CIRCADIAN CLOCK AND MUSCLE WASTING ASSOCIATED WITH IMPAIRED INSULIN SIGNALLING: *IN VITRO* EFFECT OF HYDROCORTISONE CIRCADIAN ADMINISTRATIONS.

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Introduction

Endogenous circadian clock regulates several 24-hrs physiological and behavioral processes. Circadian clock dysfunction has been associated to a wide range of disorders, including metabolic disorders such as obesity and insulin resistance. Endogenous circadian clock is regulated by a transcriptional/translational feedback loop in which, the transcriptional activator Bmal1 induces the expression of Per and Cry proteins. The latter, in turn, repressing *Bmal1* gene expression, negatively regulate the circadian clock activation. An intricate interplay between the circadian clock and the hypothalamus-pituitary-adrenal (HPA) axis has been demonstrated. In humans, HPA disrupting diseases are strongly related to circadian clock dysfunction and hypercortisolism has been associated to muscle insulin resistance and protein loss. The aim of the current study was to evaluate the effects of physiological and non-physiological hydrocortisone (HC) circadian administrations on muscle wasting focusing on the interplay between the anabolic insulin signalling and the protein loss HC dose and timing-dependent.

Results

After serum shock, clock gene *Bmal1* showed a circadian expression with a a bathyphase 20-hrs post serum free and an acrophase 12-hrs post serum free (Figures 1A and 2A). Although the different HC concentrations of exposure, no significant change in gene expression levels of *MuRF-1* and *Atrogin-1* was revealed in both *Bmal1* acrophase and bathyphase (Figure 1B). Conversely, 300 nM HC significantly inhibited mTORC1 protein expression (65%; $p<0.01$ vs 150 nM), significantly decreased pp70S6K (46%; $p<0.01$ vs 150 nM) and significantly stimulated KLF15 expression (50%; $p<0.05$ vs 150 nM) in Bmal1 bathyphase (Figure 2). Similarly, only in Bmal1 bathyphase, 300 nM HC combined with insulin strongly decreased pIRS-1 on Tyr608 and pAKT on Ser473 compared to 150 nM HC concomitantly with a strong stimulation of p110α PI3K and glucocorticoid receptor (GR) proteins expression compared to 150 nM HC (Figure 3). The transient transfection of non-synchronized C2C12, treated with HC with or without insulin, with *Bmal1* siRNA induced a knock-down of Bmal1 protein expression levels of about 50% (150 and 177 nM HC) and 75% (300 nM HC). Concomitantly with the decrease in Bmal1 expression, there was a strong decrease of pIRS-1 Tyr608, mTORC1, and pp70S6K associated with the increase of KLF15 (Figure 4).

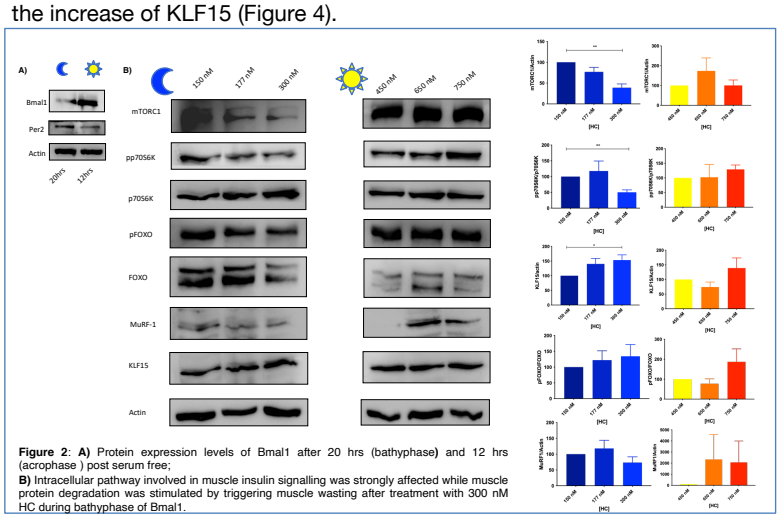
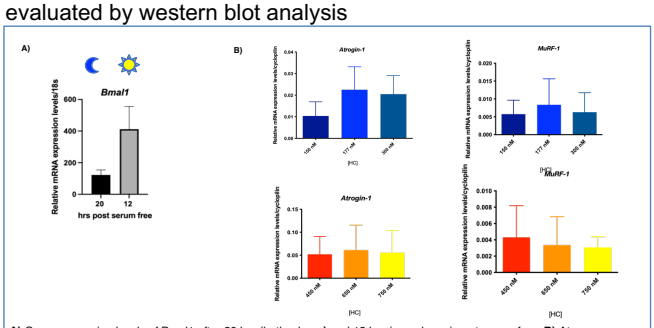


Figure 2: **A)** Protein expression levels of Bmal1 after 20 hrs (bathyphase) and 12 hrs (acrophase) post serum free;
B) Intracellular pathway involved in muscle insulin signalling was strongly affected while muscle protein degradation was stimulated by triggering muscle wasting after treatment with 300 nM HC during bathyphase of Bmal1.

Materials and methods

C2C12, a mouse skeletal muscle cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1 x 10^5 U/L penicillin and streptomycin. When C2C12 reached 70% of confluence, were differentiated into myocytes in DMEM supplemented with 2% horse serum (HS) and 1% insulin-transferrin-selenium. Differentiated C2C12 were exposed to serum shock (2 hrs DMEM with 50% HS, followed by starvation) to induce 24-hrs clock genes oscillation. Bmal1 gene and protein levels, used to set the cell circadian timing, were evaluated by RT-qPCR ad western blot analyses respectively. For each time-point, C2C12 cells were exposed for 1 hr to three different concentrations of HC to mimic physiological and non-physiological cortisol profiles. After hydrocortisone exposure, mRNA samples were collected and atrogens were evaluated by RT-qPCR. Moreover, intracellular variation of the main proteins involved in insulin signalling and in muscle protein catabolism were evaluated by western blot analysis after exposure to HC concentrations and stimulation with 50 nM insulin. Moreover, to induce a knock-down of *Bmal1*, non-synchronized C2C12 were transiently transfected (20-hrs) with negative control (CN) or Bmal1 siRNA using Lipofectamine 2000; intracellular variation of the main proteins involved in insulin signalling and in muscle protein catabolism were evaluated by western blot analysis



A) Gene expression levels of Bmal1 after 20 hrs (bathyphase) and 12 hrs (acrophase) post serum free; **B)** Atrogenes expression levels in bathyphase and acrophase of Bmal1 after HC administrations

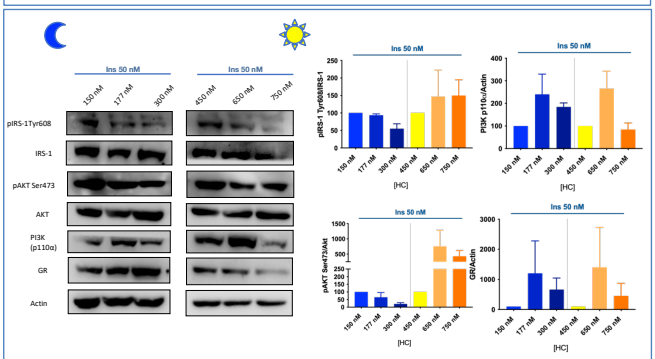


Figure 3: Intracellular insulin signalling strongly decreased after 300 nM HC in bathyphase of Bmal1 concomitantly with an increase in p100α PI3K and GR expression.

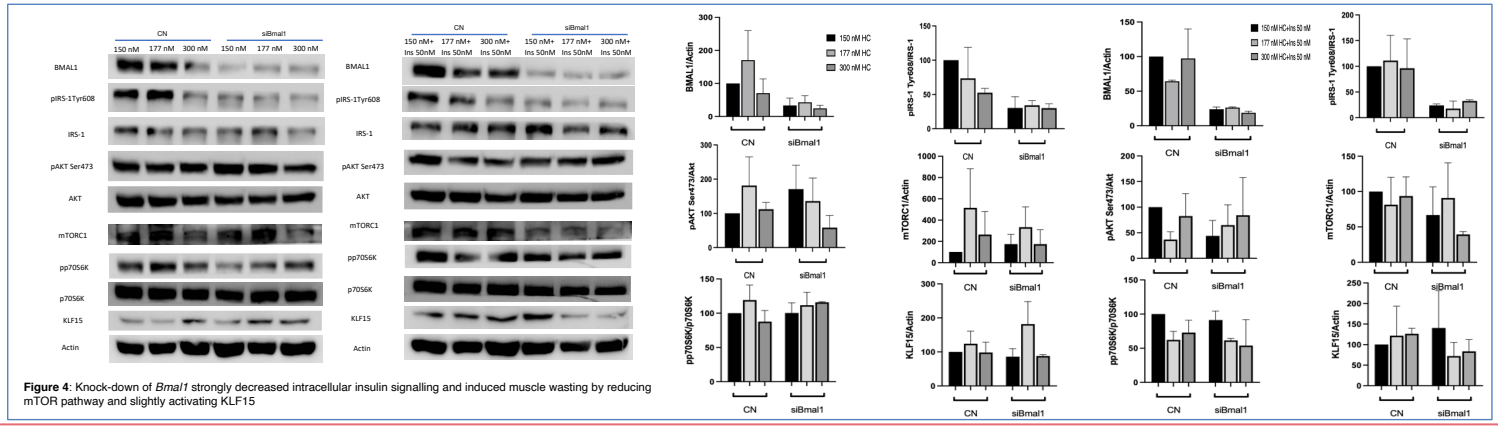


Figure 4: Knock-down of *Bmal1* strongly decreased intracellular insulin signalling and induced muscle wasting by reducing mTOR pathway and slightly activating KLF15

Conclusions

These data demonstrated that in *Bmal1* bathyphase, non-physiological HC concentration induces muscle wasting by reducing the anabolic action of insulin signalling.