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Department of Molecular and Developmental Medicine

Doctorate School of Molecular Medicine

CYCLE XXIX

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11β-HSD1: novel roles and implications in pathology and physiology of reproduction and in skin homeostasis

SCIENTIFIC FIELD: Pathophysiology of Reproduction

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Academic Year: 2015/2016

Abbreviations

11β-HSDs	11-beta-Hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
BMI	Body Mass Index
CRD	Cortisone Reductase Deficiency
CRH	Corticotropin-releasing hormone
E_2	Estradiol
GC	Glucocorticoid
GR	Glucocorticoid Receptor
H6PDH	Hexose-6-Phospate Dehydrogenase
HPA axis	Hypotalamic Pituitary Adrenal axis
IL	Interleukin
INF	Interferon
MPA	MedroxyProgesterone Acetate
MR	Mineralcorticoid Receptor
Nb-UVB	narrow-band ultra-violet Beta
PE	Preeclampsia
PUVA	Psoralen- Ultra Violet Alpha
UV	Ultra-Violet

1. Introduction

1.1 Glucocorticoids and Glucocorticoids Receptor

Glucocorticoids are part of the steroid hormones family, which is also composed by mineralcorticoids, androgens and estrogens. All these hormones are structurally similar and are generated from cholesterol through steroidogenesis, a multi-enzyme process [1]. Glucocorticoids (cortisol, corticosterone) and their analogue similar mineralcorticoid (aldosterone) are products of the adrenal glands [2]. Glucocorticoids exert a wide range of physiological roles, like the regulation of carbohydrate and amino acid metabolism, maintenance of blood pressure and regulation of inflammation and stress [3]. In contrast, mineralcorticoids role is principally to modulate epithelial sodium transport in kidney, where their receptor is located. Both of the two classes act through binding to intracellular receptors (glucocorticoid receptor, GR; mineralcorticoid receptor, MR), with subsequent positive or negative modulation of target gene transcription [4].

Cortisol is the major endogenous circulating glucocorticoid in human, produced by the adrenal cortex in response to adrenocorticotropic hormone (ACTH), released by the anterior pituitary, under the control of corticotropin-releasing hormone (CRH) [5]. ACTH stimulates glucocorticoid synthesis in the adrenal glands. The subsequent release into the circulation results in a systemic effect. The increase in circulating cortisol causes the reduction of the expression of CRH and ACTH, creating the common called 'HPA (Hypothalamic-Pituitary-Adrenal) axis', which operates as a classic negative feedback loop [2]. Cortisol plays important roles in regulating fuel metabolism, energy partitioning and body fat distribution [5], and, although GCs are regulated by circadian rhythm, increased production is associated with stress [6].

More than 90% of circulating cortisol is bound, predominantly to the α_2-globulin, cortisol-binding protein. Only the free fraction is biologically active [4].

Glucocorticoid effects are modulated by the glucocorticoid receptor (GR), the first member of

the nuclear receptor superfamily. GR protein is produced from a single gene, but alternative splicing and alternate translation initiation sites act to generate multiple isoforms. Furthermore, the GR protein can undergo post-translational modifications, such as phosphorylation, sumoylation, ubiquitination, and acetylation, with subsequent alteration of GR functions [7]. A growing number of evidence suggests that the molecular heterogeneity of GR protein influences, at least in part, the sensitivity and specificity of glucocorticoid signalling [2].

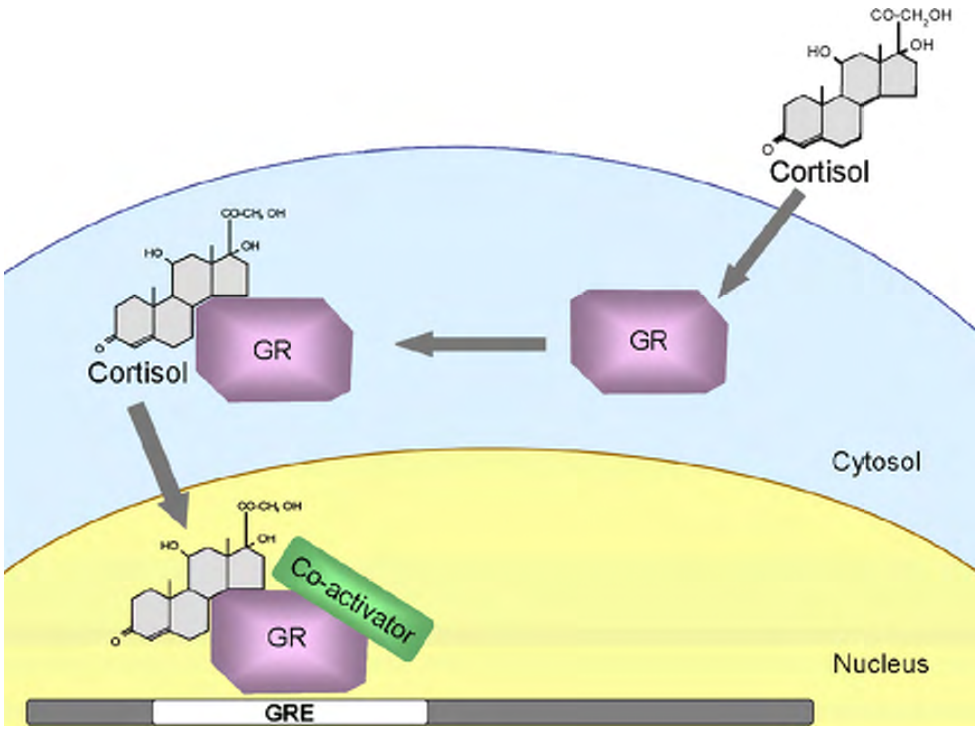

Fig. 1 Schematic representation of Glucocorticoid Receptor (GR) action. Cortisol bind the GR and allows it to move into the nucleus. There, GR binds specific glucocorticoids receptor elements (GRE) regulating transcription. The GR can also be bound by co-activators or co-repressors, adding a further level of regulation. [8]

The GR protein is composed by three domains: an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). [9] The NTD consists of most sites for post-translational modification and an activation function region (AF1); AF1 interacts with co-regulators and the transcriptional machinery. The DBD comprises two zinc-finger motifs which target the glucocorticoid receptor to DNA. The LBD consists in a hydrophobic pocket for glucocorticoid binding, and an AF2 domain for interactions with coregulators. DBD and LBD are divided by a flexible hinge region (H) [2].

In the absence of glucocorticoids, GR is mostly located in the cytoplasm in a complex with chaperones. This complex inactivates GR as a transcription factor, but it maintains high affinity for glucocorticoid ligands. Once binding to GR occurred, it induces a conformational change that dissociates the complex, with a translocation of GR into the nucleus to exert genomic effects [2].

Differences in GR proteins are mostly the result of alternative splicing. In particular, 5 splice variants have been identified. GRα is the full-length protein and represents the common mediator of glucocorticoid effects, but four additional splice variants are known: GRβ, GRγ, GR-A and GR-P. GRβ localizes to the nucleus and does not bind glucocorticoid agonists, although it has been reported to bind the partial GR antagonist, RU-486 [2;9]. Grβ originally appeared to act as a negative inhibitor of GRα [10], with mechanism like competition for GREs (Glucocorticoid Response Elements), interference with co-regulators and formation of inactive GRα/GRβ complex [11]. The other three GR splice variants, GRγ, GR-A and GR-P, were identified in glucocorticoid-resistant cancer cells and later discovered to be expressed in healthy tissues [2], with low transactivation activities [12].

GRα can be expressed as eight different translational isoforms, that originate from GRα mRNA through multiple start codons present on exon 2. These include GRα-A (94 kD) and GRα-B (91 kD), GRα-C1-C3 (82-84 kD) and GRα D1-D3 (53-56 kD). It is hypothesized that the various translational isoforms of GRα work in a tissue specific manner, regulating different transcriptional activities. [7] All GRα isoforms bind glucocorticoid ligands and interact with GREs, but transfection of individual GRα isoforms into cells has revealed different gene as target for each isoforms. [13] GRα isoforms are expressed throughout the body, but the relative abundance of each isoform varies substantially across cell types. GRα-C isoforms, for example, are abundant in the pancreas, lung and colon but are expressed at low levels in the liver. [7] GRα-D isoforms, in contrast, are highly expressed in spleen.

The understanding of the molecular mechanism behind the various glucocorticoid effects has improved with the identification of multiple GR isoforms, whose heterogeneity is further increased by the post-translational modifications. The identification of the GR isoforms expression of a tissue or a cell type could make possible to predict glucocorticoid sensitivity and physiological effects [2].

1.2 The 11β-hydroxysteroid-dehydrogenase enzyme family

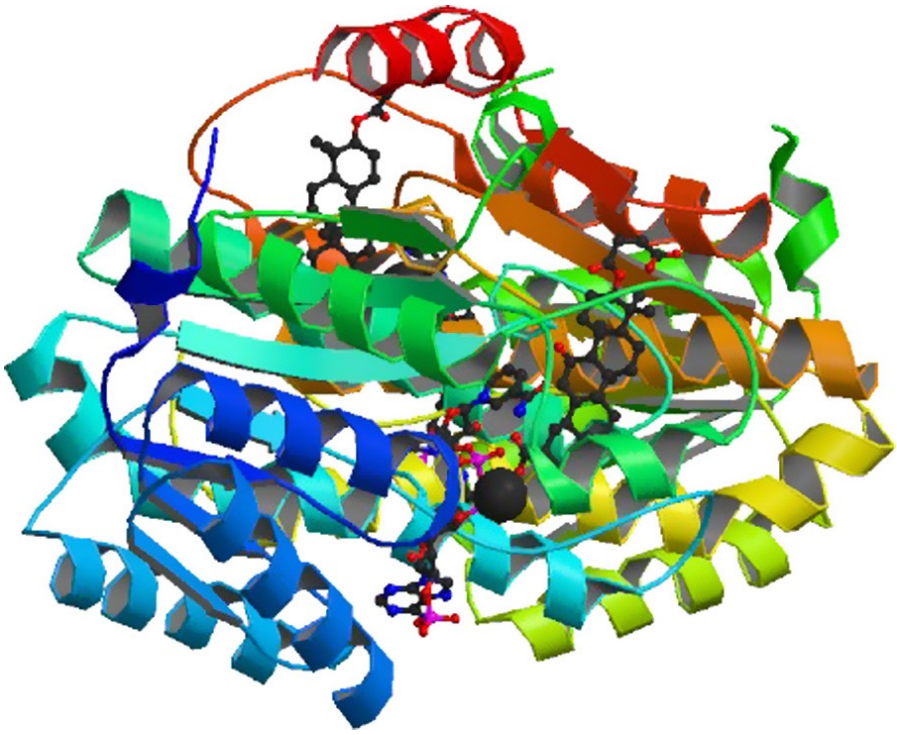

Fig. 2 Structure of a dimer of human 11β-hydroxysteroid-dehydrogenase type 1 in complex with NADP co-substrate (bottom middle) and its inhibitor carbenoxolone (top right) [14].

Although cortisol is produced by adrenal cortex under a systemic control, local availability regulation of active glucocorticoids (cortisol in human and corticosterone in rats) and their inactive metabolites (cortisone and dehydrocorticosterone, respectively) is critical for the maintenance of homeostasis, playing a major role in fundamental physiological processes, such as stress responses, energy metabolism, electrolyte levels, blood pressure, and immunity. Tissue-specific effects of glucocorticoids go far beyond their systemic levels and are modulated by a local intracellular enzyme family, 11β-hydroxysteroid-dehydrogenase (11β-HSDs) [15]. The family is composed of two different isoforms: 11β-HSD1, a low affinity NADP-dependent oxoreductase, that is distributed in most tissues including liver, kidney, decidua and placenta; 11β-HSD2, a high affinity NAD-dependent dehydrogenase present in a range of tissues, in highest abundance in kidney and placenta [16]. 11β-HSD1 is a bidirectional enzyme, acting as

both a reductase (activating glucocorticoids) and a dehydrogenase (inactivating glucocorticoids) [4]. However, it principally acts as a reductase, supported by a higher affinity for cortisone than cortisol [15]. 11β-HSD1 operates in the endoplasmic reticulum (ER) facing the lumen [8], where is abundant NADPH, produced by the activity of hexose-6-phosphate dehydrogenase (H6PDH), that converts glucose-6-phosphate to 6-phosphogluconate in a reaction that regenerates NADPH from $NADP^+$ [15]. 11β-HSD2 acts to inactivate cortisol to cortisone in humans (corticosterone to 11-dehydrocorticosterone in rodents), and was the first 11β-HSD isoform to be identified. Its tissue expression is strictly linked to the presence of mineralcorticoid receptor, for which cortisol has high affinity [15]. The inactivation of cortisol by 11β-HSD2 will prevent their binding to the mineralcorticoid receptor, leaving it available for mineralcorticoids in requiring tissues [15].

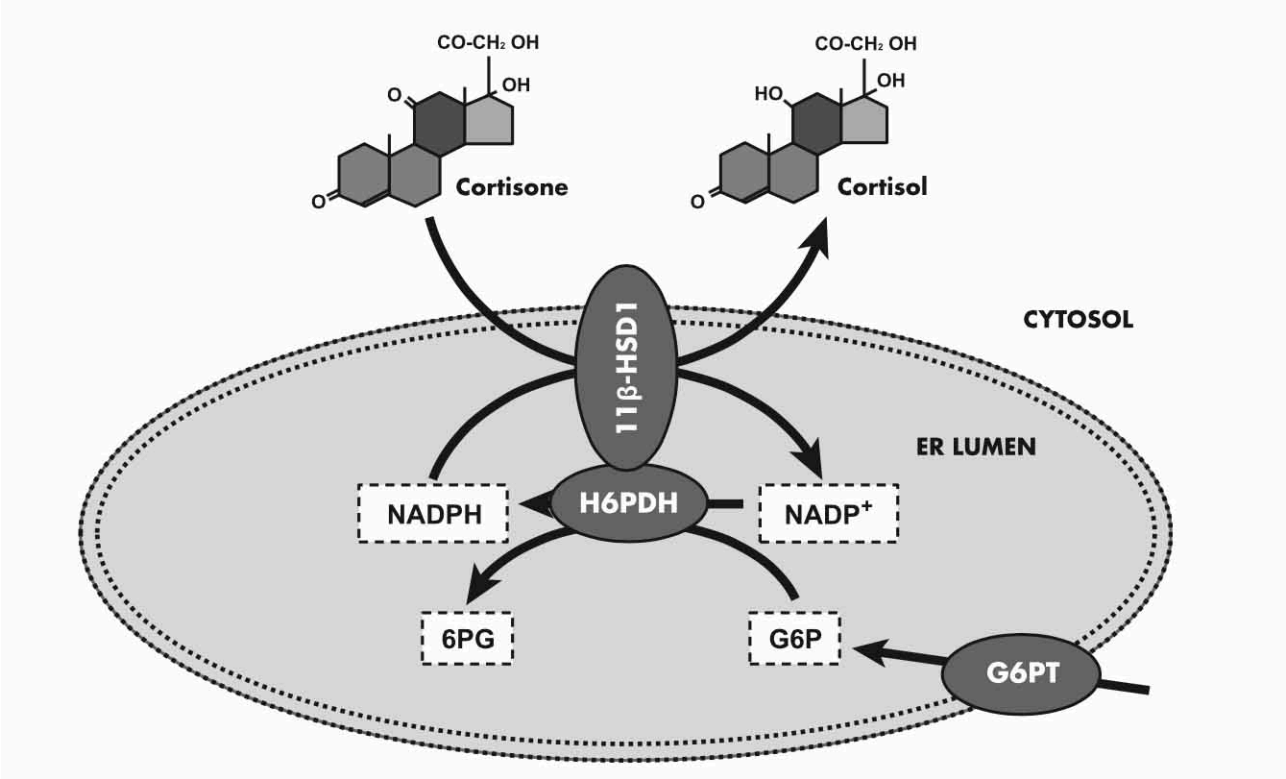

Fig. 3 Schematic representation of 11β-HSD1 and its cofactor NADPH, generated by enzyme H6PDH. Glucose-6-phosphate (G6P) is transported from cytosol to ER lumen by G6P trasporter (G6PT). In ER lumen, G6P is oxidized to 6-phosphogluconate (6PG) by H6PDH and, at the same time, $NADP^+$ is reduced to NADPH. NADPH availability allows 11β-HSD1 to behave as a reductase. [17]

In the late ‘80s, a study showed the first mammalian 11β-HSD1 cDNA cloning, obtained from a rat. The authors indicated an 861-bp open reading frame, encoding a protein of 288 aminoacids[18]–[20]. Afterwards, the same detection has been made for many species, including human. The human gene is over 30 kb in length and is composed of 6 exons and 5 introns [21]. Human expression of 11β-HSD1 is not detectable until 2-3 months of age, when it starts to increase until 1 year of age. From this moment, it maintains a stable level, with highest expression in liver, adipose tissue, gonad and brain [17]. It is also present in bone, kidney, heart and vasculature, immune system, eye, gastrointestinal tract, pancreas, skin, placenta an decidua. In all these tissues, it works to enhance local cortisol concentrations, regardless of circulating levels [4].

There is a wealth of data that implicates 11β-HSD1 deregulation in the pathogenesis of diseases, of which one of the most studied is metabolic syndrome [8].

Metabolic syndrome is one of the most frequent clinical conditions in recent times and represents a combination of cardiometabolic risk determinants, including obesity, glucose intolerance, insulin resistance, dyslipidaemia and hypertension [22]. Patients with cortisol excess, Cushing’s Syndrome, have a phenotype which shares many aspects with the metabolic syndrome [8], although circulating glucocorticoids levels are normal in patients with metabolic syndrome [23][24]. This observation leads to understand the importance of glucocorticoids in the development of the disease and the pre-receptor glucocorticoid activation by 11β-HSD1 has a key role in the process. [22]

About one of the metabolic syndrome risk determinants, formative works in the first half of twentieth century first hypothesized a role for glucocorticoids in the modulation of carbohydrate metabolism and the development of diabetes [25][26], with the subsequent discovery that glucocorticoids promote gluconeogenesis, inhibit glucose utilization and lead to insulin resistance[27][28]. Physiologically, glucocorticoids stimulate hepatic gluconeogenesis and adipose tissue lipolysis, resulting in augmented blood glucose and fatty acid concentrations [8], but, in a fed state, they are able to work together with insulin and support carbohydrate and lipid storage.

Concerning another one of the risk determinants, the visceral obesity, it is known that 11β-HSD1 has also a high expression in human adipose tissue [29], where it acts

predominantly as an oxoreductase and is induced by glucocorticoids and proinflammatory citokines [30],[31]. It has been demonstrated from a number of different models of obesity that a deregulation of 11β-HSD1 occurs in adipose tissue, with an activity increase in visceral adipose tissue [32], [33]. A large number of authors has investigated about the connection between 11β-HSD1 activity in human adipose tissue and obesity and most of these show that the enzyme expression and activity correlate positively with BMI (Body Mass Index) and insulin resistance[34],[35].

Moreover, it is known that obese subjects have higher cortisol clearence, suggestive of increased HPA axis drive [36]. Obese patients show an increase of cortisol secretion in response to ACTH administration[23],[24] and a decrease of sensitivity of negative feedback [37]. Global 11β-HSD1 activity, measured by urinary analysis, resulted decreased [38]. Although this may be a result of a combined evaluation of activity from many tissues, the decrease of 11β-HSD1 activity, in particular at liver level, is supposed to act as a compensatory and preventive mechanism to preserve insulin sensitivity and decrease hepatic glucose output through decreased local cortisol availability. In contrast, in patients with diabetes mellitus type 2 this drop is not observed [39], suggesting that failure to downregulate 11β-HSD1 activity may help to the development of hyperglycemia and diabetes in predisposed obese subjects.

A plethora of studies highlights also the role played by 11β-HSD1 in the regulation of the HPA axis[40], [41]. In particular, defects in 11β-HSD1 activities may cause CRD (Cortison reductase deficiency), in which the impossibility to regenerate cortisol from cortisone finally results in ACTH-mediates adrenal hyperandrogenism. With this disorder, male presents in early life precocious pseudo-puberty and female hirsutism, oligoamenorrhea and infertility [40].

Above all, glucocorticoids are anti-inflammatory agents, with a variety of effects on cells of the immune system, working on multiple signaling patways with targets including NF- kB and MAPK [42].

11β-HSD1 expression and activity are up-regulated by TNFα and IL-1β in a various range of stromal cells, including human osteoblast, adipocytes, amnion fibroblast, chorionic

trophoblasts, synovial, dermal and bone marrow fibroblast, embryonic fibroblast[4]. In cells with mesenchymal origin, pro-inflammatory cytokines simultaneously stimulate and block 11β-HSD1 expression through the NF-kB and p38 MAPK pathways [43].

1.2.1 11β-HSD1 and reproductive tissues

Abovementioned topics are the most studied about the physiologic and pathologic effects of 11β-HSD1 in mammals. However, the enzyme is a principal actor of further processes in other districts, in which it has been little investigated or its effects and roles are still being studied. One of these processes is the reproductive event.

Human parturition can be considered an inflammatory event, regulated by a various number of physiological factors, including steroid hormones [44]. Given the role in the mechanism of inflammation, glucocorticoids may play an essential role in the endocrinology of pregnancy [45] and 11β-HSDs, as the main local regulator of glucocorticoids availability, can be considered fundamental also for reproductive events and their deficiencies can cause problems as happens in the other districts. [46] Nevertheless, there are evidences that excess of glucocorticoids in utero may lead to irregularities of fetal development [45]. For example, pregnant women treated with glucocorticoids, in a situation of preterm birth risk, show a trend to have babies with lower weight [47] and pregnancies with intrauterine growth restriction (IUGR) manifest an increase in endogenous fetal plasma cortisol concentrations. [48] Moreover, in rat and sheep models it has also been reported an association between a prenatal glucocorticoids overexposure and increased blood pressure, due to abnormalities of kidney and cardiovascular system structure [49].

11β-HSD2 provides protection against all these problems connected with the excess of cortisol exposure, converting it into inactive cortisone [45]. In the placenta, 11β-HSD2 maintains the differential levels of glucocorticoids between mother (high) and developing fetus (low) [50]. It is known that reductions in placental expression of the 11β-HSD2 in human pregnancies can cause problems in fetal development, as IUGR [51].

Despite the importance of maintaining a low level of glucocorticoids during pregnancy to avoid complications, there appears to be a significant production activity of cortisol by the 11β-HSD1 during gestation [4]. Expression of 11β-HSD1 has been described in placenta from human [52] and rat [53], with a prevalence of reductase activity [54]. The enzyme was been found also in trophoblast, chorion, amnion and uterus [55][52][45]. The reason why glucocorticoids are required during pregnancy despite involving issues is not easy to explain, but it is to be found in the extraordinary physiology regulating gestation and labour, processes where a fetal graft is first to be tolerated, nourished, then allowed to grow and expelled, modeling the mother's body to let it all [44]. The complexity of this set of processes is possible only with the finest modulation achievable of all the actors and glucocorticoids are one of them.

Specifically, glucocorticoids play a plethora of critical roles in prenatal phases, both in structural development of organs and in functional maturation of several key tissues, notably in preparation of birth [56]. In early gestation, GCs can act as signal of suboptimal environmental conditions and modify fetal development in relation to the available resource for intrauterine growth [57]. Along the development of fetus, it is known that GCs contribute to promote the maturation of organ systems [49] and their importance during the growth has been demonstrated by the analysis of the expression patterns GR and MR. GRs are expressed in most fetal tissues including placenta from early embryonic stages [56] and mice Knock-Out for GR die shortly after birth, indicating that GCs are essential for survival [58]. In particular, in humans embryos GR expression has been detected in most tissues already since the eight week [59].

Concerning the different actors of reproduction, it is known that late human gestation is associated with an increase in the concentration of cortisol in fetal circulation and amniotic fluid, accompanied by an increase of 11β-HSD1 expression and activity in human fetal membranes [60]. This situation suggest a possible role for the enzyme as the extra-adrenal source of cortisol.

Despite the importance of local active glucocorticoid producer 11β-HSD1 in pregnancy, relatively little is known concerning its expression/activity in uterus. No definitive study have actually clarified the expression profile of 11β-HSD1 protein in uterus [46].

Thompson et al. detected 11β-HSD1 transcript in the stroma of decidua and in the epithelial cells of endometrium [61].

About the first part of gestation, it is known the wide effect of glucocorticoids on decidualization of endometrium and implantation in human [62], although the possible links between their action in uterus and the intricate endocrine system regulating the implantation are still unclear. It is known that GCs regulate several key-events of early pregnancy, including inflammatory response and trophoblast invasion and that the GR is expressed in endometrial stromal fibroblasts in first part of gestation [63]. Previous studies reported an up-regulation of 11β-HSD1 in endometrial stromal cells by steroid hormones [62], the main engines of the gestation, suggesting a possible implication of glucocorticoids local modulation in endometrial regulation.

At the end of the pregnancy, it was demonstrated a dramatically up-regulation of 11β-HSD1 in uterine myometrium of the rat in the days before the labour [64], suggesting also the existence of a role for 11β-HSD1 in myometrial contraction [64][65], final and decisive event for the labour. In particular, glucocorticoids might influence myometrial contractility by stimulation of prostaglandin production, directly involved in myometrial activation [65].

It is more difficult to have a general framework about the role of 11β-HSD1 in uterus along every stage of the gestation, especially in human, because of the nature of its long pregnancy process and this has been the focus of my work.

1.2.2 11β-HSD1 in skin

Human skin is an intricate organ acting to protect the body from the external stimuli, including water, gases e microbial invasions [66]. It represent the second largest organ, behind only the vascular system [67].

Skin is composed by two morphologically distinct compartments, the epidermis and the dermis, which contribute at different levels to set, preserve and restore tissue homeostasis [67].

Skin undergoes dramatic changes during chronological aging, that impair its functional properties. In particular, major pathophysiological damage include thinning of the epidermis and dermis, biosynthetic capacity reduction and fibroblast number

diminution, progressive disappearance of elastic tissue in the papillary dermis and skin collagen drop [68]. Skin aging is usually divided into two categories: intrinsic and extrinsic [69]. While the intrinsic aging factors are typically not editable and related to genetic characteristics, the extrinsic are more modifiable with different lifestyle choice, in particular smoking, alcohol and UV exposition [70]. Prolonged Ultra-Violet (UV) exposition cause a wide range of effects detrimental to skin structure, as rhydites, depigmented lesions and loss of tone and elasticity [69] [71].

Interestingly, many of these adverse effects are also inducible by glucocorticoids. [72] GC excess may cause severe skin damage in a manner comparable to that of aging, reducing collagen mass, hyaluronic acid and elastin synthesis, and affect keratinocytes, decreasing their size and suppressing proliferation [72]. Collectively, these actions result in epidermal thinning, with disruption of the skin barrier's function [73].

Nevertheless, GC, as the most used class of anti-inflammatory drugs, are highly effective also for the topical treatment of inflammatory skin disease [73]. The effect of topical corticosteroid is usually early, but the problems with topical corticosteroid are the late effects, as suppression of the adrenal function and skin atrophy [74]. GC levels are subjected to the local regulation by 11β-HSDs family, as in the other tissues [71]. 11β-HSD1 converts inactive cortisone to active cortisol, requiring NADPH as cofactor [4]. GC signalling is mediated through Glucocorticoid Receptor (GR, α form), as described in previous section (Chap. 1.1). In human skin, 11β-HSD1 is mostly expressed by epidermal keratinocytes, dermal fibroblasts and endothelial cells [69] , where 11β-HSD2 is expressed in a lesser amounts [75].

It is known that UV-exposition stimulates GC activation. [76] This activation involves the whole apparatus necessary for the steroidogenesis, also expressed in skin [76]. Nonetheless, previous studies reported an increased 11β-HSD1 protein expression in skin biopsied from photo-exposed compared to photo-protected controls [77][71], suggesting a contribution of the enzyme in increased GC availability after UV-exposition.

UV-exposition, as the topic glucocorticoids, is not only a problem for skin. Phototherapy is also one of the possible treatments for the severe skin diseases, such as psoriasis or atopic dermatitis, with minimal or no systemic side effects. [78]

Since early 60s, it is known that already a single dose of UV-B induces a decrease of the synthesis of DNA, RNA and proteins, that causes a reduced rate of mitosis of the cells of epidermis [79]. Two-three days after irradiation, this early effect is then inverted, the repair starts and the cells proliferate again. For this reason, an efficient UV-B phototherapy needs at least a single session for days to be effective [79]. The traditional broadband UV-B sources were the first to be used for phototherapy and they are still considered in some clinical units, but today they have lost their popularity [80]. Today, the traditional phototherapy has been replaced by narrow-band UV-B (311-312 nm) phototherapy [79], thanks in particular to its superiority for both remission time and clearing. [81] Despite narrow-band UV-B is well tolerated, it causes some side effects, however. A part the risk of erythema, there is a notable constant drying of the skin, with consequent pruritus [79].

One of the most common skin disease treated with UV-exposition is psoriasis. Psoriasis is an inflammatory skin disease, immune-mediated, with various severity range and chronic trend [82]. Psoriasis is characterized by proliferation of epidermis and the predominant form of the disease is plaque psoriasis (80% of cases) [83]. The immune system has a primary role in the development of this disease [83]. The onset of symptoms appears around 28 years, but approximately a quarter of patients reported psoriasis beginning before age 18 [83]. It may progressively aggravate with age and its severity is dependent on inheritance and environment factors [83].

Despite causes are not fully known, studies have recognized several risk factors, including family history, smoking, obesity, stress and alcohol [84]. Psoriasis is estimated to affect about 2-4% of the population in western countries [85][86].

Treatments for psoriasis include topical corticosteroids, tars, anthralin, vitamin D analogues and, as already mentioned, phototherapy with UVB, narrow-band UVB and psoralen with ultraviolet light A (PUVA) [83]. The initial dose for narrow-band UVB depends on the skin type and possible fixed increments depends on skin reactions [87]. Upon clearing, treatment is either discontinued or, in some institutions, the patients are subjected to maintenance therapy for 1-2 months [87]. Some units tend to use some adjunctive agents and combination therapies in order to improve efficacy. Narrow-band UVB has been successfully used in a variety of combination therapies, such as with

anthralin [87].

Although GC effects on skin are well documented, real mechanism of regulation of local GC by 11β-HSD1 in skin remain largely unexplored, compared to the extensively documented roles in other organs [71]. It is known only the direct activation of the enzyme in mice skin after UVB irradiation [71]. However, it remains rather unexplored in human, in particular the UVB effect on the GC pathway in skin disease, such as psoriasis. Considering the positive effect of the UV-treatment on the disease symptoms, it is conceivable a possible modulation of 11β-HSD1 after the irradiation and its possible implication in the ameliorative mechanism.

2. Aim Of The Study

Considering the profound importance of glucocorticoid modulation by 11β-HSD1 in abovementioned processes of implantation, fetal growth and labour, we investigated on 11β-HSD1 role along the pregnancy, from the implantation, passing through the first trimester, mid-pregnancy until the myometrial contraction of the labour, using a multiple-models approach; we considered the possible modulation of the enzyme by hormonal and inflammatory pathways, trying to understand its implications in physiology and pathologies of gestation.

Moreover, we studied about the possible role of 11β-HSD1 in the skin pathology of psoriasis, giving a possible explication of the UV-approach for the patients, widening our action field and highlighting the multidisciplinarity and the multitasking action of 11β-HSD1.

3.Results

3.1 Expression and regulation of 11β-HSD1 in first trimester decidua human cells: implication in preeclampsia

The aim of the work presented in this first section was to determine and characterise the expression of the 11β-HSD family in first trimester decidua, evaluating also the potential role of steroid ovarian hormones and inflammatory cytokines in modulation of the enzyme expression. On the sidelines, we have investigated on the decidual enzyme expression in preeclampsia samples, analysing its possible role in the disease.

We considered the decidual cells as primary actors of pregnancy, with a decisive role for implantation of blastocyst and for the pursuance of the first trimester of pregnancy. Despite the successful implantation depends mostly on ovarian steroids, glucocorticoids are considered important for several key-aspects of early pregnancy, including trophoblast invasion. Considering the crucial role of the 11β-HSD family for the maintenance of glucocorticoids balance, we investigate about them among the first trimester of pregnancy.

Our results showed an unbalanced distribution of the two enzyme forms in human decidua cells (11β-HSD1 43-fold increase over), suggesting a greater weight of the form 1 in the modulation of the first part of the gestation. Therefore, we explored the possible modulation of the form 1 by two protagonists of gestation, such as steroid hormones and inflammatory cytokines. We found that both hormones and cytokines had effects on the enzyme expression and activity. In particular, while E_2 (estradiol) alone had no effect, medroxyprogesterone acetate (MPA) alone and in combination with E_2 stimulate an increase in mRNA and protein expression. Moreover, MPA was able to enhance the protein activity. In addition, the analogue experiment with cytokines showed a positive modulation of 11β-HSD1 by interleukin (IL)-1β, alone and in combination with interferon (INF)-γ. Progesterone is the initiator and modulator of decidualization, regulating also the implantation. These results denote for the first time a new role for progesterone in first trimester, as modulator of the GC levels through the induction of 11β-HSD1. Such as progesterone has the role of the starter of the process, cytokines as IL-1β, with chemokines and prostaglandins, are essential for implantation, in the cross-talk between maternal decidua and embryo. Glucocorticoids assist these phases, contributing in the control of trophoblast growth and invasion and acting as anti-inflammatory mediator in several implantation-related events. Our results of 11β-HSD1 expression and activity increase after stimulation with cytokines

represented a novel mechanism in which inflammatory mediators affect key-implantation events, regulating GC levels.

Finally, we explore levels of the enzyme in preeclampsia (PE). Our results highlighting a major expression of 11β-HSD1 in decidua of PE patients, augmenting cortisol availability at the maternal-interface in PE, suggesting a role of the enzyme not only in the physiology, but also in pathological changes of trophoblast tissues.

Molecular and Cellular Endocrinology 437 (2016) 163–170

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Expression and regulation of 11β-hydroxysteroid dehydrogenase type 1 in first trimester human decidua cells: Implication in preeclampsia

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ARTICLE INFO

Article history:
Received 9 August 2016
Accepted 12 August 2016
Available online 18 August 2016

Keywords:
11beta-HSD
Decidua
Steroids
Cytokines
Preeclampsia

ABSTRACT

Glucocorticoids are implicated in successful blastocyst implantation, whereas alterations in glucocorticoid levels are associated with various pregnancy disorders including preeclampsia. Tissue concentration of active glucocorticoids depends on the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD). This study investigated the contribution of first trimester decidua to glucocorticoid availability at the fetal-maternal interface by assessing the expression and regulation of 11β-HSD in human first trimester decidual tissues and cells and by evaluating 11β-HSD levels in preeclamptic vs. gestational age-matched decidua. 11β-HSD1 was the predominant isoform in first trimester decidua. *In vitro*, decidual cell 11β-HSD1 levels and enzymatic activity were up-regulated by ovarian steroids and inflammatory cytokines. Higher levels of 11β-HSD1 were found in preeclamptic decidua compared to controls. The present study indicates the predominance of 11β-HSD oxoreductase isoform in early decidua. Observations that ovarian hormones and inflammatory cytokines up-regulate 11β-HSD1, together with increased 11β-HSD1 expression in preeclampsia, highlight a role for decidual cells in controlling biologically active glucocorticoids in early pregnancy.

1. Introduction

Decidualization of human endometrium is initiated in the late secretory phase of the menstrual cycle and involves cell differentiation, tissue remodelling and an inflammatory-like response that is required for blastocyst implantation (Strowitzki et al., 2006). In early gestation, the fetal-maternal interface consists of a decidual matrix composed primarily of resident decidual cells and an immune cell population intermixed with trophoblasts. Decidual cells are therefore ideally positioned to regulate the primary steps of implantation by controlling trophoblast behavior. Implantation in humans begins with the apposition and attachment of the blastocyst to the uterine epithelium and continues throughout the first trimester of pregnancy with extravillous trophoblast, derived from anchoring villi, invading the maternal decidua and uterine spiral arteries (Kliman, 2000). Defective trophoblast invasion is implicated in pregnancy disorders such as spontaneous preterm birth (PTB), preeclampsia (PE) and intrauterine growth restriction (IUGR) (Khong and Brosens, 2011; Romero et al., 2014). Incomplete cytotrophoblast invasion and associated re-modelling of spiral arteries is generally considered the *conditio sine qua non* of PE (Fisher, 2015). Both decidualization and embryo-decidual interactions are strongly influenced by ovarian steroids (Kodaman and Taylor, 2004) and by a complex network of peptides, hormones, inflammatory cytokines and growth factors that act by regulating endometrial receptivity and in establishing the embryo–maternal dialogue (Achache and Revel, 2006; Makrigiannakis et al., 2011).

Glucocorticoids (GCs) control several key-aspects of early pregnancy including maternal immune response, inflammatory

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http://dx.doi.org/10.1016/j.mce.2016.08.023
0303-7207/

reaction and trophoblast invasion. Glucocorticoid receptors (GR) are highly expressed in endometrial stromal fibroblast and endothelial cells (Bamberger et al., 2001), in first trimester (McDonald et al., 2006) and term (Sun et al., 1996) decidual stromal cells and in isolated population of uterine natural killer (uNK) cells (McDonald et al., 2006). GR has also been detected in term and first trimester placenta (Speeg and Harrison, 1979) and primary cytotrophoblast cultures (Driver et al., 2001). Excess GCs has been shown to lead to abnormalities in placental development indicating that tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy (Michael and Papageorghiou, 2008). Previous studies demonstrated that increased GC levels significantly reduce invasion of both human first trimester primary cytotrophoblasts and a trophoblast cell line (HTR-8/SVneo) (Librach et al., 1994). Local tissue GC levels are modulated by 11β-hydroxysteroid dehydrogenase (11β-HSD), which interconverts cortisol with its inactive metabolite, cortisone. To date, two isoforms have been described. The type I isoform (11β-HSD1) is a bidirectional enzyme, which acts predominantly as an oxidoreductase to generate the biologically active glucocorticoid, cortisol, by using nicotinamide adenine dinucleotide phosphate (NADP+/NADPH) as a cofactor. In contrast, the type II enzyme (11β-HSD2) uses nicotinamide adenine dinucleotide (NAD+) to unidirectionally inactivate cortisol (Chapman et al., 2013). Although both 11β-HSD isozymes are present at the maternal-fetal interface at term, their expression and activity have not been fully characterized in first trimester gestational tissues. Elevated levels of 11β-HSD are present in early placental tissues homogenates (Muneyyirci-Delale et al., 1996) with 11β-HSD2 being the predominant isoform expressed in first trimester trophoblasts (Arcuri et al., 1998). Increased 11β-HSD expression during *in vitro* decidualization of human endometrial stromal cells (HESC) (Arcuri et al., 1996) together with higher 11β-HSD1 mRNA levels in first trimester decidua versus non-pregnant endometrium have also been described (McDonald et al., 2006). The expression and activity of both isoforms are thought to be crucial in maintaining a GC balance at the fetal-maternal interface. Previous reports demonstrated decreased placental expression of 11β-HSD2 in early pregnancy loss (Xu et al., 2013) and in PE (McCalla et al., 1998; Schoof et al., 2001).

Systemic inflammation is widely accepted as a key contributor to the pathogenesis of PE (Lockwood et al., 2011; Schatz et al., 2016). Specifically, PE is associated with decidual hemorrhage and maternal thrombophilias and with maternal inflammatory conditions (*e.g.*, systemic lupus erythematosus, scleroderma, antiphospholipid syndrome and severe obesity) that are accompanied by excess decidual and systemic expression of the potent proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Dong and Yin, 2014; Lockwood et al., 2011; Siljee et al., 2013). The present study sought to: 1) characterize 11β-HSD1 expression in first trimester decidua; 2) evaluate the potential role of steroid ovarian hormones and inflammatory cytokines in regulating enzyme levels in cultured decidual cells and 3) assess decidual 11β-HSD1 expression in PE specimens.

2. Materials and methods

2.1. Ethical approval

First trimester decidua specimens were obtained after elective termination of pregnancy. All patients gave written informed consent and the study was approved by the Human Investigation Committee of the University of Siena. Placental biopsies were collected under the approval of the Institutional Review boards of Chang Gung Memorial Hospital (Project #104-8983B). A written informed consent was obtained from each patient.

2.2. Tissues

Decidual specimens (n = 8) were rinsed in sterile phosphate-buffered saline (PBS) at room temperature, blotted dry and carefully dissected with a razor blade. An aliquot of tissue was snap-frozen and stored in liquid nitrogen for enzyme activity analysis and RNA extraction as described below. The reminder was used for decidual cell isolation. Placental biopsies from pregnancies complicated by PE (n = 14), gestational age-matched idiopathic preterm labor or twin pregnancies (n = 11) or healthy term controls (n = 10) were collected immediately after delivery. Preeclampsia was diagnosed according to standard criteria (Committee, 2002). Clinical characteristics of patients are summarized in Table 1 (supplementary material).

2.3. Isolation and culture of first trimester decidual cells

First trimester decidual cells (FTDC) were isolated and cultured employing a procedure used in our laboratory (Lockwood et al., 2006). Tissue specimens were minced and digested with 0.1% collagenase type IV and 0.01% DNase in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of Fetal Bovine Serum (FBS) (SIGMA Aldrich, St. Louis, USA) in a 37 °C shaking water bath for 30 min.

After washing with sterile phosphate-buffered saline (PBS), the digestate was washed × 3 with PBS and subjected to consecutive filtration through 100, 70 and 40 μm filters. The isolated FTDC were then resuspended in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of FBS (SIGMA Aldrich), seeded on polystyrene tissue culture dishes and grown to confluence in a standard 95% air: 5% CO_2 incubator at 37 °C. Cultured cells were analyzed by immunocytochemistry to check purity. After 3–4 passages, cultures were found to be leukocyte free (<1%), vimentin positive (>90%) and cytokeratin negative (<10%). Cells also displayed characteristic decidualization-related morphological changes and expressed elevated levels of prolactin in response to treatment with estradiol (E2) and medroxprogesterone acetate (MPA) (SIGMA Aldrich) versus E2 alone. We employed MPA to mimic the steroid milieu of pregnancy.

2.4. Experimental cell incubations

To evaluate hormonal regulation of 11β-HSD1 expression and activity, confluent FTDC were incubated for 7 days in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of charcoal-treated FBS (ctFBS) (SIGMA Aldrich), 10^{-8} M E2, 10^{-7} M MPA, used in place of progesterone because of its greater stability in culture (Arici et al., 1999), or E2 + MPA (SIGMA Aldrich) in the presence or absence of 10^{-7} M of the antiprogestin-antiglucocorticoid, Mifepristone (RU486), or 10^{-7} M dexamethasone (DEX) (SIGMA Aldrich), a pure glucocorticoid. All steroids used to treat FTDC were added from 1000× stock solutions in ethanol and ethanol alone was used as vehicle in control samples.

To assess the effects of inflammatory cytokines on 11β-HSD1 expression, confluent FTDC cultures were incubated × 7 days in phenol free DMEM-F12 with 1% penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% ctFBS with 10^{-8} M E2 plus 10^{-7} M MPA to simulate the hormonal milieu of pregnancy; after 7 days, the cells were washed × 2 with Hanks' balanced salt solution (SIGMA Aldrich) to remove residual serum elements and then treated with steroids alone or with 1 ng/ml of IL-1β or IFN-γ, or 5 ng/ml of TNF-α, or with IFN-γ plus IL-1β or TNF-α (R&D System, Minneapolis, USA)

for 5 or 24 h (for mRNA and protein studies, respectively) in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM, 7.5% $NaHCO_3$ ITS+, 5 μmol/L $FeSO_4$, 0.5 μmol/L $ZnSO_4$, 1 nmol/L $CuSO_4$, 20 nmol/L Na_2SO_3, trace elements, 50 μg/ml ascorbic acid and 50 ng/ml epidermal growth factor (SIGMA Aldrich). After experimental incubation, the medium was removed, the cells harvested and 11β-HSD1 mRNA and protein levels as well as enzymatic activity were determined as described below.

2.5. RNA extraction and real time polymerase chain reaction

Total RNA was extracted using the SV Total RNA Isolation System (Promega Corporation, Fitchburg, USA), according to the manufacturer's instructions. RNA was quantified by UV absorption and RNA integrity was checked using a 1.5% agarose gel. To assess cDNA synthesis, 1 μg of total RNA was reverse transcribed using the ImProm-II ReverseTranscriptase (Promega Corporation).

The expression of target genes was analyzed by quantitative RT-PCR (qRT-PCR). Messenger RNA levels, normalized for GAPDH, chosen as a reference gene, were measured in triplicate using the SYBR Select Master Mix for CFX (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol, on a CFX Connect 96 (Bio-Rad Laboratories, Hercules, USA) real-time PCR. Gene-specific primers used for amplification by polymerase chain reaction (PCR) were the following. 11β-HSD1 5′ primer was 5′-AGAG-CAATTTGTTGCCCAAG-3′, the 3′ primer was 5′-AATGCTTC-CATTGCTCTGCT-3′. 11β-HSD2 5′ primer was 5′-ACGCAGGCCACAATGAAGTAG-3′, the 3′ primer was 5′-GCA-CAGGCTGGATGATG-3′. Glyceraldehyde 3-phosphate dehydrogenase 5′ primer was 5′-GAAGGTGAAGGTCGGAGTC-3′ the 3′ primer was 5′-GAAGATGGTGATGGGATTTC-3′. Characteristics of PCR products were initially determined by direct DNA sequencing. For each RNA specimen, a negative control was prepared by omitting the reverse transcriptase. Amplification was carried out at 95 °C for 5 min, followed by 10 s at 95 °C and 30 s at 60 °C for 40 cycles, with fluorescence detection performed at the end of each extension step. For each run, melting-curve analysis was used to confirm the specificity of the amplified products and the absence of primer-dimers formation. Calculations of relative gene expression of 11β-HSD1 were conducted via the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001) using GAPDH as reference gene. Relative expression was normalized to control samples.

2.6. Western blotting

Cell pellets were lysed in a 20 mM Tris-HCl pH 8, 135 mM NaCl, 10% glycerol, 1% NP-40 and 10 mM EDTA, together with protease inhibitors (Thermo Fisher Scientific) for 30 min on ice. After centrifugation for 10 min at 1000 rpm, the supernatant was assessed for total protein content using the Bradford method (Quantum Protein Kit; Euroclone, Milan, Italy).

Western blotting was used to detect 11β-HSD1 in first trimester decidua sample. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose. Membranes were blocked for 1 h at room temperature with 2.5% dry skimmed milk and 2.5% BSA in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and then probed overnight at 4 °C with anti-11β-HSD1 Polyclonal Antibody (Cayman Chemicals, Ann Arbor, USA) or anti-α-tubulin rabbit polyclonal antibody (Genetex Inc. Irvine, USA) as a housekeeping protein. Immunoreactive bands were detected after 1 h incubation with anti-rabbit IgG HRP-conjugated antibody (Cell Signaling Technology, Danvers, USA) followed by detection with ECL Western Blotting Detection Reagent (GE Healthcare, Wauwatosa, USA).

2.7. Enzymatic activity

Enzymatic activity was assessed in cell homogenates using cortisone and NADPH as substrate and cofactor, respectively. Each cell pellet was resuspended in 110 μl 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (20 mM pH 7.2) containing 100 mM KCl, 20 mM NaCl and 1 mM $MgCl_2$ in presence of 1 μM cortisone and 2 mM of NADPH and 0.5% of Triton X100 (SIGMA Aldrich) and incubated at 37 °C for 120 min under shaking. Cortisol levels were measured with an ELISA kit (Enzo Life Sciences, Farmingdale, USA), according to the manufacturer's instructions in a VICTOR^3 1420 Multilabel Plate Counter (PerkinElmer, Waltham, USA). Results were normalized to 1×10^6 cells.

2.8. Immunofluorescence

Serial sections (2 μm) of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. Antigen retrieval was carried out by incubating sections in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven at 750 W for 5 min. Sections were incubated with 5% normal horse serum in a humidified chamber for 30 min at room temperature and then exposed to rabbit polyclonal anti-human 11β-HSD1 (Santa Cruz Biotechnology, Santa Cruz, USA) or to a mouse monoclonal anti-vimentin antibody (Clone: Ab-2V9, Neomarkers, Fremont, USA), using a 1:100 dilution of both antibodies, overnight at 4 °C. Normal rabbit or mouse IgG isotype were each used as a negative control at the same concentration as the primary antibodies. Secondary chicken anti-rabbit fluorescein isothiocyanate conjugate (FITC)-conjugated IgG or chicken anti-mouse rhodamine-conjugated IgG (Santa Cruz Biotechnology), at 1:50 dilution, was added. Nuclei were stained with 6-diamino-2-phenylindole (DAPI) in mounting medium (Santa Cruz Biotechnology).

2.9. Immunohistochemistry

Deparaffinized formalin-fixed sections (2 μm) were subjected to antigen retrieval by boiling in citrate buffer (10 mM, pH 6.0) for 15 min. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 12 min. Slides were incubated in a humidified chamber with 5% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, and then exposed to a rabbit polyclonal anti-human 11β-HSD1 (Santa Cruz Biotechnology) with a 1:100 dilution overnight at 4 °C in a humidified chamber. Non-immune rabbit IgG was used as an isotype control. Antigen-antibody complex was detected using a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA), the avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) with light hematoxylin counterstaining. The intensity of IHC staining was assessed with a computer-generated HSCORE ranging between 0 and 300 in 10 separate areas in each slide using a Leica QWin image analysis system (version standard V 2.5; Leica Imaging Systems).

2.10. Statistical analysis

Normal distribution of data was assessed by the Shapiro-Wilk test. Results were analyzed by One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls method for multiple comparisons. Statistical significance assumed P values <0.05. Statistical calculations were performed using SigmaPlot version 12.0 (Systat Software, San Jose, USA). Data are expressed as mean ± SEM.

3. Results

3.1. 11β-HSDs mRNA expression levels in first trimester decidua

The expression of 11β-HSD1 and 11β-HSD2 was evaluated in first trimester decidua tissues and in primary cultures of FTDC. As shown in Fig. 1, 11β-HSD1was the dominantly expressed isoform in first trimester decidual tissues (43-fold increase over 11β-HSD2 levels, $p < 0.001$; Fig. 1A), as well as in cultured FTDC (13-fold over 11β-HSD2, $p < 0.01$; Fig. 1B). These results indicated the importance of assessing the effects of potential regulatory factors on the type 1 enzyme.

3.2. Steroid hormones effect on decidual 11β-HSD1 expression and enzymatic activity

The effects of steroid hormones on 11β-HSD1 expression in FTDC ($n = 8$) are shown in Fig. 2. While E2 did not affect 11β-HSD1 mRNA levels (Panel A), MPA induced a significant increase over control cultures (13 fold; $p < 0.05$). In co-incubations with E2 + MPA the stimulatory effect was significantly higher than that of MPA alone (40-fold vs. control; $p < 0.001$ and 3.0-fold vs. MPA; $p < 0.05$). Co-treatment with the antiprogestin RU486 blocked almost all of this MPA enhancing action, whereas RU486 alone did not exert an agonistic effect. In contrast to the marked effect of MPA, the synthetic glucocorticoid DEX did not significantly alter 11β-HSD1 mRNA levels indicating a progestational effect.

To determine whether steroid-mediated enhancement of 11β-HSD mRNA expression is accompanied by protein changes, 11β-HSD1 levels were measured in FTDC by Western Blot. As shown in panel B, corresponding protein results were obtained with E2 + MPA eliciting the strongest induction. Unexpectedly, in cells treated with DEX, augmented 11β-HSD1 protein expression was observed despite the absence of a corresponding induction of 11β-HSD1 mRNA. Finally, the same pattern observed by Western blotting was obtained when 11β-HSD1 enzymatic activity was measured in treated cells (Fig. 2 Panel C). Accordingly, E2 + MPA synergistically increased 11β-HSD1 activity (2.5-fold vs. control; $p < 0.001$) and 11β-HSD1 activity was also significantly augmented by DEX (2.7-fold vs. control; $p < 0.05$). The inductive effects of MPA were completely abrogated by co-treatment with RU486.

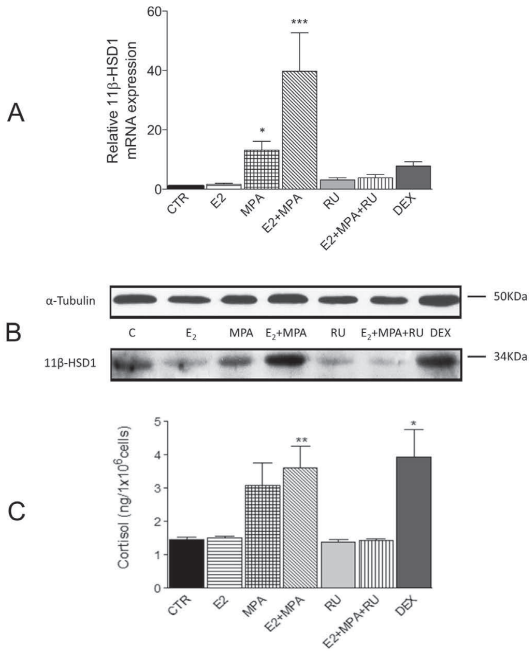

Fig. 2. 11β-HSD1 expression and activity in hormone-treated FTDCs. Effects of steroid hormones on 11β-HSD1 mRNA levels (Panel A; $n = 8$), protein levels (panel B; one representative blot out of four is shown) and enzymatic activity (Panel C; $n = 6$) are shown. Data were normalized to GAPDH (Panel A) and cell number (Panel C) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs.control). α-Tubulin was used as loading control. The position of molecular weight markers is shown on the left (Panel B).

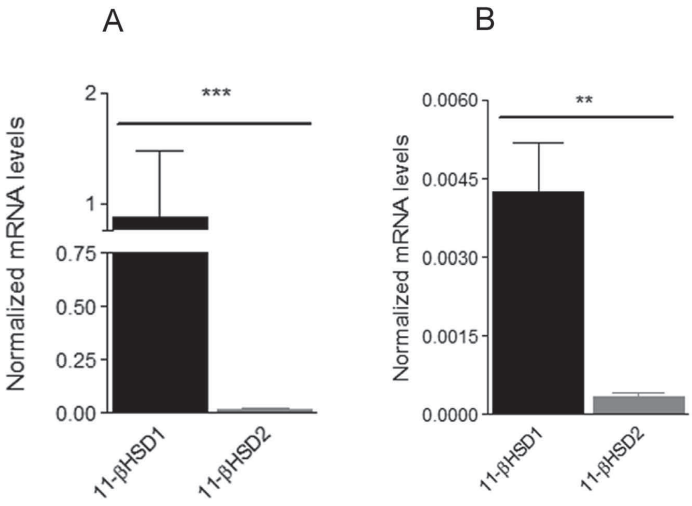

Fig. 1. 11β-HSD1 and 2 messenger RNA expression in extracts of first trimester decidua (A; $n = 6$) and in confluent cultures of purified first trimester decidual cells (FTDCs) (B; $n = 6$) cultured as described in Materials and methods (2.3). Messenger RNA levels were normalized to GAPDH as described in Materials and methods (***$P < 0.001$; **$P < 0.01$).

3.3. Effects of inflammatory cytokines on decidual 11β -HSD1 expression and enzymatic activity

The individual effects of IL-1β, TNF-α or IFN-γ and the combined effects of IFN-γ with either IL-1β or TNF-α on 11β-HSD1 mRNA levels are shown in Fig. 3 (Panel A; n = 8). In cultured FTDC incubated with E2 + MPA, the addition of IL-1β significantly up-regulated steady-state levels of 11β-HSD1 mRNA (32-fold vs. control; $p < 0.01$), whereas neither TNF-α nor IFN-γ elicited a statistically significant effect when used alone or in co-incubations of IFN-γ with either IL-1β or TNF-α (Panel A).

Fig. 3 also displays the effects of pro-inflammatory cytokines, either added alone or in combination, on 11β-HSD1 protein levels (Panel B) and enzymatic activity (panel C). Consistent with mRNA results, IL-1β induced a statistically significant increase in both 11β-HSD1 protein levels and enzymatic activity (for the latter, 2.3-fold vs. control; $p < 0.05$), whereas neither TNF-α nor IFN-γ exerted a significant effect when added alone. However, in incubations combining IFN-γ and IL-1β, a limited, albeit significant further augmentation of enzymatic activity was observed compared with IL-1β-treated cultures ($p < 0.05$).

3.4. Decidual expression of 11β-HSD1 in PE placenta

Fig. 4 displays 11β-HSD1 and vimentin immunostaining in placentas from women with PE vs. gestational age-matched controls.

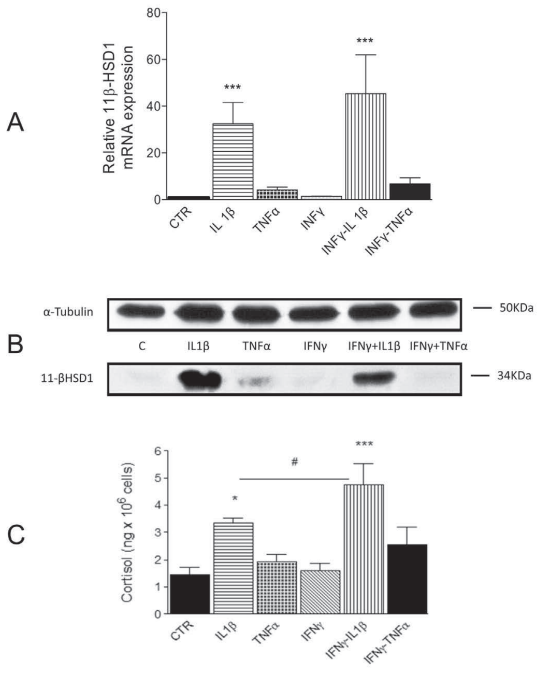

Fig. 3. 11β-HSD1 expression and activity in cytokine-treated FTDCs. The effects of inflammatory cytokines on 11β-HSD1 mRNA (Panel A; n = 8), protein expression (panel B; one representative blot out of four is shown) and enzymatic activity (Panel C; n = 5) are shown. FTDCs were treated with vehicle alone (CTR), IL-1β, TNF-α, IFN-γ, or with IFN-γ plus IL-1β or TNF-α for 5 (Panel A) or 24 h (Panels B and C). Data were normalized to GAPDH (Panel A) and cell number (Panel C) (*P < 0.05; **P < 0.01; ***P < 0.001 vs. control; #P < 0.05). α-Tubulin was used as loading control (Panel B). The position of molecular weight markers is shown on the left.

As shown in panel A, 11β-HSD1 (green fluorescence) is present in the vimentin-positive decidual cells (red fluorescence) of both PE complicated placentas and healthy controls. However, the staining intensity for 11β-HSD1 appears to be stronger in PE placental tissues as indicated by yellow-orange staining in the merged image. Confirming this assessment, immunostaining for 11β-HSD1 was more intense in the extended-fusiform decidual cells in PE (Fig. 5A) vs. control, irrespective of the gestational age (panels B and C). The HSCORE measurements, summarized in panel D, were 247.4 ± 2.7 (mean ± SE) for the PE specimens vs. 55.0 ± 3.3 of preterm control ($P < 0.001$) and 52.8 ± 1.8 of term controls ($P < 0.001$).

4. Discussion

This study demonstrates clear dominance of 11β-HSD1 over 11β-HSD2 in first trimester human decidua. Specifically, 11β-HSD1 mRNA levels are 43 and 13-fold higher than those of 11β-HSD2 in decidual tissues and cultured primary decidual cells, respectively. Our results extend previous observations by McDonald and co-workers that demonstrated a significant increase of 11β-HSD1 mRNA over the type 2 isoform in first trimester decidua compared to cycling endometrium (McDonald et al., 2006). Previously, our laboratory reported that the oxidative activity of 11β-HSD2 is predominantly expressed in first trimester placental villous tissues (Arcuri et al., 1998). These earlier results considered together with the present observations indicate that 11β-HSD isoforms are differentially distributed in specific cell types at the fetal-maternal interface in early human pregnancy. The physiological impact of differential tissue localization of these two isoforms is currently unclear and requires further investigation. While the oxidative activity of 11β-HSD2 in first trimester placenta may protect the fetus from maternal glucocorticoids (Arcuri et al., 1998), the expression of the oxoreductase isoform in first trimester decidua may augment local cortisol levels to act as an autocrine/paracrine regulator of implantation and/or placentation. Consistent with both actions, induction of 11β-HSD1 in primary HESC cultures increases cortisol availability, which, in turn, regulates distinct GR- and mineralocorticoid (MR)-dependent gene networks involved in key embryo-endometrial interactions (Kuroda et al., 2013).

The current study also demonstrates that MPA significantly induces 11β-HSD1 expression and activity. Although. E2 was ineffective when administered alone, co-treatment with E2 + MPA exerted maximal effect on mRNA levels, protein expression and cortisol formation, whereas the antiprogestin RU486 counteracted E2 + MPA effects on 11β-HSD1 expression, thus supporting progesterone receptor-mediated control of 11β-HSD1 expression in early decidua. Ovarian steroids modulate differentiation of human endometrial stromal cells and are involved in recruiting a subset of uNK cells and macrophages (Gibson et al., 2015; Szekeres-Bartho et al., 2009). Progesterone initiates and maintains decidualization of human endometrial stromal cells and ensures the integrity of the feto-maternal interface at implantation site thereby regulating trophoblast invasion (Halasz and Szekeres-Bartho, 2013). The results of the present study indicates the existence of previously unrevealed mechanism of progesterone action in first-trimester decidua by demonstrating that progesterone modulates GC levels through the induction of 11β-HSD1. Worth noting is that in FTDC, DEX did not affect 11β-HSD1 mRNA, whereas it up-regulated protein levels and activity. These paradoxical observations are consistent with a report of augmented 11β-HSD1 protein levels in human endometrial stromal cells treated with DEX (Kuroda et al., 2013) and support the existence of a GC-dependent post-transcriptional mechanism that regulates 11β-HSD expression in both cycling and pregnant endometrial cells.

A specific temporal–spatial release of pro-inflammatory

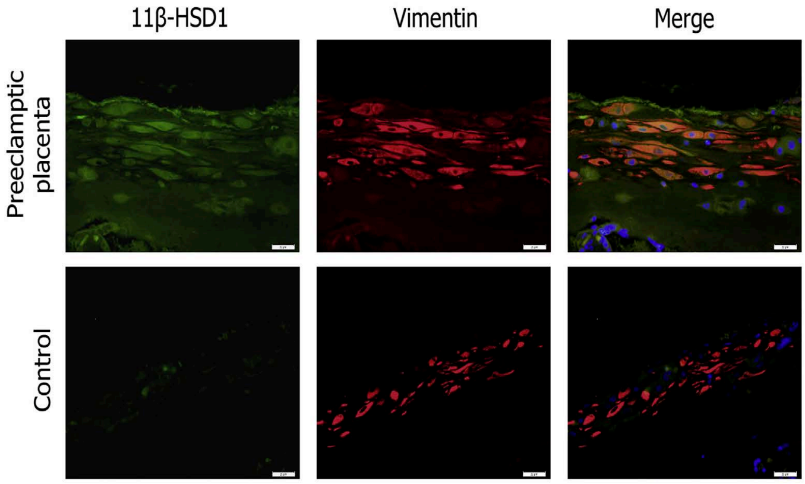

Fig. 4. 11β-HSD1 immunostaining in PE decidua and healthy controls. Representative photomicrographs of immunofluorescence staining of 11β-HSD1 (green staining) and vimentin (red staining) are shown. Cell nuclei are stained in blue. Merge (yellow staining) indicates co-localization of 11β-HSD1 and vimentin in decidual cells (400× original magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

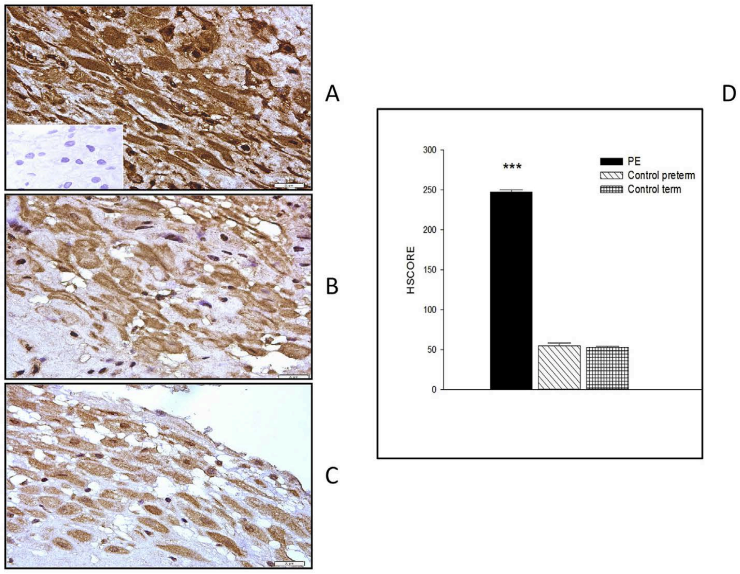

Fig. 5. Representative photomicrographs of immunohistochemical staining for 11β-HSD1 in PE (A), preterm control (B), and term control (C) decidua. Inset in (A), isotype control. Bar = 20 μm (400× original magnification). Panel D: HSCORE (mean ± SEM) of 11β-HSD1 immunoreactivity in the decidual cells of PE (n = 14), preterm (n = 11) and term controls (n = 10) (***P < 0.001 vs. control).

cytokines, chemokines and prostaglandins is an essential component of cross-talk between the implanting embryo and maternal decidua. Its deregulation leads to adverse gestational outcomes including spontaneous abortion, PTB, PE, and IUGR (Vannuccini et al., 2016). The IL-1 system plays key roles in early pregnancy. Thus, IL-1β expression is increased during *in vitro* decidualization of HESCs (Sharma et al., 2016) and IL-1β, IL-1 receptor type I and IL-1 receptor antagonist are expressed by villous trophoblast and decidual cells in early implantation (Librach et al., 1994; Simon et al., 1994). Furthermore, IL-1β modulates trophoblast invasion (Karmakar and Das, 2002; Librach et al., 1994), promote angiogenesis (Choi et al., 2002) and mediate macrophage infiltration of the decidua (Huang et al., 2006; Lockwood et al., 2006). Glucocorticoids exert a marked influence on the implantation process by stimulating the secretion of hCG, suppressing uNK cells infiltration and controlling trophoblast growth and invasion. Furthermore, the

anti-inflammatory effects of GCs modulate several implantation-related events (Michael and Papageorghiou, 2008). The IL-1β-dependent enhancement of decidual 11β-HSD1 mRNA, protein levels and activity shown in the present study may represent a novel mechanisms by which inflammatory mediators can affect implantation by increasing the availability of biologically active GCs at the fetal-maternal interface. Interestingly, IFNγ, an inflammatory cytokine abundantly secreted in early pregnancy, significantly augmented IL1β-dependent induction of 11β-HSD1 activity. We previously showed that IFNγ interacts with either IL-1β or TNF-α to synergistically enhance the production by human FTDCs of specific uNK-cell recruiting chemokines (Lockwood et al., 2013). Taken together, these results suggest that the synergic interaction of IFNγ with other inflammatory mediators in early decidua represents a common regulatory mechanism of key-implantation events.

Elevated expression of 11β-HSD1 in decidua from women with PE is a main finding of the current study. Although PE symptoms generally become evident as pregnancy progresses, this syndrome is thought to develop in the first trimester in response to shallow trophoblast invasion of the maternal decidua (Fisher, 2015). Defective implantation is associated with a strong inflammatory response (Redman and Staff, 2015). Previous studies indicated involvement of inflammatory cytokines in the pathogenesis of PE and suggested that IL-1β may be primarily responsible for the endothelial dysfunction and enhanced prostaglandin levels observed in the preeclamptic placenta (Hefler et al., 2001; Munno et al., 1999; Rinehart et al., 1999). More recently, IL-1β serum levels were reported to be an early onset biomarker of PE (Siljee et al., 2013), whereas an earlier study found that IFNγ levels are significantly elevated in both the plasma and decidua of pre-eclamptic women (Sargent et al., 2006).

Prior reports indicated the potential involvement of GCs in the etiology of PE. Thus, elevated cortisol levels were observed in PE-associated pregnancies compared to normotensive pregnancies (Aufdenblatten et al., 2009). Moreover, early onset PE is associated with altered DNA methylation of cortisol signaling (Hogg et al., 2013). In rats, experimentally-induced PE is related to abnormal GC synthesis in the placenta (Wang et al., 2014), whereas GC exposure during early placentation induces PE by inhibiting trophoblast proliferation, migration and invasion (Zhang et al., 2016). Two studies found that 11β-HSD2 expression and activity are significantly reduced in preeclamptic trophoblast tissues compared to controls (McCalla et al., 1998; Schoof et al., 2001), with McCalla et al. observing that this reduction in 11β-HSD2 expression is reflected in higher cord serum cortisol levels (McCalla et al., 1998). Our results demonstrating a cytokine-dependent 11β-HSD1 induction in cultured FTDCs, and augmented expression of the decidual 11β-HSD1 protein *in vivo*, suggest a novel mechanism by which inflammation-induced decidual 11β-HSD1 expression, together with decreased 11β-HSD2 levels, may augment local cortisol availability at the fetal-maternal interface in PE.

In conclusion, results presented in the current study reveal that 11β-HSD1 is the predominant decidual isoform expressed *in vivo*, and that *in vitro* expression of 11β-HSD1 is regulated by both steroid hormones and inflammatory cytokines. Moreover, our data show that PE is characterized by elevated decidual 11β-HSD1 levels. These observations highlights a role of decidual 11β-HSD in controlling biologically active GCs in early pregnancy, and in both the physiology and pathology of placentation and in pathological changes associated with the later development of PE.

Conflict of interest

None.

Acknowledgments

This study was supported in part by a grant from the Italian Government (PRIN) to F.P.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.08.023.

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3.2 11-HSD1 along pregnancy: implications in timing of labour and in myometrium contraction

With this second work, the attention was moved along gestation, shifting from first trimester to mid-pregnancy until the labour. In particular, it was explored the time-course of 11β-hsds expression and localization in murine uterus, comparing the form 1 trend in the last part before the labour with the same enzyme in human myometrium. In addition, we investigated on the effect of 11β-HSD1 inhibition on the contractile activity of a human myometrial cell line.

Our results showed for the first time the spatial and temporal localisation of murine 11β-hsd1 in uterus at pregnancy. While 11β-hsd2 remain stable and low during all the pregnancy, 11β-hsd1 exhibited a day-specific progress, with comparable mRNA and protein data. The expression was low until mid-pregnancy and started to increase progressively from day 8 of gestation until day 18, close to the moment of labour. Interestingly, at the onset of labour we observed a significant decrease of the 11β-hsd1 expression. This decrease at the moment of labour appears to be comparable to that we observed in myometrial human sample, comparing term non-labouring myometrium samples to term labouring.

Additional results *in vitro* showed that a non-selective inhibitor of 11β-HSD, carbenoxolone, used for treatment on a human myometrial cell line, apparently suppressed the cells contraction.

According to these results, we proposed a novel theory where 11β-HSD1 has a decisive role in the timing of pregnancy phases, labour included, helping and facilitating the myometrium activation and then contractions. We suggest that this role is due by the stimulating role of 11β-HSD1 for estrogen and prostaglandins. Estrogen enhance is decisive for the myometrium priming and activation in the phases before the labour and glucocorticoids may influence this augment through stimulation of aromatase expression, final producer of estrogen. Prostaglandins appear essential for successive myometrium contractions and glucocorticoids 11β-HSD1-dependent strong influence these events.

Concerning the final fall in the expression at the onset of labour, observed both in mouse than in human, we suggest a connection with the inflammatory nature of the labour. As part

of their anti-inflammatory actions, glucocorticoids are able to inhibit a various range of inflammatory genes. The breakdown 11β-HSD1 at the onset of labour may prevent the anti-inflammatory glucocorticoids action, consequently promoting the pro-inflammatory events, fundamental for the main events of parturition and the post-natal phase.

Article published on Molecular and Cellular Endocrinology

11β-hydroxysteroid dehydrogenase type 1 and pregnancy: role in the timing of labour onset and in myometrial contraction

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Abstract

Glucocorticoids play a primary role in the maturation of fetal organs and may contribute to the onset of labour. Glucocorticoid activity depends on the 11β-hydroxysteroid dehydrogenase family (11β-HSDs), catalysing the interconversion between "active" cortisol into inactive cortisone. No definitive study exists on 11β-HSD expression profile in human decidua and myometrium during pregnancy.

We investigated the implications of 11β-HSD1 in the regulation of uterine activity in pregnancy, examining its role on contraction of a myocyte cell line and murine 11β-hsd1 levels in utero. Murine 11β-hsd1 mRNA and protein levels in utero progressively increased until the last day of gestation and significantly decreased at the onset of labour ($P < 0.0001$) (n= 3 to 5 in the various gestational days analysed). Experiments on human myometrial samples confirm the significant fall

in 11β-hsd1 mRNA levels at labour, compared to end pregnancy samples (*n*= 5 to 8). *In vitro* experiments showed that human myometrial contraction is inhibited by using a non-selective inhibitor of 11β-HSD1. The present study shows the temporal localisation of 11β-HSD1 in uterus, highlighting its importance in the timing of gestation and suggesting its contribution in the myometrium contraction

1. Introduction

The 11β-hydroxysteroid dehydrogenase family (11βHSDs) consists of two enzymes that act on glucocorticoid metabolism to regulate the availability of active glucocorticoids (cortisol in humans and corticosterone in mice) and of their inactive metabolites (cortisone and dehydrocorticosterone, respectively)(McDonald et al., 2006). The type 1 enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1) is bi-directional but operates mostly as an oxo-reductase, facilitating the production of active cortisol (or corticosterone, murine equivalent), in a NADPH-dependent manner. The type 2 (11β-HSD2) is a NAD^+-dependent enzyme, uni-directional and inactivating cortisol to cortisone (or corticosterone to dehydrocorticosterone) (Michael et al., 2003; Murphy and Clifton, 2003).

During pregnancy, cortisol is synthesised in the adrenal glands (Terao and Katayama, 2016) and placenta (Gangestad et al., 2012) and plays a primary role in the maturation of fetal organs at the end of pregnancy, thus contributing to the onset of labour (Alfaidy et al., 2003; Kamel, 2010). Glucocorticoid action is dependent upon the availability of glucocorticoids in the plasma and local metabolism by the two forms of 11βHSDs (White et al., 1997). 11β-HSD1 is the predominant enzyme expressed in the decidua, chorion, amnion and vascular endothelial cells (Burton and Waddell, 1999; Thompson et al., 2002; Tomlinson et al., 2004). In addition, the biochemically distinct activities of the two 11βHSDs are present in the placenta, where the balance between the reductase activity of 11β-HSD1 and the oxidative activity of 11β-HSD2 changes at different stages of gestation (Murphy and Clifton, 2003). Specifically, 11β-HSD2 appears to be the major form

expressed in the human placenta (Brown et al., 1993), but the mRNA expression of rat 11β-hsd1 increases in the placenta at later stages of gestation (Burton and Waddell, 1999), suggesting a potential importance of local glucocorticoids increase in the placenta in preparation for labour.

The expression of 11β-HSDs in the myometrium and decidua of the pregnant uterus has received less scrutiny. Both 11β-hsd1 and 11β-hsd2 transcripts have been detected in the murine decidual stroma, co-localized with Glucocorticoid Receptor (GR) in the cytoplasm. Given the role of glucocorticoids in a number of significant functions in the murine uterus, such as eosinophil infiltration and stromal cell prostaglandin synthesis (Thompson et al., 2002), the presence of the enzymes co-localized with the GR suggests that 11β-hsd expression may be important in decidual glucocorticoid signalling. Indeed, an increase in the concentration of 11β-HSD1 has been demonstrated in the human decidua in late gestation (Chan et al., 2007) and low concentrations of 11β-HSD1 in fetal membranes overlying the decidua has been associated with poor response to induction of labour (Novembri et al., 2013), suggesting a role of the enzyme in the mechanism of labour.

Recent reports have shown that the expression of 11β-HSD1 is up-regulated by steroid hormones, both in rat (Tomlinson et al., 2004) and in human (Funghi et al., 2016; Tomlinson et al., 2004), and by TNFα and IL-1β in human in a large range of stromal cells, including amnion fibroblasts and chrorionic trophoblast (Gathercole et al., 2013) and decidua (Funghi et al., 2016). This latter regulation appears to have a functional relevance, augmenting the expression of GCs-regulated genes, such as IL-6 (Gathercole et al., 2013). Arcuri and collaborators showed the effects of various steroid treatments on the expression of 11β-HSD1 in endometrial stromal cells (Arcuri et al., 1996). They found that progesterone alone or in combination with estrogen increased the expression of 11β-HSD1 whereas estrogen alone had no effect. The same study proposed also a link between 11β-HSD1 activity and matrix metalloproteinase (MMP) action (F. Arcuri et al., 1996). This is potentially important because MMP action is crucial for trophoblast invasion (Zhang et al., 2016),

in both physiological and pathological conditions such as gestational trophoblastic diseases (GTDs) and preeclampsia (Rahat et al., 2016). Additionally, a direct role for 11β-HSD1 in the etiology of pathological changes of preeclampsia has also been suggested (Funghi et al., 2016).

Despite the importance of 11β-HSDs in the physiology and pathology of gestation, the expression profile of 11β-HSDs has never been explored in detail in the uterine compartments throughout pregnancy. Myometrium is a highly dynamic and adaptable tissue, which plays a pivotal role in pregnancy maintenance and parturition. A plethora of hormonal stimuli, including glucocorticoids, may influence myometrial function during all gestational phases (Burton et al., 1996). A study conducted on pregnant rats showed that 11β-hsd1 expression was induced in the period leading up to parturition and suggested that glucocorticoids might regulate myometrial contractility, through the up-regulation of prostaglandin synthase (PTGS)-2 gene expression, with an increased production of prostaglandins, involved in the myometrial contraction stimulation (Challis et al., 2000).

We hypothesised that 11β-HSD1 and 11β-HSD2 would display distinct expression patterns at different times of pregnancy, and that these expression patterns might contribute to the regulation of uterine activity during pregnancy. To address our hypothesis, we aimed to a) characterise the expression of 11β-hsd1 and 11β-hsd2 in the mouse uterus throughout gestation and in the human myometrial biopsies derived from term and preterm pregnancies and b) determine the effect of 11β-HSD1 inhibition on the contractile activity in a human myometrial cell line obtained from term pregnancy.

2. Materials and Methods

2.1 Human tissue

Following informed written consent, biopsies were obtained from the upper margin of the lower segment of myometrium from women undergoing elective caesarean section (ELCS) at the Simpson Centre for Reproductive Health at the Royal Infirmary of Edinburgh. West of Scotland Research Ethics Committee 4 (09/S0704/3) granted ethical approval to the Edinburgh Reproductive Tissue BioBank. Biopsies were collected from lean (LN; 19 kg/m^2<BMI<25 kg/m^2) women delivering at term (>37 weeks of gestation) prior to the onset of labour (TNL, *n*=8) or during active labour (TL, *n*=8) and preterm (<37 weeks of gestation) during active labour (PT, *n*=5). Patients with twin pregnancies and pregnancy complications were excluded.

2.2 Mouse tissue

Uterus was obtained from C57BL/6 female mice, 6-8 weeks old, housed at the animal facility at the University of Edinburgh. All animal care and experimental protocols were performed according to United Kingdom Home Office guidelines under license 60/4241 to J.E.N. Mice were time-mated with plug day considered to be day 1 of gestation. Mice were sacrificed and uterus was collected on gestational day 5 (D5), 8 (D8), 12 (D12), 15 (D15), 17 (D17), 18 (D18) and during active labour (LB) usually on day 19/20 of gestation (n= 3-6/group). (D8, D12, D17 *n*= 5; D5, D18 *n*=4; D15, LB *n*=3). In addition, uterus from non-pregnant (NP) mice (*n*=5) was used as control/calibrator.

2.3 PHM1-41 cell line

PHM1-41 cells, originally derived from pregnant human myometrial cells, were a kind gift from B.Sanborn (Colorado State University, Fort Collins, Colorado). Cells were maintained in DMEM high-glucose medium with phenol red (Lonza) in the presence of 10% fetal bovine serum,

penicillin/streptomycin and L-glutamine (all Lonza), together with Geneticin (Gibco) and Normocin (InvivoGen).

2.4 Sample processing

Immediately after collection, samples were placed in RNALater solution (Life Technologies) for RNA extraction, in 10% neutral-buffered formalin for immunohistochemical analysis or on dry ice for protein extraction. RNA and protein samples were stored at -80ºC before further processing. Frozen mouse tissue samples were disrupted and homogenized using a Mixer Mill MM 300 (QIAGEN). Total RNA was extracted with TriReagent (Sigma-Aldrich), followed by RNeasy mini kit (QIAGEN) with on-column deoxyribonuclease digestion. Extractions were performed according to the manufacturers' instructions. RNA quality and quantify was determined using Nanodrop (Celbio). Total protein was extracted in RIPA buffer (Sigma) containing one cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche). RNA and protein was stored at -80ºC.

2.5 Quantitative RT- PCR

cDNA was prepared from 300 ng RNA using the High Capacity cDNA Reverse Trascription kit (Applied BioSystem). Predesigned gene expression assays from Applied BioSystems were used to examine the expression of 11β-HSD1 (Mouse: Mm00476182_m1, Human: Hs01547870_m1), 11β-HSD2 (Mouse: Mm01251104_m1) and β-actin (Mouse: 4352341E, Human: 4326315E). Experiments were done in duplicate on 384-well optical PCR plates (Applied Biosystems), optimised to the universal PCR protocol of the manufacturer, with a TaqMan Universal PCR Master Mix (Applied Biosystems). The negative control for each reaction consisted of amplification performed in the absence of RT and blank samples in absence of RNA samples (H_2O RT). Target gene expressions was normalized using β-actin and the expression in each sample was calculated relative to the average of NP samples for mouse and TNL for human, using the $2^{-\Delta\Delta Ct}$ method of

analysis. All qRT-PCR analysis was performed on an ABI 7900HT (Applied Biosystem), according to the standard protocol. The negative control for each reaction consisted of amplification performed in the absence of RT and blank samples in absence of RNA samples (H_2O RT).

2.6 Western Blot Analysis

Western Blot was used to detect 11βHSD-1 protein. Protein was separated by SDS-PAGE and was then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 hour at room temperature with 5% dry skimmed milk in 50 mM Tris-HCl, ph 7.5, 150 mM NaCl, 0.2% Tween-20, and were then probed overnight at 4°C with generated in-house sheep anti-11β-HSD1 antibody (1:200) (kindly provided by Dr Scott Webster), and monoclonal T9026 anti-α-tubulin (1:5000) (Sigma Aldricht) as a quantification control, followed by extensive washing.

Immunoreactive materials were detected by 1 hour incubation with secondary antibodies (1:10000) IRDye 800 CW Donkey AntiGoat IgG (H+L) Highly Crossed Adsorbed (Li-cor) for 11βHSD-1 and with IRDye 680 RD Donkey (polyclonal) Anti-Mouse IgG (H+L) Highly Crossed Adsorbed (Li-cor) for α-tubulin.

A Li-Cor Odyssey scanner (Li-cor) was used for membranes analysis.

2.7 Immunohistochemistry

To evaluate the localization of 11β-HSD1 in mouse uterus, immunohistochemistry was carried out on 5-μm-thick sections, obtained from paraffin-embedded samples; sections were dewaxed in xylene and rehydrated in graded ethanol. Antigen retrieval was performed in boiling 0.001 M citrate buffer (PH 6.0). Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol at room temperature for 30 minutes. The ImmPRESS Kit (Vector) was used as per manufacturers' protocol. Slides were incubated overnight at 4°C with an in-house generated sheep anti-11βHSD-1 purified antibody, used 1:10000 in Normal horse serum (NHS, ImmPRESS kit). A negative control

(primary antibody absent) was included. Positive staining was detected with 3,3'-diaminobenzidine (DAB Peroxidase (HRP) substrate kit (Vector). Sections were counterstained in hematoxylin, dehydrated in ethanol, and mounted. Images were obtained using a PROVIS microscope (Olympus Optical, Hamburg, Germany) and AxioVision Rel 4.8 software (Zeiss, Cambridge, U.K.).

2.8 Collagen gel assay

PHM1-41s were embedded in type I collagen in 24-well plates at 10^5 cells/well as previously described (Hutchinson et al., 2014; Makieva et al., 2016). Briefly, the collagen/cell suspension was allowed to polymerise and the gels were detached and incubated at 37ºC for 24, 48 and 72 hours with treatments prepared in DMEM high-glucose medium with phenol red (Lonza) in the presence of 10% fetal bovine serum, penicillin/streptomycin and L-glutamine (all Lonza) (Makieva et al., 2016). Treatments included carbenoxolone (Sigma-Aldrich), a non-selective inhibitor of 11β-HSDs (Stewart et al., 1990), used at 500μM and 1 mM, and medium (vehicle). Untreated or vehicle-treated cells developed a basal contraction, which manifested as a decrease in the gel area and was first evident 24 hours post detachment. Gels were photographed using a Leica MZ6 microscope with Firecam camera system at 24, 48, 72 hours after treatment. The measurement (pixels) for each gel area was reported, performed with Adobe Photoshop CS3. Each treatment was performed in 6 replicates for three different times. The aggregate of replicates for each experiment was calculated and used for the statistical analysis.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6. Normally distributed data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests between each group to identify significant differences. Error bars denote ±SEM. Significance was assumed whenever $P < 0.05$.

3. Results

3.1 11βhsd-1 increases in the mouse uterus from early to term-pregnancy to reach a peak expression in preparation for labour

11β-hsd1 mRNA was detected in the mouse uterus in early pregnancy (defined as day 1-6), mid-pregnancy (defined as days 7-12) and term-pregnancy (defined as day 13-labour). The gene expression of the enzyme was the lowest early in pregnancy on day 5 (D5) and progressively increased in mid-pregnancy from day eight (D8) to day 12 (D12) of gestation to reach a peak at term-pregnancy on day 18 (D18) of gestation, one day prior to the onset of labour. Specifically, 11β-hsd1 mRNA concentration was significantly higher on D8 compared to D5 ($P < 0.0001$, 10 fold lower), higher on D12 ($P< 0.0001$, 7 fold higher) compared to D8 and higher on D18 compared to D12 ($P < 0.0001$, 4 fold higher) (Figure 1A). The concentration of mRNA did not change between D18 and day of labour (LB).

Albeit 11β-hsd2 mRNA was detected in the mouse uterus, its expression was very low and no statistically significant differences in expression were recorded between the different gestational days (Figure 1B).

Western Blot analysis (Figure 1C) confirmed the mRNA results. Specifically, a 34-KDa 11βhsd-1 band was evident from D8 to LB but was undetectable on D5. The concentration of 11β-hsd1 protein was significantly higher on D12 compared to D8 ($P < 0.0001$, 19 fold higher), lower on D12 compared to D15 ($P < 0.0001$, 1.5 fold lower) and higher on D18 compared to D17. The signal of 11β-hsd1 was the highest on D18 and a significant decrease occurred with the onset of labour (D18 vs LB $P < 0.0001$)

Immunohistochemical staining of 11β-hsd1 in the uterus revealed the localisation of the enzyme (Figure 2). Positive staining was evident in the cytoplasm of decidua epithelial cells from D8 (Figure 2D) of gestation onwards, whilst appeared weak or absent on D5 (Figure 2C), similar to the NP (Figure 2B) and the negative control (Figure 2A). Decidual 11β-hsd1 expression was observed throughout gestation, and in active labour (Figures 2F - I). In the myometrium, staining was absent or weak until D15 onwards (Figure 2F).

3.2 11β-HSD1 expression is higher at term gestation before onset of labour in the human myometrium.

11β-HSD1 mRNA was also detected in human myometrium. Expression of 11β-HSD1 mRNA differed depending on stage of pregnancy and labour status. Specifically, the relative expression of 11β-HSD1 was significantly greater in the TNL group when compared to both the TL and to the PT groups (Figure 3A).

Western blot analysis (Figure 3B) revealed 11β-HSD1 in the positive control (decidua), with a 34 kDa immunoreactive band. No significant difference in 11β-HSD1 protein expression was found when the TNL and TL groups were compared.

3.3 Carbenoxolone inhibits contraction of human myometrial cells in vitro

We examined the effect of 11βHSD inhibitor carbenoxolone (carb) on the contraction of PHM1-41s. PHM1-41s embedded in the collagen gel spontaneously contracted, which manifested as a significant decrease in the gel area at 24h and 48h (Figure 4A, vehicle lane). The addition of carbenoxolone (500 μM and 1mM) abolished the baseline contraction (Figure 4A) suggesting that

the compound inhibited contraction of PHM1-41 cells. Specifically, carbenoxolone significantly reduced the contraction of the collagen gels, both at 24h and 48h (1/3 fold lower than the vehicle, $P < 0.0001$) (Figure 4B).

4. Discussion

We report for the first time the temporal localisation of 11β-hsd1 mRNA and protein in the murine uterus at pregnancy, highlighting the potential involvement of this enzyme in regulating local glucocorticoid concentration/action and its role in the timing of labour. In contrast to 11β-hsd2 mRNA, whose expression remained low and virtually stable along the pregnancy time-points investigated, 11β-hsd1 exhibited a remarkable gestation-specific progress. The expression of murine 11β-hsd1 was low (and similar to the non-pregnant levels) until D5 and rose significantly from mid-pregnancy (D8) until the last day of pregnancy (D18, term non-labouring time). Once delivery started, a relative decrease in 11β-hsd1 expression was observed.

In addition, this is the first evidence of altered 11β-HSD1 expression in the human myometrium during pregnancy. In line with the murine 11β-hsd1, the concentration of human 11β-HSD1 mRNA was low in the preterm myometrium, and high in the term myometrium. We also observed a significant fall in human 11β-HSD1 mRNA in labour (compared to end of pregnancy samples), a pattern that was mirrored in murine 11β-HSD1 protein expression. Myometrial biopsies from early/mid pregnancy are extremely hard, if not impossible, to obtain and thus we cannot determine whether human 11β-HSD1 mRNA rises progressively in early pregnancy. Although we could not examine the localisation of 11β-HSD1 within the human myometrium, due to technical problems with the nonspecific antibody, we confirmed the presence of 11β-HSD1 mRNA and protein. Immunolocalisation studies of the murine uterus suggested that the decidua is a major contributor for the high expression of 11β-hsd1 in mouse, although 11β-hsd1 protein expression was also

present in murine myometrium from mid pregnancy to labour. Given that the cell mass of the myometrium is far greater than the decidua epithelium, we suggest that the contribution of myometrium in the observed increase of the enzyme should not be underestimated.

The present evidence for a high expression of 11β-hsd1 in murine uterus and in human myometrium in the preparatory phase before the labour are consistent with findings suggesting that a general system of glucocorticoids up-regulation is operative in late gestation. For example, an increase in the expression of 11hsd-1 during pregnancy has been previously reported in the placenta of various mammals, including the rat (Burton and Waddell, 1999; Klemcke and Christenson, 1996). In these species, glucocorticoid concentration increases in both maternal and fetal circulation as well as the amniotic fluid as pregnancy approaches parturition (Li et al., 2014).

Challis and colleagues previously proposed an association between the activity of 11β-HSD1 and parturition. (Challis et al., 2000) In particular, they hypothesized that cortisol generated by 11β-HSD1 in the myometrium and decidua could increase the concentration of uterine prostaglandins (PGs) namely E2 (PGE2) and F2alpha (PGF2α), through an up-regulation of prostaglandin H synthase 2 (PGHS2) gene expression (Challis et al., 2000). Indeed, PGE2 concentration progressively increases in the fetal circulation over the last 15-20 days of sheep gestation (Challis et al., 2000). Based on evidence that the elevation in uterine PGs stimulates myometrial contractions (Challis et al., 2001) and that 11β-HSD1 has been previously described to facilitate the PGs increase in fetal membranes (Alfaidy et al., 2001), we propose a novel hypothesis whereby 11β-HSD1 has a similar role in the regulation of myometrium. Given that 11β-HSD1 is lower once labour starts, it may be that 11β-HSD1 is important in the initiation but not the maintenance of labour.

Additional results *in vitro* with a cell line obtained from human pregnant myometrium (PHM1-41) also support a role for 11β-HSD1 in myometrial contraction, since the inhibition of this enzyme with carbenoxolone strongly reduced the contraction capability of myometrial cells. Although

multiple mechanisms underlie myometrial cells contraction, we can speculate that the inhibition of cortisol production might result in a loss of PG production, which appeared to be essential for the *in vitro* contraction of this cell line. (Hutchinson et al., 2014) Although detailed investigation of these mechanisms is out of the scope of the present work, it deserves further exploration in future studies.

There is additional evidence to propose that GCs trigger parturition in some mammals, including sheep (McLaren et al., 2000) and cow (Shenavai et al., 2012), by evoking a major event facilitating the shift from a progesterone-dominated inactive myometrium to an estrogen-activated uterus at the end of gestation (Challis et al., 2000). Specifically, it is known that in placenta GCs induce the expression of the enzyme cytochrome P45017αhydroxylase (P450c17), which converts C21 steroids such is progesterone into P19 steroid aromatase precursors (estrogens). In all species, estrogen holds a key role in myometrium activation for labour by stimulating expression of contraction associated proteins, such as oxytocin receptor (OTR), connexin 43 (Cx43) and prostaglandin (PG) receptors in myometrium (Challis et al., 2000). Nevertheless, the pathway to estrogen production in humans does not include the induction of P450c17. Glucocorticoids in human, instead, may influence this myometrial priming by increasing the conversion of dehydroepiandrosterone to estrogen, via induction of aromatase expression in human placenta (Wang et al., 2012). Thus, the observed increase of 11β-hsd1 expression in the second part of mouse gestation in our study might reflect the effort of GCs to achieve reasonable estrogen concentrations in the uterus to control the myometrial transition. The importance of this local regulation in uterus driven by 11β-HSD1 may be even greater in human myometrium, considering the critical differences in the parturition-specific high concentration of estrogens between human and the most other mammals. In humans, serum estrogen concentration is high throughout pregnancy and gradually increases at term (Tulchinsky et al., 1972), causing that a further estrogen activation may also occur, linked to a functional progesterone withdrawal, to achieve the same myometrial activation (Mesiano et al., 2002). In this situation, the 11β-HSD1 increase could be

critical for the estrogen-mediated myometrial switch and our murine and human results are in line with this hypothesis.

Over the last years, there have been many new data proposing that the main events in human parturition are inflammatory (Golightly et al., 2011). This idea is supported by the demonstration that neutrophils and macrophages invade the myometrium, cervix and fetal membranes at the onset of the labour, with an infiltration of inflammatory mediators, including plasminogen activators, eicosanoids, collagenase and elastase (Thomson, 1999). At the same time, an increased expression of pro-inflammatory cytokines including IL-1β and TNFα, affect both the myometrium and the cervix (Osman et al., 2003) and facilitate tissue remodelling (Sennström et al., 2000). In particular, the increase of pro-inflammatory cytokines at the onset of the labour potentiates myometrial contraction. For example, IL-1β induces calcium entry in myometrial smooth muscle cells, with a resulting effect on their contractile potential (Tribe et al., 2003).

As part of their anti-inflammatory action, glucocorticoids inhibit the expression of multiple inflammatory genes (cytokines, enzymes, receptors and adhesion molecules)(Barnes, 1998). The falling uterine 11β-hsd1 murine and human expression at the onset of the labour may reflect the decrease of active glucocorticoids. Their breakdown could prevent their anti-inflammatory effects and promote pro-inflammatory events, which may be important immediately after the switch from quiescent to contracted myometrium, for the immediate postnatal phase and involution.

Collectively, the present data strongly suggest that 11β-HSD1 plays a main role in modulating active glucocorticoids concentration in different pregnancy phases, promoting transitions between these stages, thus contributing to the regulation of uterine activity during gestation, both in mouse and in human. According to our results, active glucocorticoids derived by 11β-HSD1, expressed in the various tissues/organs involved in gestation and labour (myometrium, decidua, fetal membranes), can promote in paracrine/autocrine fashion myometrial contraction. In addition, the

rapid decrease in the 11β -HSD1 concentrations at the moment of labour fits well with the current view of labour as an inflammatory event.

Acknowledgements

We would like to thank Professor Angiolo Benedetti for constructive discussions and text reviewing.

Figure legends

Figure 1: 11βhsd-1 (A) and 11βhsd-2 (B) mRNA expression and 11βhsd-1 protein expression (C) in mice uterus. 11βhsd-1 (A) and 11βhsd-2 (B) mRNA expression in mice uterus on different days of gestation (non pregnant (NP), days 5 (D5), 8 (D8), 12 (D12), 18 (D18), and labour (LB)), investigated by RT-PCR. Values are the mean $\pm$ SEM (NP, D8 D12 n= 5; D5, D18 n=4; LB n=3). **** $P < 0.0001$ (C) 11βHSD-1 protein expression in mice uterus on different days of gestation (NP, D5, D8, D12, D15, D17, D18 and LB), investigated by Western Blot. Values are the mean ± SEM (NP, D18 n=4; D5, D8, D12, D17 and LB n=5; D15 n=3). Liver from wild type mouse was used as positive control (PC (WT)). The red signal consists of α-tubulin, used for quantification; the green signal consists of 11βHSD-1. **** $P < 0.0001$; *** $P = 0.0009$; * $P = 0.0474$

Figure 2: Representative images of immunolocalization of 11βHSD-1 in mice uterus during gestation (Non-pregnant (NP), day 5 (D5), 8 (D8), 12 (D12), 15 (D15), 17 (D17), 18 (D18) and moment of labour (LB)). The first image is the negative control. Note positive staining in the endometrium from the day 8. Staining is very strong from D15 until labour. Note the total absence

of the myometrium staining until D15. Scale bars, 100 μm. All images taken with a x10 objective lens.

Figure 3: 11βHSD-1 mRNA (A) and protein (B) expression in human myometrium. (A) 11βHSD-1 mRNA expression in human myometrium on different groups (term non labouring (TNL), term labouring (TL) and PreTerm group (PT)), investigated by RT-PCR. Values are the mean ± SEM (TNL n= 8; TL n=7, PT n=8). *** P = 0.0001; ** P = 0.0024. (B) 11βHSD-1 protein expression in human myometrium. Digital image of Western blot results of 11βHSD-1 in human myometrium on different samples (term non labouring (TNL), term labouring (TL)). Far right is the positive control (TNL decidua sample). Red banding at 50 kDa indicates the loading standard α-tubulin (50 kDa). Green banding at 35 kDa indicates expression of 11βHSD-1 protein. Graph displaying analysed results from western blot gel (TNL:n=5; TL : n=5). *** P = 0.0001; ** P = 0.0024.

Figure 4: Effects of carbenoxolone (carb) on contractility of PHM1-41s embedded in collagel gel at 24 and 48 hours. Gel area was significantly greater using carbenoxolone at 500 μM and 1mM then the vehicle without treatment after 24 hours ($P < 0.0001$). Gel spontaneously twitched, and the contraction increases in a significant manner after 48 hours. After 48 hours, in the collagen gel with treatments no significant change were observed. Data are the mean ± SEM, 6 wells each experiment (n= 3 veh, carb 1mM, carb 500μM). **** $P < 0.0001$; * P = 0.028.

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Figure 1

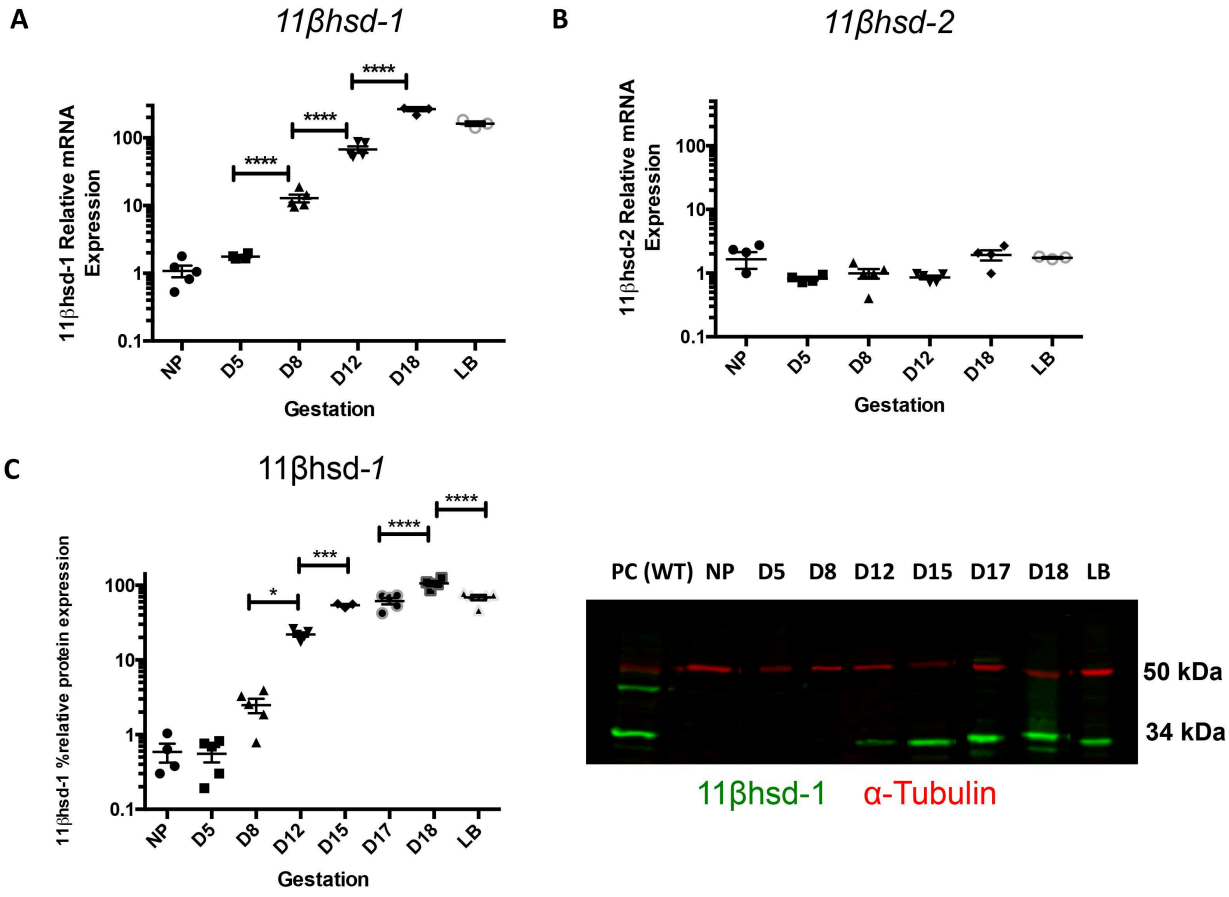
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Figure 2

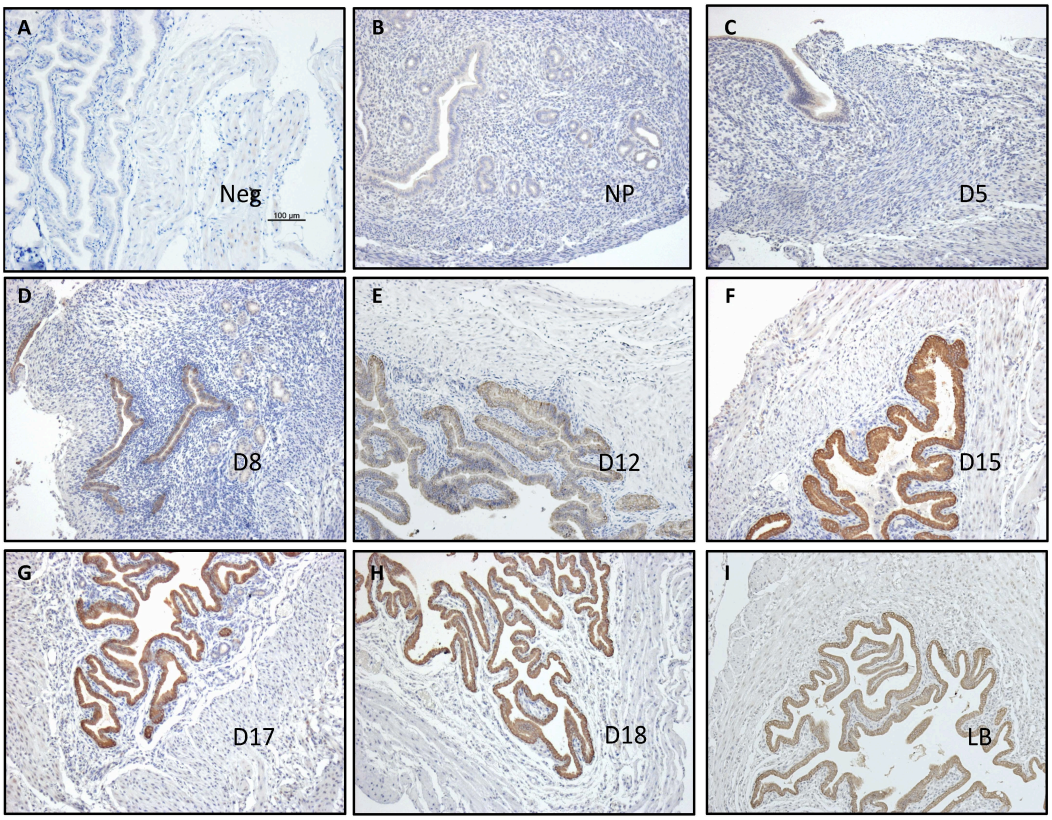

Figure 3

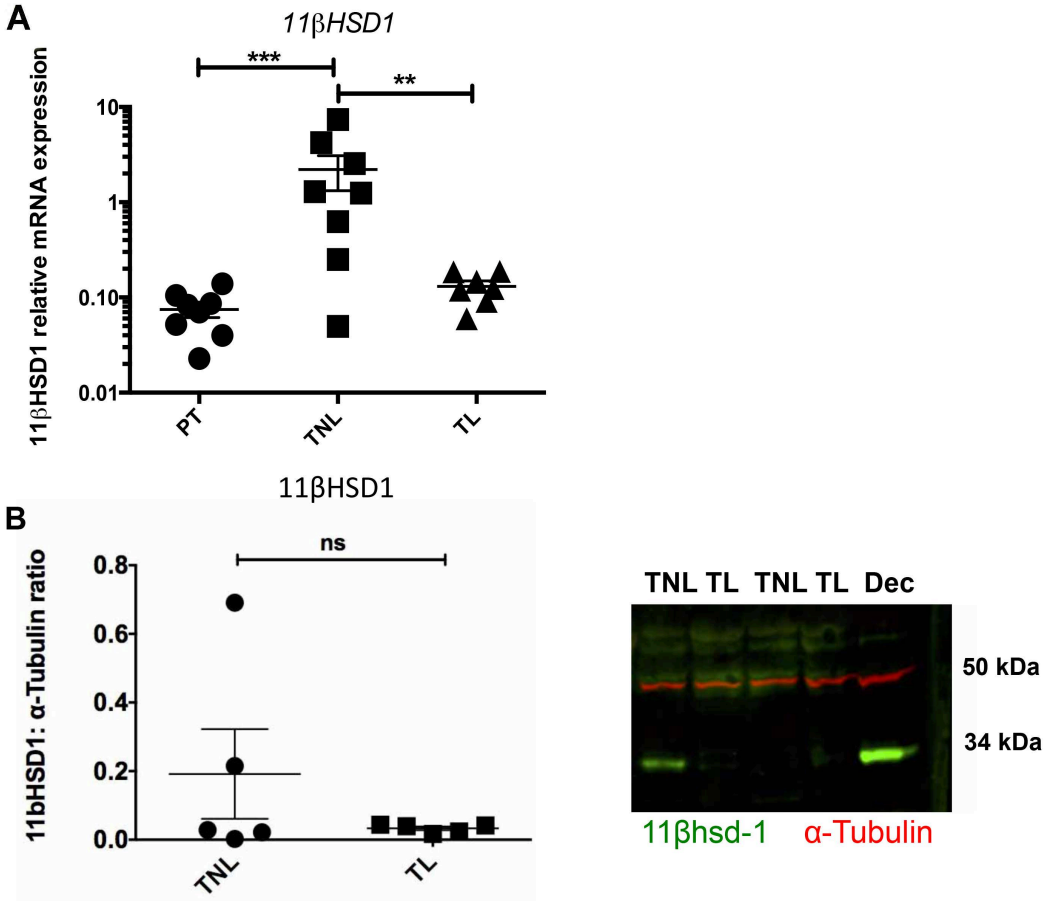
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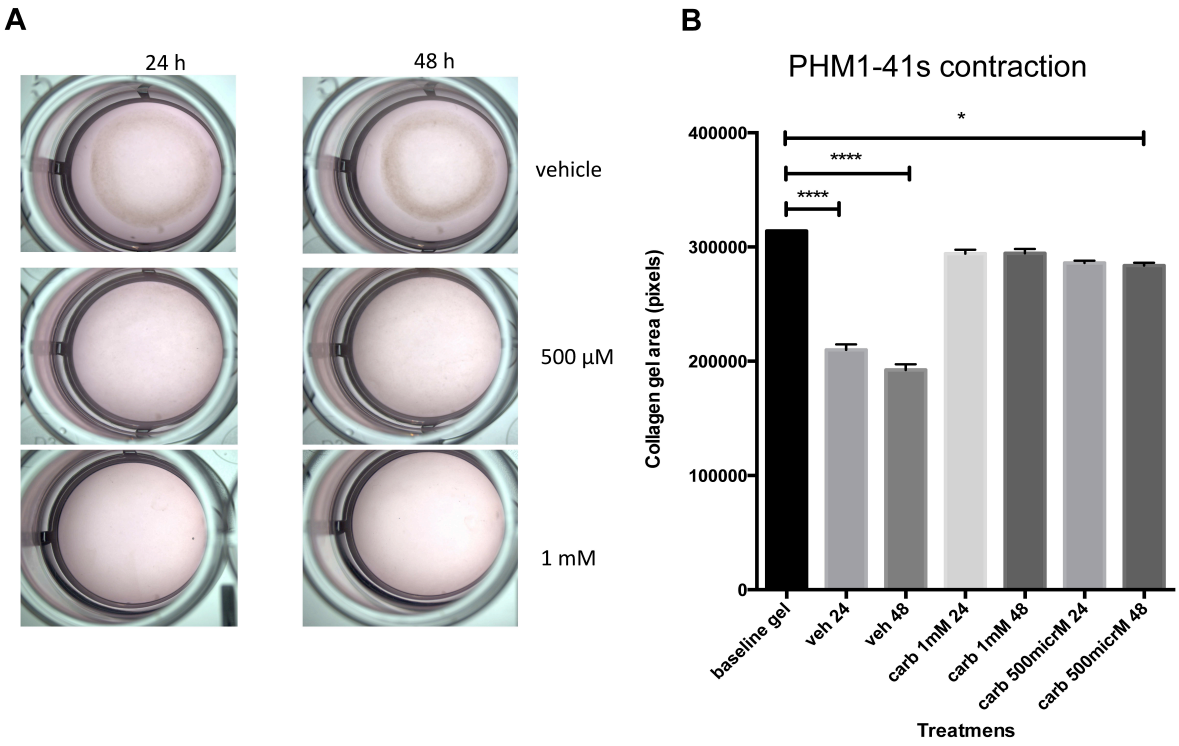
Figure 4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carb 500micrM 48
Treatmens

3.3 Nb-UVB treatment of psoriasis: possible implications of 11β-HSD1 in disease improvement

The aim of this third work was to move from the reproduction area and investigate the role of 11β-HSD1 in skin, focusing our attention on its implication in psoriasis, one of the most common skin disease.

Psoriasis, an inflammatory disease accompanied by a broad spectrum of pathological manifestation, is commonly addressed through the application of topic glucocorticoids or oral immune-suppressors. Given the long list of side effects that these treatments lead, phototherapy has been established as an important treatment alternative. Among phototherapy approaches, narrow-band (nb) phototherapy is one of the most used, due to its effectiveness and the few contraindications. Despite the large use, it is still unclear the mechanism through which nb-phototherapy has ameliorative effects on psoriatic plaques. Recent studies have shown an augmented production of glucocorticoids (GCs) in healthy subjects after UVB exposition, with an increase in the local modulator of GCs, 11β-HSD1. Given the improvement of symptoms in patients with psoriasis after nb-UVB, we investigate about the possible modulation of 11β-HSD1 in fibroblasts from psoriatic subjects, before and after treatment cycle with nb-UVB, comparing the data with normal subject values. Our preliminary results showed an apparent similar expression level between psoriatic subject before treatment and healthy control. Interestingly, after the treatment cycle, resulting in improvement of symptoms, we observed a 5-fold increase in mRNA expression in fibroblasts of patients, compared with the levels before the treatments. Our preliminary Western Blot showed an analogue result for the protein levels of the enzyme, suggesting that an effective modulation of the enzyme occurred by the nb-UVB irradiation.

Our results need substantial increase of the subjects included in the study, to confirm the validity of the reported data, but preliminary observations seem direct us to a glucocorticoids-mediated improvement of psoriasis symptoms, after nb-UVB treatments.

Preliminary paper

Quantitative evaluation of 11- beta Hydroxysteroid Dehydrogenase type 1 in cutaneous fibroblasts cultures of psoriasic lesional skin before and after narrow band -UVB phototherapy

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Abstract

Psoriasis is an inflammatory skin disease, associated with manifestation such as diabetes or metabolic syndrome, involving also psychological distress in patients. One of the most common therapeutic approach for psoriasis is topic glucocorticoid application. However, side effects of topic and oral treatments brought to develop new therapeutic methods, such as narrow band (nb) -UVB treatments. Despite the excellent results and the minor unwanted consequences, molecular mechanism of nb-UVB ameliorative effects on psoriasis plaques are still unclear. The purpose of this work was to explore the possible mechanism underlying nb-UVB therapy in psoriasis, investigating on the modulation by the local regulator of glucocorticoids, 11β-HSD-1, before and after improvement due to nb-UVB treatments. Although our studies are still uncompleted, our results showed an increase of the mRNA (5-fold increase) and protein expression after the treatments cycle, suggesting a possible role of the enzyme in the mechanism of symptoms suppression after nb-UVB approach.

Introduction

Topic glucocorticoids are a class of steroids structurally similar to the endogenous cortisol. As from a long time, glucocorticoids are still today the first therapeutic choice in the vast majority of dermatitis requiring anti-inflammatory and immunosuppressive activities. Atopic dermatitis and psoriasis constitute auto-inflammatory disease responsive to a topic glucocorticoids therapy. In addition to the immunosuppressive induction, glucocorticoids effects include epidermis thinning, erythema suppression and impairment of melanogenesis [1][2].

It is known that cortisol in skin is made from cholesterol, in a slower process than the one of adrenal cortical [3]. Cortisol acts binding glucocorticoid receptor (GR), but its availability

depends on the pre-receptor level regulation of 11β-HSD enzyme family[1][2]. The family is composed by the 11β-HSD1, that works activating cortisone to cortisol, and the 11β-HSD2, catalysing the opposite reaction, inactivating the molecule.[1]

Over the last years, 11β-HSDs family has been subject of a plethora of studies, with characterization in liver, ovary, lung, Central Nervous System (SNC), adipose tissue, placenta, colon and kidney [4][5][6][7]. In these studies, it has been shown the role of 11β-HSD1 in obesity, metabolic syndrome and diabetes. Recently, it was investigated its function in skin. 11β-HSD1 has been identified in epidermis, specifically in keratinocytes culture [8] and keratinocytes-melanocytes co-culture [9], and dermis, specifically in fibroblasts[10] and endothelial cells [11].

Psoriasis is an inflammatory skin disease with a chronic-progressive trend, characterized for its association with other pathologies, such as hypertension, diabetes, metabolic syndrome and for the psycho-social implications of patients [12][13]. The epidemiological estimates are variant in the studies, but the incidence of the disease appears increasing in the last 40 years, with values between 1.8-5% in adults in the world (2-4% in western countries) and 0.2-1% in children [12][13][14]. Etiology of the disease is multifactorial and well known. However, pathogenic molecular mechanisms are still unclear. In particular, few data are available about the specific role of cutaneous glucocorticoids homeostasis in the development of psoriasis. Nevertheless, studying the steroids metabolism in skin represents a crucial step to understand pathogenesis, physiopathology and therapeutic response of many skin disease, included psoriasis. Recently, a study demonstrated that keratinocytes from lesional skin of psoriatic patients showed a lower expression of CCHR1, steroidogenesis promoter, compared to control subjects [15], suggesting that a steroidogenesis deregulation occurs in psoriasis.

It is known the immunomodulator effect of UVB therapy, able to induce disease remission in 60-70% cases of moderate forms of psoriasis [16]. However, the mechanism action of narrow-band UVB (nb-UVB) on psoriatic skin is still unclear [16]. Over the last years, Tiganescu et al. investigated on the stimulator effect of UVB on 11β-HSD1, both in murine and in human models [11]. In both models, it has been found a higher expression of 11β-HSD1 in photo-exposed skin compared with photo-protected skin [11], [17]. Similar expression levels were found in keratinocytes cultures, after UVB and UVA expositions [9]. However, 11β-HSD1 expression modifications have not yet been studied in psoriasis. Given these premises, we aimed to evaluate the 11β-HSD1 expression on lesional psoriatic skin, before and after nb-UVB treatments, in order to identify a possible deregulation of glucocorticoids homeostasis

in psoriasis and the phototherapy effects on it.

Materials and Methods

Patients recruitment and treatment

Six psoriatic patients, between 18 and 80 years of age, were recruited among the consecutive patients attending the nb-UVB cyclic treatments at Dermatology Unit of AOUS – Policlinic "Santa Maria delle Scotte" of Siena. All enrolled patients gave signed, informed consent after receiving written and oral information on the study. The project was approved by the local ethics committee and conducted according Helsinki Declaration ethical conditions.

Patients performed two weekly sessions for 7 months, with increasing nb-UVB doses (from 500 to 1300 mj/cm^2). For each subject, it was performed a 4 mm punch biopsy in photo-protected cutaneous area. Psoriatic patients have undergone one biopsy before the phototherapy and one at the end of the treatment period. Along the treatment, no topic glucocorticoids have been applied.

Concerning the control subjects (n=6), it was performed a 4 mm punch biopsy in a photo-protected cutaneous area only one time.

Cell Culture

Tissue samples from biopsy were washed with DPBS (*Dulbecco's Modified Buffered Saline, Gibco)* and fragmented in small pieces (< 1 mm), together with DMEM (*Dulbecco's Modified Eagle's Medium, Sigma-Aldrich),*10% FBS (*Fetal Bovine Serum, Euroclone)*. Pieces were seeded in a flask and, after adhesion, were maintained with DMEM, 10% FBS, 5mM Penicillin/Streptomycin (*Euroclone)* and 2mM L-Glutamine *(Euroclone)* at 37°C. In 3-4 days, fibroblasts were the only cells in adhesion. Subculture was initiated after reaching semi-confluence. Detaching was performed through Trypsin-EDTA (*Euroclone)* and a 600 g centrifuge for 8 minutes.

Fibroblasts freezing and pellet preparation

Fibroblasts were frozen in a freezing medium composed by 10% DMSO (*Sigma-Aldrich)* and 90% DMEM and stored in cryovials at -80 °C. For each sample, a pellet from approximately 3-4 x 10^6 cells was obtained after two consecutive centrifugation at 600xg for 8 minutes.

Rna extraction and Real-Time PCR

Total RNA was extracted from fibroblasts using NucleoSpin RNA (*Macherey-Nagel*) kit, according to the manufacturers' istructions. RNA quality and quantify was determined using *Gene QuantPro*

(Pharmacia Biotech) and stored at -80°C. cDNA was prepared from 500 ng RNA using the *SuperScript III First-Strand Synthesis Super Mix* for qRT-PCR (*Invitrogen by Life Tecnologies*) kit. Predesigned gene expression assays were used to examine the expression of human 11β-HSD1 (forward: CCAGCAAAGGGATCGGAAGAG; reverse: GTAGTGTGCTGAGGCTGCTCC) and human GAPDH (forward: TTCACCACCATGGAGAAGGC; reverse: GGCATGGACTGTGGTCATGA). Experiments were done in duplicate with the PCR protocol of the manufacturer of *TransStart Top Green qPCR SuperMix* (*Transgen Biotech*, Cina). The negative control for each reaction consisted of amplification performed in the absence of RT and blank samples in absence of RNA samples. Target gene expressions was normalized using GAPDH and the expression in each sample was calculated relative to the average of control samples, using the $2^{-\Delta\Delta ct}$ method analysis. All qRT-PCR analysis was performed on *MJ Research PTC-200 (BioRad)*, according to the standard protocol.

Sample Processing

Fibroblasts were lysed by a Lysis Buffer (LB) (20 mM Tris-HCl, 135 mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 10 mM EDTA, pH 8, togheter with proteases inhibitor cocktail (*Thermo Scientific*), at the concentration indicated by manufacturers). We added 75 µl of LB for 10^6 fibroblasts and samples were left 30 minutes on ice. After centrifugation at 1000xg for 10 minutes, supernatant was recovered and protein was quantified by using *QuantumMicroProtein (*EuroClone).

Western Blot

Western Blot was used to detect 11β-HSD1 protein. Protein was separated by SDS-PAGE (Precast gel Bolt 4-12% Bis-Tris Plus, *Invitrogen by Thermo Fisher Scientific*) and then transferred to a nitrocellulose membrane (*GE Healthcare by Life Science*). Membranes were blocked for 1 hour at room temperature with 2.5% dry skimmed milk and 2.5% Bovine Serum Albumin Fraction V (BSA) (*AppliChem Panreac*) in 0.05% Tris-Buffered Saline Tween (TBST) and were then probed overnight at 4°C with anti-11β-HSD1 antibody 1:200 (*11β hydroxysteroid dehydrogenase (Type 1) Polyclonal Antibody, Cayman Chemical*) and monoclonal anti-α tubulin antibody (*Alpha Tubulin antibody, GeneTex)*, used as a quantification control, followed by extensive washing.

Immunoreactive materials were detected by 1 hour incubation with secondary antibody 1:1000 (*Anti-rabbit IgG HRP-linked Antibody, Cell Signaling*). After extensive washing, membranes were incubated with *SuperSignal West Femto Maximum Sensitivity Substrate* (*Thermo Scientific*) for 5 minutes, according with manufacturers' indications and were finally analyzed by *ChemiDoc (Bio-Rad)*.

Preliminary Results

11β-HSD1 increases after nb-UVB treatments in psoriatic subjects

11β-HSD1 mRNA was detected in the human skin fibroblasts analysed. The gene expression appeared low and generally similar between patients before treatments(n=7, called PSO) and control subjects (n=8, CTR). In contrast, average of the samples after nb-UVB treatment (N=5, PSO post) showed a significant higher expression of the enzyme compared with the same samples before the treatment ($P = 0.0015$, 5 fold higher).

Western Blot analysis confirmed the mRNA results. Specifically, a 34-KDa immunoreactive band was evident in all samples and the normalization through α-tubulin intensity band evidenced an higher expression in psoriatic samples after nb-UVB treatments (n=2), compared with the same samples before the treatment and the control subjects (n=4).

Discussion (Draft)

The role of 11β-HSD1 was widely studied in a plethora of organs/tissues [18], but its role in skin homeostasis is still unclear, despite various recent studies suggested an implication in cutaneous functions. We aimed to clarify its possible role in psoriasis, investigating on the mechanism underlying improvement after one of the possible psoriasis treatments. Psoriasis is a frequent skin disease and the most common way to treat psoriasis is topic glucocorticoid [19]. Nowadays, phototherapy nb-UVB seems to be one of the best approach to ameliorate the symptoms, suppressing the possible collateral effects (ref), but molecular mechanism at the basis of the improvement are still unclear.

For that reason, we compared the expression of the enzyme in psoriasis patients, before and after a treatments cycle with nb-UVB irradiation, until the improvement of the symptoms. Our experiments evidenced a significant enhance of 11β-HSD1 mRNA after phototherapy cycle (Figure 1) and similar trend is visible in preliminary Western Blot data (Figure 2). Given the decrease of lesions extent and thickness that patients of the study showed, we suggest that 11β-HSD1 is involved in the symptoms resolution-nb-UVB mediated. Recents *in vivo* studies showed an augmented 11β-HSD1 expression in normal murine skin after UVB phototherapy [17]. An analogue treatments in psoriatic patients may augment in a similar trend 11β-HSD1 levels and, probably, concur to the ameliorative process of psoriasic plaques. In this context, local glucocorticoids production 11β-HSD1-mediated may influence the lesion evolution, thus supporting topic

glucocorticoids use in the treatment of psoriasis. Thanks to their anti-inflammatory and immunosuppressive activity [2], topic glucocorticoids are largely used in a plethora of skin disease, such as atopic dermatitis and allergic dermatitis [20]. It is known that psoriasis is an inflammatory chronic skin disease[12][21], characterized by an immune disorder, principally involving T $CD4^+$ cells, and a remarkable production of pro-inflammatory cytokines (IL-6,IL-18,TNFα) [22]. Nevertheless, glucocorticoids are anti-inflammatory agents [23][24] able to inhibit transcription factors involving in inflammation, such as Nf-kB and AP-1, and with effects on keratinocytes and on immune-system cells infiltrating in skin [20]. Therefore, anti-inflammatory activity of glucocorticoids may contrast manifestation of skin disease characterized by chronic-relapsing flogosis inflammatory reactions.

A recent study showed that an increase in 11β-HSD1 expression amplify local tissue-specific activity of glucocorticoids [25]. It is known that glucocorticoids induce thinning of dermis and epidermis, with elasticity reduction [9] [10][26]. Given the peculiar skin thickness of psoriatic subjects, superior to the normal one due to an atypical keratinocytes differentiation [27], glucocorticoids activity may reduce thickness of psoriatic plaques, normalizing keratinocytes metabolism.

Finally, we can assert that data obtained suggest a sensibility of psoriatic lesions to phototherapy, probably due to the local glucocorticoids production by 11β-HSD1 nb-UVB-induced. Nevertheless, it will be critical making further experiments, to increase samples number and confirm the real relationship between phototherapy and psoriasis regression. To reach this goal, it will be probably helpful investigate on keratinocytes, repeating the experiments carried out on fibroblasts. Furthermore, experiments on Glucocorticoid Receptor (GR) expression before and after phototherapy could be a good chance, to actually understand if the augmented expression of 11β-HSD1 reflects an increase in cortisol activity.

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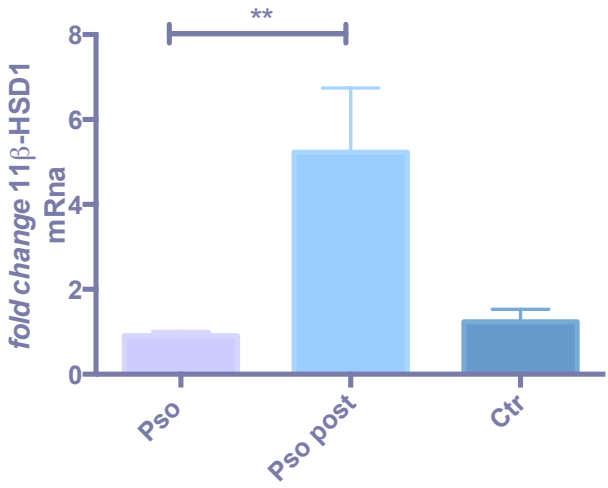

Figure 1. 11β-HSD1 mRNA expression in cutaneous fibroblasts. A comparison between mRNA expression levels in psoriasis subjects fibroblasts before nb-UVB treatments (PSO, n=7) and after nb-UVB treatments (PSO post, n=5) and control subjects (CTR, n=8). Values and the mean ± SEM.

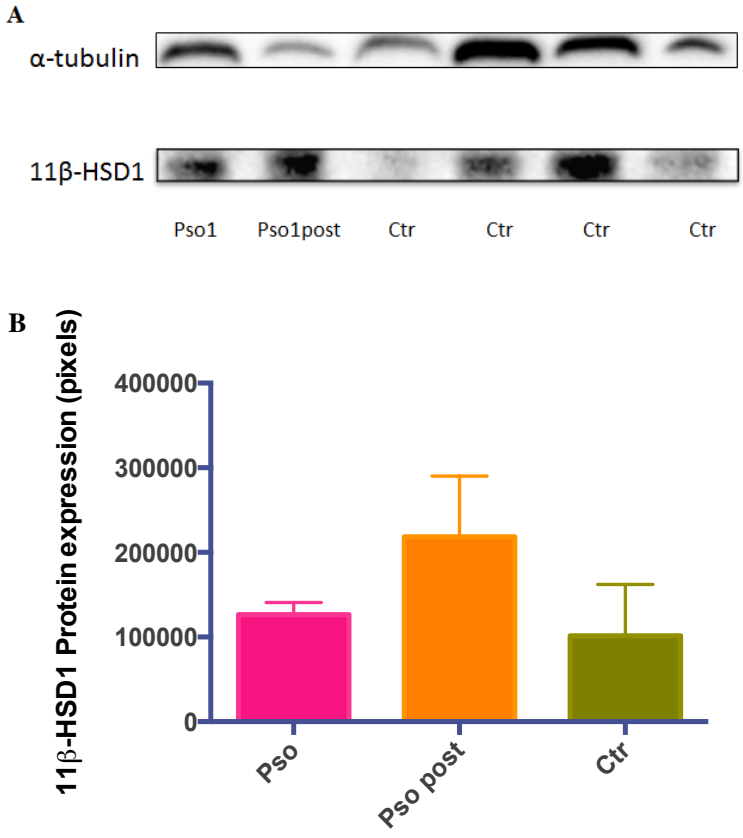

Figure 2. 11β-HSD1 protein expression in cutaneous fibroblasts. A comparison between protein expression levels in psoriasis subjects fibroblasts before nb-UVB treatments (PSO, n=2) and after nb-UVB treatments (PSO post, n=2) and control subjects (CTR, n=4). Panel **A** is an example of Western blot of a sample (PSO1) before and after treatment and of 4 controls. Higher bands represented the housekeeping levels (α-tubulin); lower bands represented 11β-HSD1. Panel **B** is the schematic representation of normalized data, expressed in pixels. Values and the mean ± SEM.

4. General Discussion

The aim of my three year-work was to expanding the knowledge of roles, implications and action mechanism of 11β-HSD1 in tissue/organs in which the enzyme has not yet been fully and clearly studied. Most of work was focused on reproduction regulation, investigating on the 11β-HSD1 role along the pregnancy phases, from the early stages after implantation until the last steps of myometrial contraction and labour. Finally, the attention was moved to the skin, another unexplored district concerning 11β-HSD1, where we evaluate its possible role in pathologic states, focusing the work on psoriasis and strategical approaches to treat it.

Our evidences have shown that pregnancy is strongly influenced by 11β-HSD1 in different moments and ways during the various stages of gestation. From the early events, pregnancy modulation shows 11β-HSD1 as a significant actor of the gestation, modulated by steroid hormones and inflammatory cytokines, the real leaders of the physiological process.

In fact, it is known that both ovarian steroids and inflammatory cytokines decisively influence decidualization and interactions between embryo and decidua [88], primary processes for blastocyst implantation and proper development of the fetus. After the attachment of blastocyst, implantation is characterized by the extravillous trophoblast invasion of the decidua [89]. Defects in the invasion may lead gestational disease, such as preeclampsia (PE) [90]. Our work gave a new glucocorticoid-central vision of the preliminary pregnancy phases, in which decidua is still modulated by hormones and cytokines, but through the mediation by glucocorticoids and their local regulator, 11β-HSD1. Estrogens and progesterone together work enhancing 11β-HSD1 expression and activity in decidua in first trimester, highlighting the importance of the enzyme in the economy of the development of the fetus. Moreover, our results showed a possible involvement of the enzyme not only in physiologic process, but also in pathology, demonstrating a higher expression of 11β-HSD1 in decidua in the development of preeclampsia (PE).

The importance of the enzyme is undoubtedly confirmed by our evidences in murine experiments. 11β-HSD1 in uterus has a constant and repeatable trend during all murine gestation, with a first silent phase and then a progressive increase of the expression until the last day of pregnancy, tying in a clear manner the advancement of the process

with 11β-HSD1 expression levels. Human experiments gave us similar results, confirming the endometrium high expression levels of the enzyme immediately before the labour. We suggested a role for the glucocorticoids 11β-HSD1-dependent augmented expression in prostaglandin stimulation, involved in turn in myometrium contraction activation [91], given also the *in vitro* experiments with PHM1-41s. In fact, confirming our conclusions, embedding myometrial cells in a collagen gel, we observed an inhibition of the spontaneous contraction after treating them with carbenoxolone, an inhibitor of 11β-HSD1. Moreover, myometrium activation is directly dependent on estrogen levels in uterus [65] and glucocorticoids are able to influence positively estrogen concentration, both in human [92] and in mouse [93]. Therefore, we suggested that the augmented expression of 11β-HSD1 observed in uterus during the second part of gestation has as its ultimate goal the estrogenic increase needed for myometrial activation.

In addition, our evidences gave further importance to the enzyme, not only for its presence, but also for its absence. In fact, our murine and human experiments showed a dramatic drop of the enzyme expression at the moment of the labour, compared with the phases and days immediately before. This behaviour is probably due to the inflammatory condition of the labour. In fact, the main events of the labour are inflammatory [44] and glucocorticoids have anti-inflammatory actions by definition [42]. Given this impossible convergence, 11β-HSD1 drop may be crucial to achieving the appropriate inflammatory levels of the labour, promoting the importance of the absence of the enzyme for the final event of the gestation.

In view of the above, 11β-HSD1 appear to be a key focus for starting and maintaining gestation, stimulating the end of pregnancy and strategically decreasing when its presence would be detrimental.

Changing perspective, but keeping the lens on the same actor, we analysed the role of 11β-HSD1 in skin. In particular, we move our attention on psoriasis and one of the most used approach to treat it, nb-UVB. Given the unclear mechanisms underlying the improvement in psoriasis symptoms after nb-UVB cycle treatments, we investigated on the possible changes in the expression of 11β-HSD1. The work was done based on previous evidences of the enhanced expression of the enzyme in photo-exposed skin, compared with photo-protected [71] and on the previous hypothesis of a

steroidogenesis deregulation occurred in psoriasis [94]. Our evidences in cutaneous fibroblasts showed a substantial equity in the enzyme expression values between patients with psoriasis and control subjects. However, we observed a notable augmented expression of 11β-HSD1 after nb-UVB treatment. Considering the improvement of the symptoms, we suggested a possible implication of glucocorticoids and its local regulator in the ameliorations, as it occurs with topic glucocorticoid treatments. Despite the study still needs further experiments and a significant increase in samples number, the evidences highlight an additional role for 11β-HSD, drawing a picture in which the enzyme is able to act in different ways, times and districts.

Globally, my work highlights the extreme importance of local modulation, confirming what happens in a plethora of diseases, such as metabolic syndrome, where a pathologic situation does not necessarily correlate with a systemic deregulation (normal levels of circulating cortisol), but is connected to a malfunction of the local regulator, 11β-HSD1. In our observations, the local modulation of glucocorticoids decisively influences physiology and pathology of reproduction and skin homeostasis, as and, perhaps, even more than systemic glucocorticoids production and modulation.

My work has then tried to wide horizons for 11β-HSD1, revealing new functional perspective and possible implications yet unknown in physiological and pathological processes. Future work on this enzyme in reproductive processes could open roads to better understand disease processes such as preterm labour, preeclampsia or defects in implantation; equally, a better understanding of the underlying mechanisms of psoriasis and of the methods to relieve its symptoms, could lead to refine treatments and to improve the general health of subjects affecting by psoriasis.

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