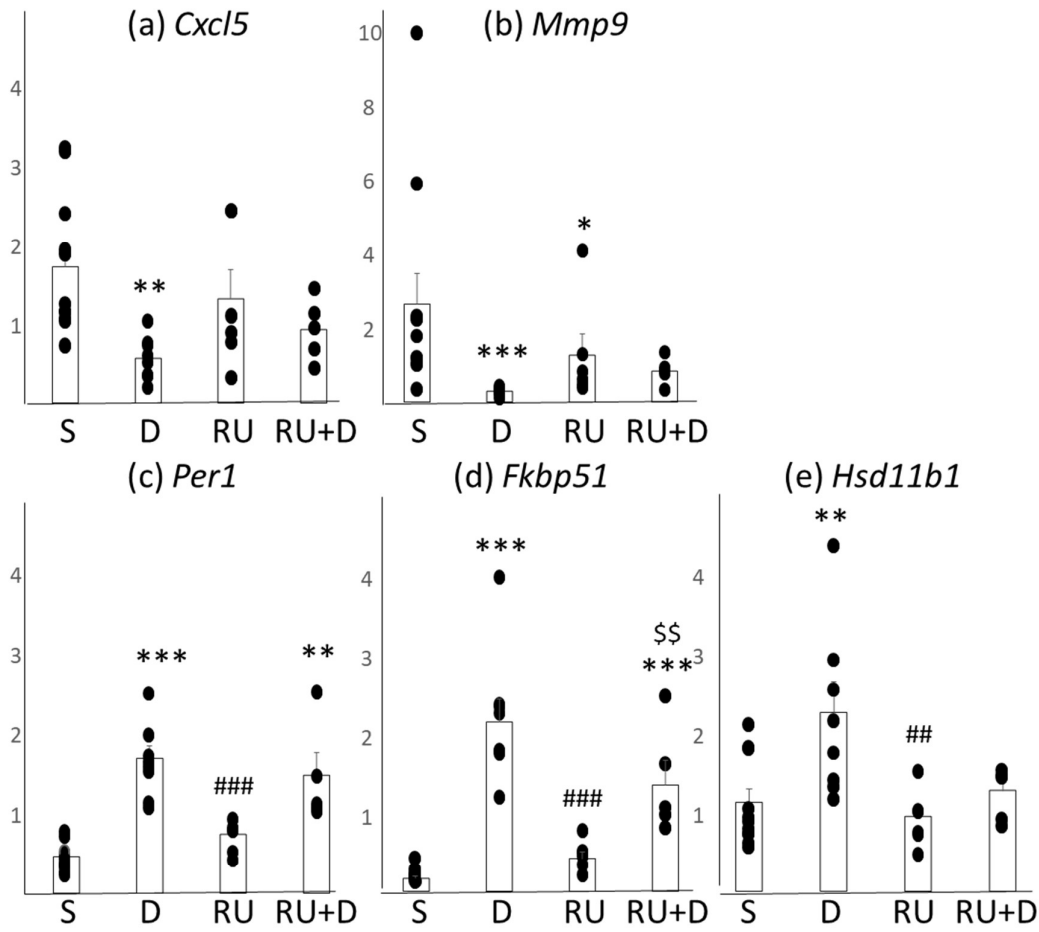


Figure 5:

Effect of RU38486 on gene regulation by dexamethasone



Effect of RU38486 on gene regulation by 5 α THB

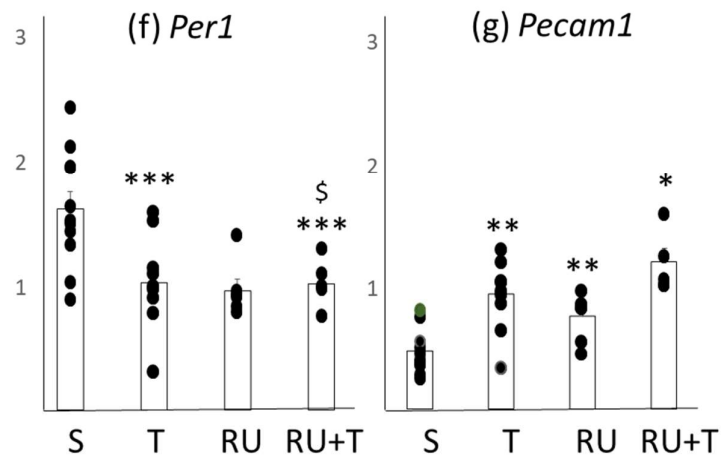


Table 1:

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)
<i>Cxcl5</i>	cagtgggttgagaacaccata	ctggaggctcattgtggac	25	NM_009141	114
<i>Per1</i>	gcttcgtggacttgacacct	tgcttagatcggcagtggt	71	NM_011065	100
<i>Hsd11b1</i>	tctacaaatgaagagttcagacca g	gccccagtgacaatcacttt	1	NM_008288	62
<i>Fkbp51</i>	tggtcaagaagttcgagagc	ccttctgctcccagcttt	69	U16959	63
<i>Actb</i>	ctaaggccaaccgtgaaaag	accagaggcatacagggac a	64	NM_007393	114

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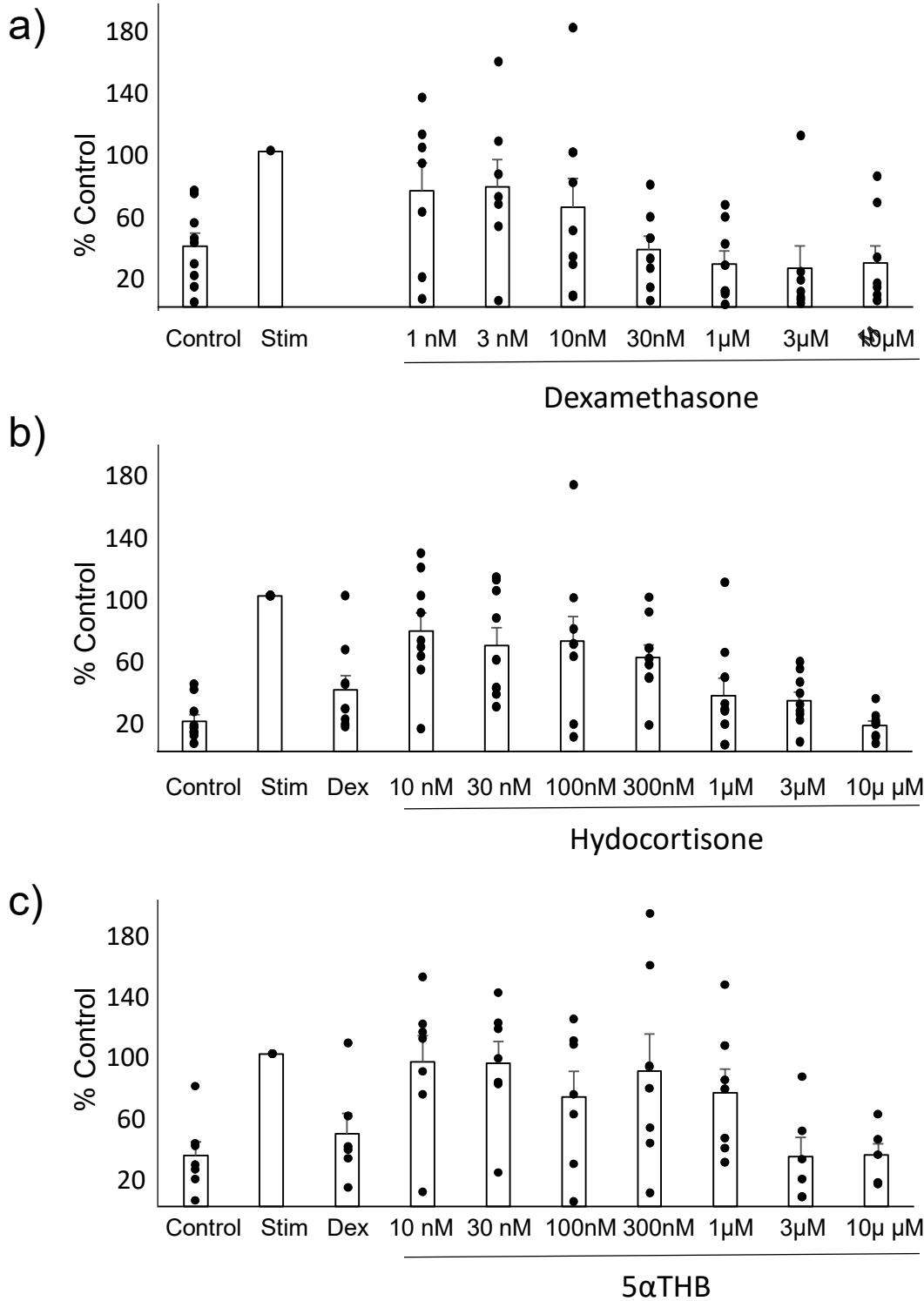
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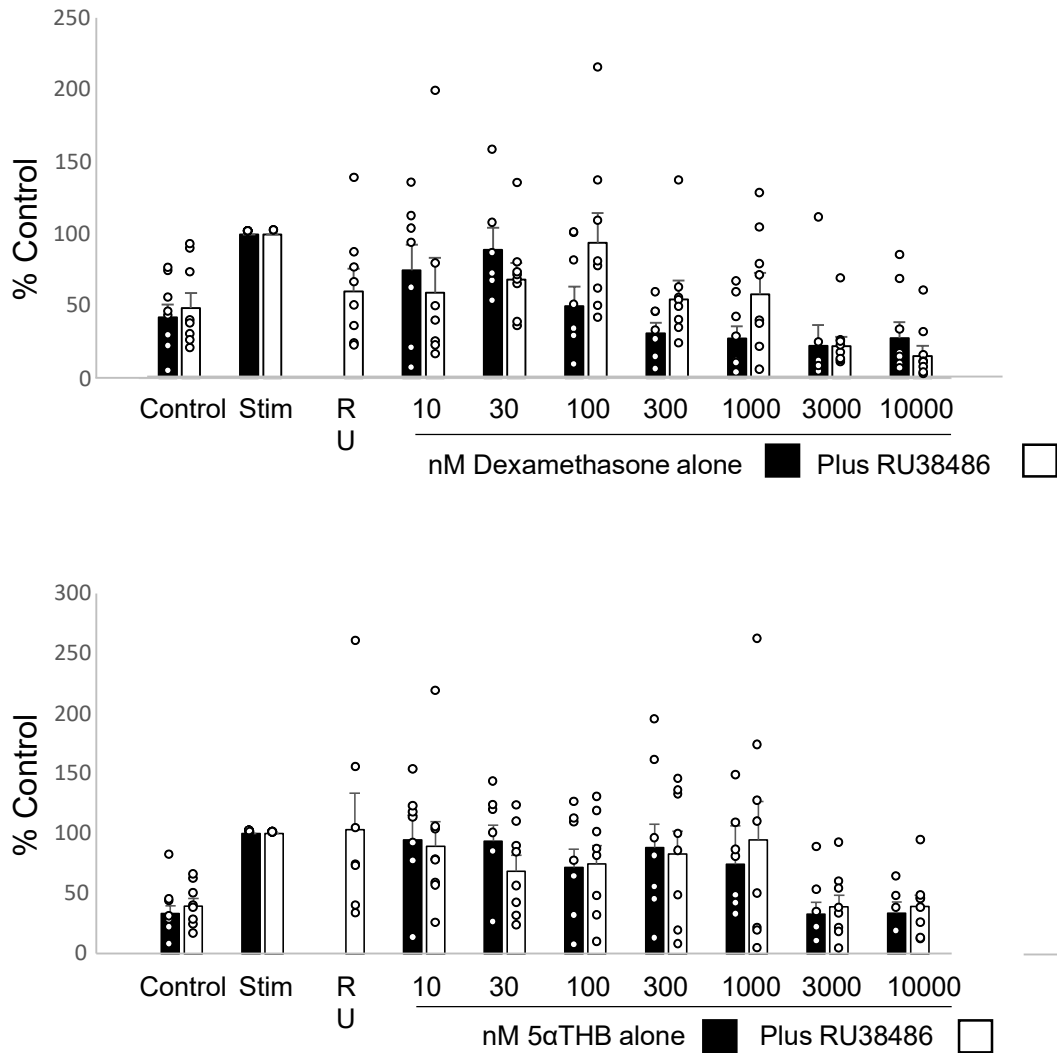
Abernethie et al Supplement Figure 1



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 \pm SEM of n=8 experiments.

Abernethie et al Supplement Figure 2



RU38486 did not antagonise 5 α -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 5 α -tetrahydrocorticosterone (5 α THB; 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) 5 α THB. Whereas RU38486 antagonized the effect of low concentrations of dexamethasone, it did not antagonise the effect of 5 α THB. Graphs show individual data points and mean \pm SEM of n=8, except 5 α THB which is n=7.