Mineralocorticoid receptor blockade improves glucocorticoid-induced skin atrophy but partially ameliorates anti-inflammatory actions in an irritative model in human skin explants

Julia Boix[1], Van Tuan Nguyen[2,3], Nicolette Farman[2], Sélim Aractingi[4], Paloma Pérez[1*]

[1]Instituto de Biomedicina de Valencia, Consejo Superior de Investigaciones Científicas

(IBV-CSIC), Valencia, Spain

[2] UMR 1138, INSERM, Centre de Recherche des Cordeliers, Paris, France

[3] Basic Science Department, Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam

[4] Hôpital Cochin Tarnier, Département de Dermatologie and Université Paris Descartes, Paris, France

* Corresponding author: Paloma Pérez, IBV-CSIC, Jaime Roig 11, E-46010-Valencia, Spain.

Tel +34 96 339 1766 Fax +34 96 369 0800

pperez@ibv.csic.es

Short title: MR blockade improves GC-induced skin atrophy in human damaged skin

Abstract

We recently demonstrated that blockade of the mineralocorticoid receptor (MR) effectively ameliorated GC-induced skin atrophy in healthy human skin explants and epidermal MR knock-out mice. However, whether MR blockade improves the therapeutic index of GCs in skin pathology was not investigated.

We assessed the effects of GCs, MR antagonists (MRA), or both, in SDS-treated human skin explants. All treatments restored SDS-augmented epidermal thickness but only GC plus MRA restored the expression of *COL1A1*. However, MRA alone or in combination with GCs may exert a dual role in regulating inflammatory cytokines. Thus, although combined treatment may be beneficial to improve irritative skin, extensive *in vivo* testing is required to establish whether the anti-inflammatory effects of GCs are maintained in the presence of MRA.

Background

Skin inflammatory diseases are commonly treated with topical glucocorticoids (GCs) because their anti-proliferative and anti-inflammatory effects (1). However, their use is limited due to adverse side effects such as skin atrophy characterized by tissue thinning caused by inhibition of keratinocyte and fibroblast proliferation and a reduction in the collagen synthesis (1,2). Also, local steroidogenic activity is key to homeostatic prevention or attenuation of skin pathology (3,4).

GCs bind and activate two closely related receptors, the GC receptor (GR) and the mineralocorticoid receptor (MR). Our recent work using genetic and pharmacological approaches in mouse skin and healthy skin of volunteers and human skin explants has shown that MR inactivation ameliorates GC-induced skin atrophy (5,6). In both settings, MR blockade improved the epidermal thinning partially reversing the keratinocyte proliferation inhibition induced by topical GC treatments (5,6). However, GCs similarly reduced *Col1a1* mRNA levels in MR epidermal knock-out and control mice indicating that inactivation of dermal MR is also necessary for the effective reversion of skin atrophy (5).

Questions addressed

We have analyzed whether GC treatment combined with pharmacological MR inhibition achieves a better therapeutic index in an irritative model in human skin explants.

Experimental design

Human skin explants culture and treatments
Human skin biopsies were obtained from three different subjects. After adipose tissue excision, 6 mm full-thickness punches were obtained of each subject, placed in organotypic culture floating with the dermis embedded into the medium, and used for one independent experiment. Human skin explants were cultured in medium DMEM 1x with GlutaMAX, 10% charcoal-stripped fetal bovine serum and 1% Penicilin/Streptomicin, and incubated at 37ºC, 5% CO_2 (6). SDS (15 μg/ml; Merck, Darmstadt, Germany) was added to the medium during 3 days; then, washed, and explants were treated with 100 nM clobetasol propionate, 10 μM canrenoate, 100 nM clobetasol plus 10 μM canrenoate (Sigma, St Louis, MO), or vehicle, during 4 days.

Results

Using human skin explants, we have mimicked an irritative pathology by adding SDS to the medium (7). After 3 days, SDS-induced tissue damage was revealed in hematoxylin-eosin stained sections by cell ballooning (Fig.1a, arrows), increased dermal cellularity (Fig.1a) and augmented epidermal thickness (Fig.1a,b). These features were similar to those observed in human skin topically treated with SDS (8,10). However, SDS did not affect collagen deposition or mRNA expression levels relative to vehicle-treated samples (Fig.s1a,b).

We assessed the therapeutic effectiveness of the GC clobetasol, the MRA canrenoate, or both, in skin explants incubated with SDS. Clobetasol improved the SDS-induced effects by reducing

cell ballooning, dermal cellularity, and epidermal thickness (Fig.1a,b). As expected, clobetasol also reduced collagen deposition as shown by the Masson trichrome staining and the 70% reduction in *COL1A1* mRNA levels (Fig.s1a,b). Treatment with canrenoate elicited similar effects than GCs in terms of epidermal thickness and *COL1A1* expression, with a reduction of approximately 60% in both parameters (Fig.1a,b; s1a,b). This is consistent with previous data reporting that the induced expression of *COL1A1* in human dermal fibroblast was effectively blocked by treatment with the MRA spironolactone (9). Importantly, the combined treatment using clobetasol and canrenoate was also effective in decreasing the SDS-induced epidermal thickening (Fig.1a,b). In addition, this treatment was the only that improved the collagen deposition and the *COL1A1* mRNA levels as compared to each compound used separately (Fig. s1a,b).

We also evaluated the expression of keratin (K)6, a marker of keratinocyte hyperproliferation that is found in healthy palmar and plantar skin as well as diseased skin, in explants treated with SDS alone or in combination with the indicated compounds. After SDS treatment, all epidermal layers stained positive for K6; this expression was significantly reduced by clobetasol, which was confined to the upper epidermal layers, but unaffected by canrenoate (Fig.2a). The combined treatment reduced the effectiveness of clobetasol alone as it produced an intermediate effect with K6 being expressed in several suprabasal layers (Fig.2a). These results suggest that complete K6 inhibition by GCs requires functional MR.

Next, we assessed whether the anti-inflammatory effects of GCs were preserved after blocking MR. We analyzed the expression of the pro-inflammatory cytokines *IL-6* and *IL1A* and a key enzyme in prostaglandin biosynthesis, *COX-2,* which are up-regulated after SDS treatment in human keratinocytes (11) or skin (8,12). Clobetasol significantly reduced the expression of SDS-induced *IL-6*, *IL1A,* and *COX-*2 mRNA levels, as expected (Fig.2b). However, canrenoate triggered both anti-inflammatory effects, with decreased expression of *IL-6* and *COX-2*, and also pro-inflammatory, with up-regulation of *IL1A* mRNA levels (Fig.2b). The use of clobetasol plus canrenoate resulted in attenuated or lack of repression of *IL-6* and *IL1A*, respectively, while it had no effect in the inhibition of *COX-2* relative to clobetasol alone (Fig.2b). Also, consistent with its reported induction by GCs (13), Clobetasol up-regulated the mRNA levels of the anti-inflammatory cytokine *IL-10* in SDS-treated skin explants (Fig. 2b). While canrenoate did not affect *IL-10* mRNA levels, clobetasol plus canrenoate resulted in a trend towards increased transcript levels without reaching statistical significance, suggesting that MR blockade affects GC-dependent induction of *IL-10*. These results show that MR may exert a dual role in skin inflammation.

Conclusions

SDS-treatment in human skin explants induced tissue damage as demonstrated by cell ballooning, increased epidermal thickening and dermal cellularity. In this clinically relevant setting, MRA alone was as effective as GCs in normalizing epidermal thickness although it was unable to control up-regulation of K6 expression and exerted dual pro- and anti-inflammatory actions. The combined use of GCs plus MRA had several advantages relative to individual treatments since it restored epidermal thickness, partially reversed K6 up-regulation, and normalized collagen deposition. However, in this model of human diseased skin, the anti-inflammatory effects of GCs, in the case of *IL6* and *IL1A*, were not fully preserved in the

presence of MRAs. Overall, our data show that addition of MRA to GCs may be beneficial to improve irritative skin, but extensive *in vivo* testing is required to establish whether the anti-inflammatory effects of GCs are maintained in the presence of MRA.

Acknowledgements

Conflict of interest

The authors have declared no conflict of interests.

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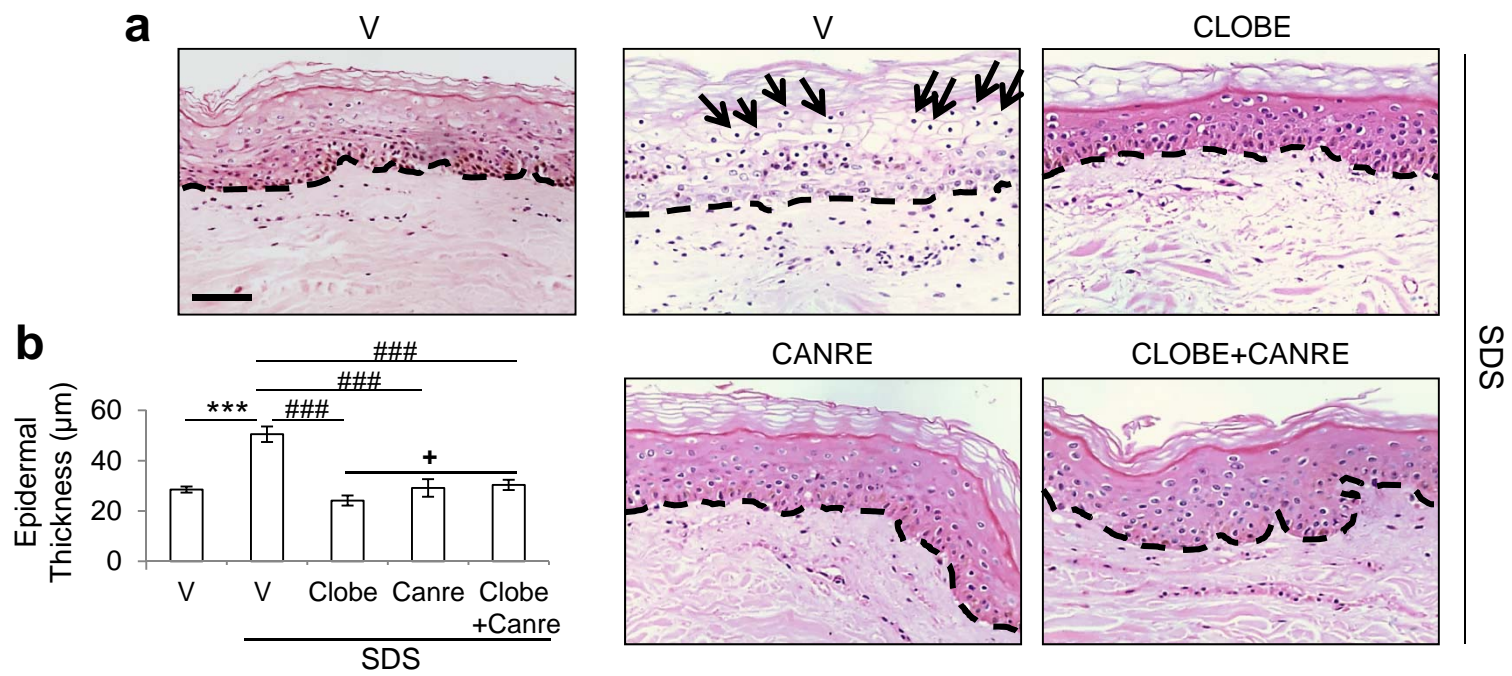
Figure legends

Figure 1. Combined treatment using GCs (Clobetasol) and MR antagonists (Canrenoate) in human skin explants confers an overall protection against SDS-induced skin damage.
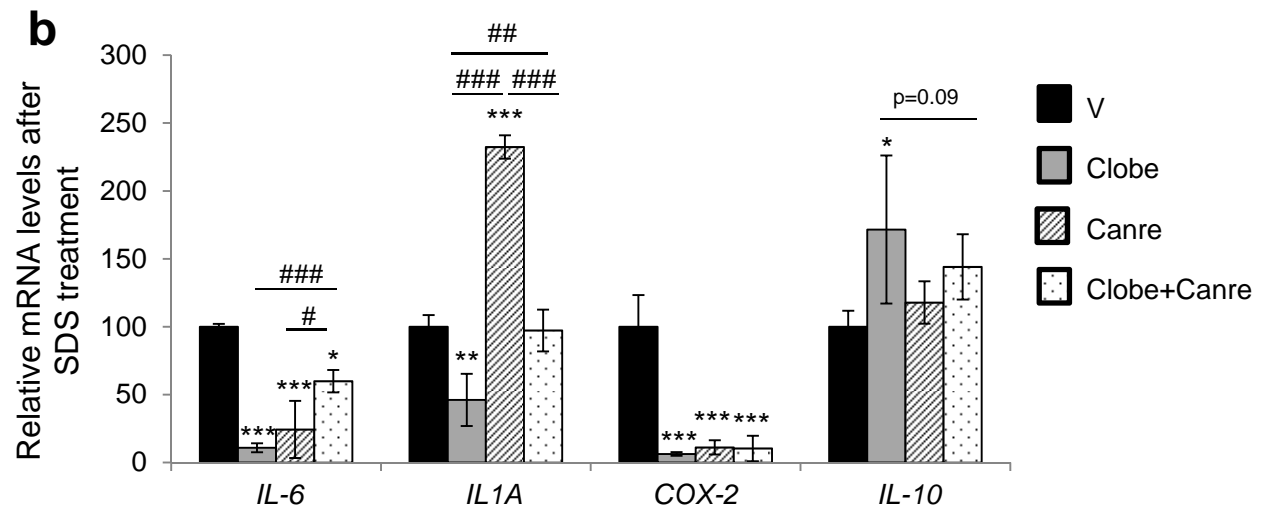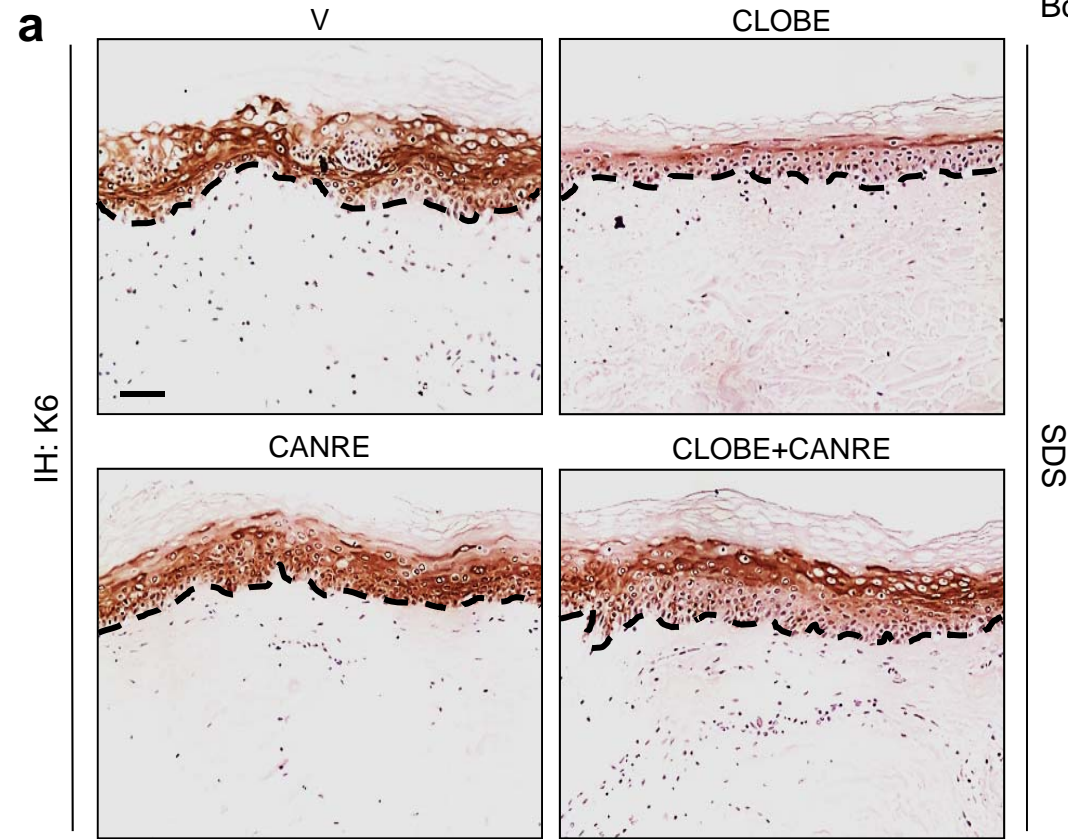
a) H&E staining of human skin explants treated with vehicle only or SDS plus vehicle (V), clobetasol (Clobe), canrenoate (Canre), or Clobe+Canre. Broken lines separate epidermis from dermis. Arrows indicate cell ballooning of suprabasal keratinocytes induced by SDS. Bar: 50 μm.

b) Epidermal thickness quantitation was assessed in the same groups as in a). ANOVA, post hoc Tukey test: n=3 of each treatment, *** $p<0.001$, differences versus vehicle only; ### $p<0.001$, differences versus SDS+V; + $p<0.05$, differences between other treatments.

Figure 2. Epidermal damage induced by SDS in human skin explants is not completely restored by Clobetasol upon MR pharmacological blockade.

a) Immunohistochemistry for K6 of human skin explants treated with SDS plus vehicle (V), clobetasol (Clobe), canrenoate (Canre), or Clobe+Canre. Broken lines separate epidermis from dermis. Bar: 50 μm.

b) Relative mRNA levels of *IL-6*, *IL1A*, *COX-2*, and *IL-10*, were assessed by RT-qPCR in the same groups as in a). ANOVA, post hoc Tukey test: n=3 of each treatment, * $p<0.05$, ** $p<0.05$, *** $p<0.001$, differences versus V; # $p<0.05$, ## $p<0.01$, ### $p<0.001$, differences between other treatments.

Boix FIG. 1

Boix FIG. 2

a

IH: K6

V   CLOBE

CANRE   CLOBE+CANRE

SDS

b

Relative mRNA levels after SDS treatment

- V
- Clobe
- Canre
- Clobe+Canre

IL-6   IL1A   COX-2   IL-10

Supplementary Information

Supplementary Materials and Methods

Skin samples

Human skin was obtained from patients subjected to surgery. Patients were informed and had no objection that the skin discarded from surgery could be used for research purposes. Written patient consent was not required because French law considers that it is not mandatory for non-interventional biological collection for immunological and molecular research. This procedure was approved by the Comité de Protection des Personnes Ile de France V.

Sample processing, and histological analysis

After the treatments, each sample was divided in two parts. One was frozen at -80ºC for RNA isolation and RT-qPCR, and the other was fixed in 70% ethanol followed by paraffin embedding. 4 μm skin sections were obtained and stained with hematoxylin-eosin, and Masson's trichrome (Sigma, St Louis, MO), or used for K6 immunohistochemistry (PRB-169P, Biolegend, San Diego, CA), as described (5). Epidermal thickness measurement was performed using the software IMAGE J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland). At least five photomicrographs were taken of each section and at least five measurements were taken from each photomicrograph using a Leica DM1000 microscope, a Leica EC3 camera and Leica LAS EZ software (Leica Microsystems, Wetzlar, Germany).

RNA isolation and RT-qPCR

RNA was isolated using Trizol (Thermo Fisher, Thermo Fisher Scientific, Waltham, MA) and reverse transcribed using oligo-dT and RevertAid H-minus Reverse Transcriptase (Fermentas Inc., Burlington, Canada). qPCR was conducted using specific oligonucleotides (Table 1) and FastStart Universal SYBR Green Master ROX (Roche) in an Applied Biosystems 7500 Fast real time PCR system, as described (5). Data were normalized to *RPLP0* expression.

Table 1. RT-qPCR primers.

Gene symbol	Forward sequence 5'→ 3'	Reverse sequence 5'→ 3'	product bp
COL1A1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC	140
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	81
IL1A	TGGTAGTAGCAACCAACGGGA	ACTTTGATTGAGGGCGTCATTC	215
COX-2	CAGAGTTGGAAGCACTCTATGG	CTGTTTTAATGAGCTCTGGATC	306
IL-10	GTGGAGCAGGTGAAGAATGC	GCCACCCTGATGTCTCAGTT	137
RPLP0	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC	59

Statistical Analysis

Experimental data were analyzed using IBM SPSS Statistics 23 software as described (5). In all graphs, mean values ± SD are shown. Prior to parametric testing, data were subjected to logarithmic transformation. For comparisons among more than two experimental groups, we used the one-way ANOVA which if statistically significant was followed by a post hoc Tukey multiple comparison test. p values less than 0.05 were considered statistically significant.

Supplementary Figures

Supplementary Fig. 1. Combined use of Clobetasol and MRA Canrenoate in human skin explants improved the collagen deposition and the COL1A1 mRNA levels relative to individual treatments.

a) Masson trichromic staining of human skin explants treated with vehicle only or SDS plus vehicle (V), clobetasol (Clobe), canrenoate (Canre), or Clobe+Canre. Broken lines separate epidermis from dermis. Bar: 50 μm.
b) Relative *COL1A1* mRNA levels were assessed by RT-qPCR in all groups in a). ANOVA, post hoc Tukey test: n=3 of each treatment, * $p<0.05$, ** $p<0.01$, differences versus vehicle only; # $p<0.05$, ## $p<0.01$, differences versus SDS+V.

a

V	V	CLOBE

CANRE	CLOBE+CANRE

SDS

b

Relative *COL 1A1* mRNA levels

*

**

#

##

150

100

50

0

V V Clobe Canre Clobe +Canre

SDS