Regulation of p38 MAPK and glucocorticoid receptor activation by hydrocortisone in mono-and co-cultured pancreatic acinar and stellate cells

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Short title: hydrocortisone modulates P38 MAPK and GR activation in vitro

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Running head: regulation of p38 MAPK and GR activation by hydrocortisone in the exocrine pancreas

Abstract

Background/objectives: Acute pancreatitis develops as an inflammatory response to pancreatic tissue injury. Postoperative pancreatitis has Postoperative pancreatitis has recently been associated with increased incidence of complications.been associated with increased occurrence of complications. Activation of the mitogen-activated protein kinase p38 (p38 MAPK) pathway occurs early in acute pancreatitis and its inhibition has been suggested to alleviate pancreatic inflammation. Glucocorticoids are potent anti-inflammatory steroids whose use in the management of acute pancreatitis remains controversial. Our aim was to examine the effect of crosstalk between pancreatic acinar cells (PACs) and stellate cells (PSCs) on p38 MAPK and glucocorticoid receptor (GR) activation and to assess the impact of hydrocortisone on these events.

Methods: The long-term co-culture setting for mouse PACs and PSCs developed in our laboratory was used. Parallel 4d mono- and co-cultures with or without 10nM hydrocortisone were performed followed by immunocytochemical analysis of nuclear GR and phospho-p38 MAPK (pp38 MAPK).

Results: Hydrocortisone inhibited pp38 MAPK up-regulation evoked by co-culture in PACs and PSCs and increased nuclear translocation of GR in PAC monocultures and in co-cultured PACs and PSCs. In PSC monocultures and co-cultured PACs, ligand-independent expression of nuclear GR was observed. In the former no change in nuclear GR but a significant decrease in total GR as analysed by Western blot was caused by hydrocortisone.

Conclusions: Cellular microenvironment plays a significant role on p38 MAPK and GR activation in PACs and PSCs. Hydrocortisone is an effective means to inhibit p38 MAPK activation in PACs and PSCs. Both ligand-dependent and -independent regulatory roles for hydrocortisone are suggested in the exocrine pancreas.

Key words: acute pancreatitis, glucocorticoids, inflammation, paracrine interaction, prophylactic

Introduction

Acute pancreatitis is a necroinflammatory disease of the pancreas caused by chemically or physically inflicted tissue damage. Postoperatively occurring acute pancreatitis has recently been associated with increased incidence of complications after pancreatic surgery Postoperatively occurring acute pancreatitis has recently been associated with increased incidence of complications after pancreatic surgery [1-3].

Ectopic zymogen activation and activation of the NFkB inflammation cascade are parallel processes within pancreatic acinar cells (PACs) characterizing the early pathophysiology of acute pancreatitis [4]. During the course of the disease PACs assume inflammatory cell-like properties and produce chemokines and cytokines which mediate the local inflammatory reaction and contribute to systemic dispersion of the disease. Tissue injury and cytokines activate pancreatic stellate cells (PSCs) to synthesize fibrous proteins for tissue reparation which, if persistent, may lead to the development of fibrosis [5,6]]. In addition to profibrogenic properties, proinflammatory characteristics similar to those observed in PACs have been described for activated PSCs [7].

Many signaling pathways have been implicated in the pathogenesis of pancreatic inflammation and fibrosis, including the p38 mitogen-activated protein kinase (p38 MAPK) signaling cascade [4, 8-11]. p38 MAPK is activated in response to stress factors and proinflammatory cytokines and controls cell behavior and gene expression by modulating the activity of downstream transcription factors and protein kinases by phosphorylation [12]. Early activation of p38 MAPK in acinar cells has been shown in experimental models of acute pancreatitis [13-16] and its inhibition has been observed to down-regulate acinar cell cytokine expression and to ameliorate pancreatic injury [15,17,18]. Protective effects for p38 MAPK signaling after caerulein hyperstimulation have also been reported [19]. In addition to PACs, p38 MAPK is suggested to be closely involved in the activation of PSCs [8-10,20,21]. Modulation of the profibrogenic and proinflammatory actions of PSCs by inhibitors of the p38 MAPK pathway has been reported [9].

Glucocorticoids play a central role in immune function and inflammation [22]. Because of their ability to interfere with the cytokine network, their therapeutic use in the treatment of various inflammatory conditions is well established. In animal models of acute pancreatitis both beneficial [23-25] and inefficient outcomes [26] have been reported after therapeutic treatments with glucocorticoids. Their prophylactic administration, on the other hand, has generally been shown to be protective against pancreatitis-associated tissue injury [27-30]. In humans, glucocorticoids have been successfully used to alleviate autoimmune pancreatitis [31,32] and severe acute pancreatitis [33]. Our group has recently reported that perioperative administration of hydrocortisone is able to reduce major complications after pancreatic surgery-inflicted acute pancreatic inflammation [1,3].

Glucocorticoids exert their actions via binding to their cognate receptors, glucocorticoid receptors (GRs), which translocate into the nucleus to regulate GR-responsive genes [22] In addition to genomic regulation, various non-genomic actions for GR have been described [22]. GR function can be modulated under proinflammatory conditions by increased expression of GR and/or its ligand-independent activation in response to inflammatory mediators [34] p38 MAPK has been shown to be able to alter the phosphorylation state of GR, thereby modulating its regulatory potential [35,36].

Isolated pancreatic acini [13,15] and PSC monocultures [8,9,20,21] have previously been used as models to study p38 MAPK activation *in vitro*. This study was undertaken to elucidate the effect of crosstalk between PACs and PSCs on p38 MAPK as well as GR activation in both cell types *in vitro* and to assess the influence of hydrocortisone on their pattern of expression. The experimental setting was the long-term co-culture model for mouse PACs and PSCs developed in our laboratory [37] performed here with reference to parallel PAC and PSC monocultures.

Materials and methods

Cells and culture design

Pancreatic acinar cells (PACs) and PSCs were prepared from the pancreatic tissue of 6-7-week-old male mice of the strain C57BL/6JOlaHSd (Envigo, the Netherlands) and cryopreserved and thawed for experiments according to previously published protocols [37-39]. PACs representing passage 1 and PSCs in passages 5-8 were used for this study. Triplicate experiments were performed with independent cell lots. The use of mice as donors of pancreatic tissue for cell culture purposes was approved by the Institutional Animal Welfare Committee.

Co-culture

The long-term co-culture model for mouse PACs and PSCs developed in our laboratory [37] was used to study the effect of crosstalk between PACs and PSCs on the nuclear localization (activation) of GR and p38 MAPK and to examine the effect of hydrocortisone on these phenomena *(Fig 1)*. In the double chamber co-culture system PACs are grown in the wells of a 24-well plate and PSCs in cell culture inserts (Greiner Bio-One, Frickenhausen, Germany) whose membrane pore size of 8 µm enables humoral interaction between the two cell compartments. PACs and PSCs were maintained for 4d in parallel mono- and co-cultures in the presence or absence (vehicle only) of 10 nM hydrocortisone (Sigma-Aldrich, St. Louis, MO). All experiments were performed using PAC-specific medium [38] supplemented with 0.1% BSA. Half of the medium in each culture was replaced daily by fresh medium.

Immunocytochemistry

Antibodies against GR and phospho-p38 MAPK (pp38 MAPK) (both from Cell Signaling Technology, Danvers, MA) were used to analyze by immunocytochemical means the extent of nuclear expression of the antigens in mono- and co-cultures of PACs and PSCs with or without hydrocortisone treatment. Prior to successive

incubations with primary and secondary antibodies, the cells were fixed in 4% formaldehyde and permeabilized in 94% ethanol as previously described [37]. Immunoreactive proteins were visualized with diaminobenzidine. Hematoxylin was used as counterstain.

All cells in six randomly picked microscopic areas (200x magnification) were counted and the percentage of immunopositive nuclei was calculated. The data are expressed as mean ±SEM of three independent experiments. Statistical analysis was performed using the two-tailed T-test for paired samples. Differences were considered statistically significant at a p value of <0.05

SDS-PAGE and Western blot

In addition to immunocytochemistry, PSC monocultures were subjected to immunoblotting analysis of GR and pp38 MAPK. For this, PSCs were allowed to reach near confluency in DMEM/F12 medium supplemented with 10% fetal calf serum after which the cells were detached, suspended into PAC-specific medium containing 0.1% BSA and counted. The cells were plated at equal seeding densities into T25 culture bottles and allowed to attach for 48 h. The media were replaced, and 10 nM hydrocortisone was added. Parallel cultures treated with vehicle only served as controls. The cells were cultured for 4d with one medium change after 48 h.

Protein extraction was accomplished with the M-PER® (Pierce, Rockford, IL) reagent modified with protease inhibitors (Complete Mini Protease inhibitor cocktail tablets; Roche Diagnostics GmbH, Indianapolis, IN, USA). Total protein concentrations were measured using the BCA Protein Assay Kit (Pierce). Fifty micrograms of total protein were loaded onto 10% polyacrylamide gel, fractionated, blotted onto PVDF membranes (Millipore, Billerica, MA, USA) and exposed overnight to 1:1000 dilutions of primary antibodies against pp38 MAPK and GR. After thorough washings the membranes were incubated for 1h with horse radish peroxidase-conjugated anti-rabbit antibody (1:2000; Cell Signaling Technology) and thereafter subjected to enhanced chemiluminescence reagents (ECL Western Blotting Detection Reagents, GE

Healthcare, Buckinghamshire, UK). Densitometric analysis of the immunoreactive protein bands was performed using the ImageLab software (BioRad, Hercules, CA). Protein densities were equalized with reference to β-actin (Sigma-Aldrich).

Results

Activation of p38 MAPK and GR in PACs

Compared to the low expression of unliganded nuclear GR in PAC monocultures, an approximately 10-fold increase in the number of cells exhibiting nuclear GR was detected after treatment with 10nM hydrocortisone. The low basal level of pp38 MAPK in monocultured PACs remained unchanged in the presence of hydrocortisone. In the absence of hydrocortisone co-cultured PACs showed a trend towards increased nuclear localization of unliganded GR as well as pp38 MAPK compared to corresponding monocultures ($p = 0.071$ and 0.079, respectively).

Hydrocortisone evoked a further 2.5-fold increase in nuclear GR and down-regulated the percentage of pp38 MAPK-positive cells approximately 2-fold.

Activation of p38 MAPK and GR in PSCs

In control monocultures of PSCs *(Fig 2c)*, unliganded GR was detected in 37.6±7.4% (mean±SEM) of the cell nuclei. Hydrocortisone had little effect on nuclear GR. The expression of pp38 MAPK in PSC monocultures remained at a low level both with and without hydrocortisone supplementation. A total of 10.6±3.0% of PSCs co-cultured with PACs *(Fig 2d)* expressed unliganded GR in their nuclei. In the presence of ligand, the percentage of PSCs with GR-positive nuclei increased 6.4-fold. Co-culture without hydrocortisone significantly increased the number of pp38 MAPK-positive cells compared to parallel monocultures. The observed up-regulation was significantly attenuated by hydrocortisone treatment.

As no significant effect was seen in the nuclear localization of GR in monocultured PSCs, the cells were further examined by immunoblotting *(Fig 3a)*. The analysis revealed a significantly higher (p= 0.0026) amount of total GR (nuclear plus cytoplasmic) in the absence of ligand than in its presence. Western blot analysis of the nuclear form of p38 MAPK *(Fig 3b)* was in line with its respective immunocytochemical analysis.

Discussion

PACs and PSCs and their mutual interactions in pancreatic tissue microenvironment are central players in the initiation and progression of acute pancreatitis after tissue injury. In the present *in vitro* study we examined the effect of crosstalk between mouse PACs and PSCs on p38 MAPK and GR activation in both cell types and assessed the impact of hydrocortisone on these events. The results showed that hydrocortisone was able to effectively inhibit p38 MAPK activation stimulated by coculture in PACs and PSCs. The experimental setting of parallel monocultures and co-cultures also revealed cell type- and culture context-associated differences in ligand dependent and independent nuclear translocation of GR.

The culture setting used in this study was the long-term co-culture model developed in our laboratory [37]. Reciprocal stimulation of PACs and PSCs has previously been shown to occur in this culture system involving activation of NFκB in PACs and increased production of extracellular matrix proteins in PSCs [37]. The model has also been shown to closely recapitulate *in vitro* changes in Wnt/β-catenin signaling during fibrotic remodeling of the pancreas [40]. The PSCs used in this model represent cells that have become preactivated by propagation through several passages on cell culture plastic [5]. That PSCs of this phenotype are capable of further activation by exposure to cytokines [5] and to interactions with PACs [37] has previously been shown. Despite preactivation, a similar low basal level of pp38 MAPK expression in PSC monocultures as in monocultured PACs was observed in the present study. This indicates that no external stress signals that might have activated p38 MAPK were inflicted on the cells by the monoculture environment, the observed p38 MAPK activation in cocultures being, therefore, the result of PAC-PSC

crosstalk only. As hydrocortisone was added simultaneously with the onset of co-culture, it was able to target the very earliest processes of reciprocal stimulation. The 10 nM concentration of hydrocortisone used in the present study was significantly lower than the lowest normal serum cortisol level of approximately 140 nM in human sera [41].

In the present study, hydrocortisone was observed to significantly decrease co-culture-induced pp38 MAPK expression in both PACs and PSCs. p38 MAPK activation has previously been shown to occur within hours after the onset of acute pancreatitis not only in the pancreas but also in extra-pancreatic organs [16,42]. Noxious stimuli such as hyperthermia [14], osmotic stress [13] and reactive oxygen species [43] have been shown to activate p38 MAPK in pancreatic tissue and PACs in various experimental settings. Activation of p38 MAPK in PACs has also been detected after secretagogue stimulation [13,15,17] suggesting an important role for p38 MAPK in secretagogue-stimulated overproduction of cytokines [17]. In PSCs, activation of p38 MAPK has been shown to precede profibrogenic activation in response to ethanol and acetaldehyde [20] and to high extracellular glucose [21]. The use of compounds that prevent p38 MAPK activation (SB203580 and CNI-1493) has been observed to ameliorate pancreatic injury [18] and to attenuate the severity of pancreatitis-induced adult respiratory distress syndrome [42] in animal models of experimental pancreatitis.

In PAC monocultures and in co-cultured PSCs hydrocortisone significantly increased nuclear GR expression from the low x% levels in vehicle controls. This observation conforms to the canonical mode of transcriptional regulation by glucocorticoids involving ligand binding to GR, translocation of the ligand-GR complex to the nucleus and its binding to glucocorticoid response elements or to other transcription factors [22]. In PSC monocultures and co-cultured acinar cells, however, approximately 30% of cells were observed to express unliganded GR in their nuclei. It has become evident that GR does not always require binding of ligand for nuclear localization and transcriptional activation [34,44]. Increases in receptor density as well as the effect of cellular stressors and proinflammatory cytokines have been suggested to contribute to the

shift of unliganded GR into the nucleus in order to either modulate the transcription of selected genes or to sensitize the cells to lower glucocorticoid concentrations [45]. Whereas in co-cultured PACs hydrocortisone further increased the level of nuclear GR, in PSC monocultures no apparent change was elicited by ligand. Closer analysis of the latter by Western blot revealed drastic downregulation of total GR in response to hydrocortisone. Previously downregulation of GR has been shown to be a common response to glucocorticoid exposure in a variety of tissue types [46-48].

Prophylactic administration of glucocorticoids appears to have protective effects against pancreatitis-associated tissue injury as shown in various animal models of experimental acute pancreatitis [27-30]. Yubero et al. [30] showed that dexamethasone was able to inhibit p38 MAPK activity in mild but not in severe acute pancreatitis when administered 1h after experimental induction of pancreatic inflammation in rats. Our group has previously shown that in acinar cell rich (more than 40% acinar cells at the cut edge of the pancreas) human pancreatic tissue the inflammation cascade begins early after surgical trauma with the peak activation of inflammation markers observed after 4h of the injury [1]. In these cases, perioperative administration of hydrocortisone was shown to reduce major complications after pancreaticoduodenectomy [1] and distal pancreatectomy [3]. It was suggested that postoperative pancreatic inflammation may be a possible mediator of complications after pancreatic surgery and prophylactic anti-inflammatory treatment with hydrocortisone, therefore, an effective means of their prevention. Involvement of the p38 MAPK signaling pathway in these events is now known. Given the early activation of p38 MAPK in the pancreas and in extra-pancreatic organs in acute pancreatitis [16,42] and the observation that inhibition of p38 MAPK is able to attenuate pancreatic injury [18] as well pancreatitis-associated pulmonary dysfunction [42], a potential role for p38 MAPK signaling cannot be ruled out.

In conclusion, hydrocortisone turned out to be an effective means to attenuate p38 MAPK activation evoked by co-culture in PACs and PSCs. Nuclear translocation of unliganded GR in PSC monocultures as

well as in co-cultured PACs suggests modulation of the GR signaling mechanism in these microenvironments. This may entail increased sensitivity of the cells to glucocorticoids or ligand-independent gene regulatory functions for GR. p38 MAPK inhibition may play a role in the observed complication-reducing effect of perioperatively administered hydrocortisone.

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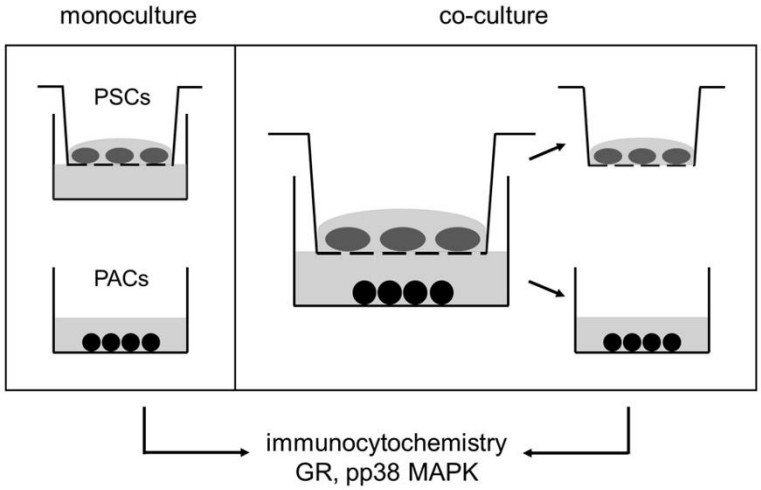
Figure legends

Figure 1. Schematic illustration of the *in vitro* procedure. PACs and PSCs were maintained for 4d in parallel mono- and co-cultures with or without 10 nM hydrocortisone and thereafter subjected to immunocytochemical staining with antibodies against GR and pp38 MAPK.

Figure 2. Nuclear localization of GR and pp38 MAPK in PACs (a,b) and PSCs (c,d). The cells were probed with antibodies specific to GR and pp38 MAPK after 4d culture with 10 nM hydrocortisone (HC) or vehicle control. All cells in 6 randomly picked microscopic areas (200x magnification) were counted and the percentage of immunopositive nuclei was calculated. Representative immunocytochemical stainings are shown below the bars. Statistical analysis was performed using the two-tailed T-test for paired samples. The quantitative data in bars are expressed as mean ±SEM of three independent experiments . $^{\star}p<0.05$ vehicle vs HC; $^{\#}p<0.05$ GR in PSC monocultures vs cocultures; $^{\circ}p<0.05$ pp38 in PSC co-cultures vs monocultures.

Figure 3. Western blot analysis of the effect of hydrocortisone on GR (a) and pp38 MAPK (b) expression in PSC monocultures. PSCs were maintained for 4d in the absence or presence of 10 nM hydrocortisone prior to protein extraction. The data represent results (mean±SEM) from three independent PSC lines. (a) Hydrocortisone significantly ($^{\star}p<0.05$) down-regulated total GR. (b) No significant change in pp38 MAPK was elicited by hydrocortisone.

Figure 1


monoculture
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PSCs
PACs
immunocytochemistry
GR, pp38 MAPK

Figure 2

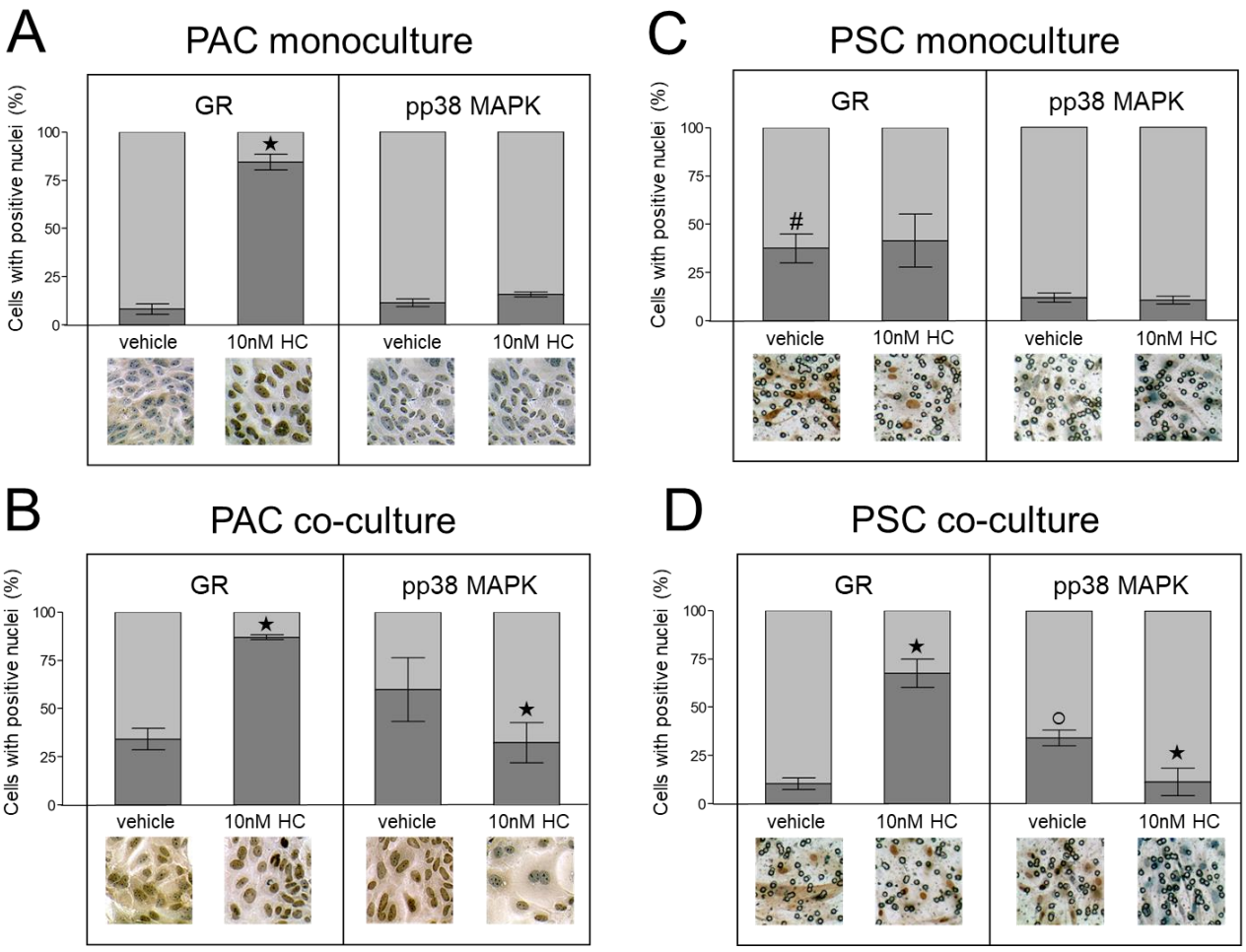
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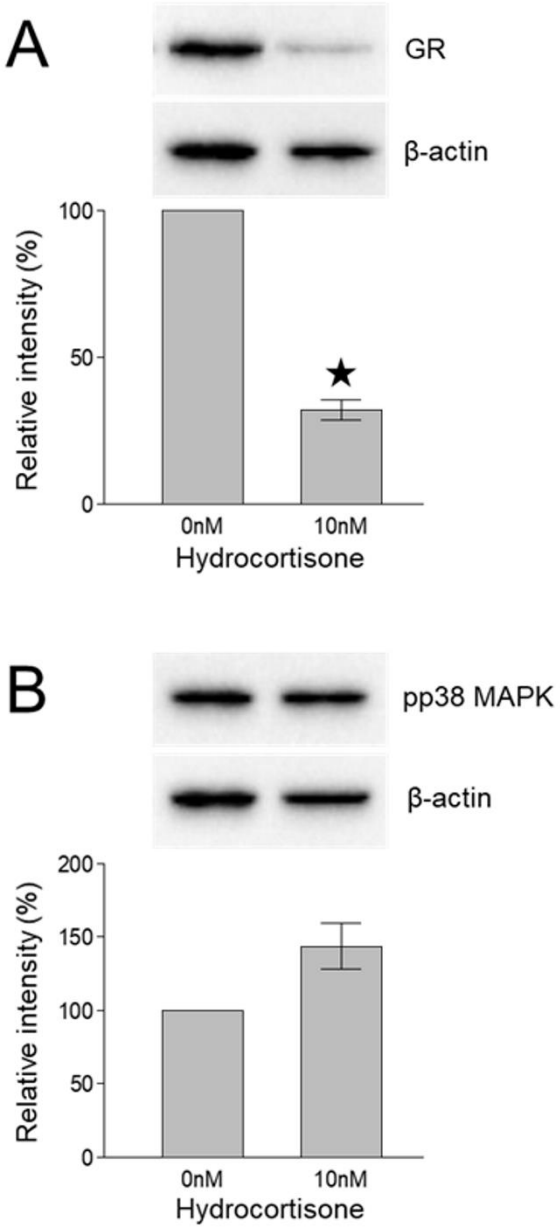
Figure 3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