

GLUCOCORTICOID RECEPTOR SIGNALLING AND THE EFFECT OF
INTERLEUKIN 1 β ON GLUCOCORTICOID MEDIATED GENE EXPRESSION
IN INTESTINAL EPITHELIAL CELL LINES CACO-2 AND IEC-6

Dissertation

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AP-1	activating protein 1
ATF-2	activating transcription factor 2
bp	base pair
CLSM	confocal laser scanning microscopy
CREB	cAMP response element binding protein
CSF	colony stimulating factor
Cox	cyclooxygenase
Dex	dexamethasone
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GR	glucocorticoid receptor
GRE	glucocorticoid receptor responsive element
hGR α	human glucocorticoid receptor alpha
hGR β	human glucocorticoid receptor beta
hsp	heat shock protein
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
I κ B	inhibitory kappa B
IFN γ	interferon gamma
IL-1 α	interleukin 1 alpha
IL-1 β	interleukin 1 beta
IL-2	interleukin 2

IL-6	interleukin 6
IL-8	interleukin 8
kD	kilodalton
LPS	lipopolysaccharid
MAP kinase	mitogen-activated protein kinase
MCP-1	monocyte-chemoattractant protein 1
MR	mineralocorticoid receptor
mRNA	messenger RNA
NF- κ B	nuclear factor kappa B
NF- κ B-RE	nuclear factor kappa B responsive element
OHSD	11 β -hydroxysteroid dehydrogenase
PBS	phosphate buffered saline
PKA	protein kinase A
RT-PCR	reverse-transcription polimerase chain reaction
S	substrate
SEM	standard error of means
SRC-1	steroid receptor coactivator 1
TGF β 1	transforming growth factor beta 1
TNF α	tumor necrosis factor alpha
U	unit

1. SUMMARY

Background and aims: It has been reported that epithelial cells are involved in the process of inflammatory bowel disease (IBD) producing proinflammatory cytokines and chemokines. Glucocorticoids, being the most effective drugs for IBD patients, seem to have influence not only on the infiltrated inflammatory cells but also on epithelial cells. It is a clinical observation that about 20 % of IBD patients are resistant to glucocorticoids and requires alternative treatment.

Since there are only few data regarding influence of glucocorticoids on intestinal epithelial cells, the aim of this work was to characterize the glucocorticoid receptor signalling pathway in the human colon carcinoma cell line Caco-2 and rat intestinal cell line IEC-6. The second aim of this study was to test the hypothesis that IL-1 β , a major cytokine in IBD may inhibit glucocorticoid receptor action.

Methods: The subcellular distribution of glucocorticoid receptor was studied by Western blot and immunostaining procedures using antibodies against rat and human GR. Its quantity was determined by radiobinding assay in both cell lines.

The regulatory (activating and repressing) effects of dexamethasone on gene transcription in presence or absence of IL-1 β were analyzed by reporter gene assay using either glucocorticoid receptor-(pGRE-SEAP) or nuclear factor kappa B responsive element (pNF- κ B-SEAP) carrying constructs.

The effect of IL-1 β on receptor translocation was investigated by immunostaining. The repressing effect on gene transcription was further investigated by RT-PCR for TNF α , a cytokine typically down-regulated by glucocorticoids.

In Caco-2 GR quantity was increased by the GR overexpression vector pRShGR α .

Results: The subcellular distribution of GR was found to be different in the human and rat cells. In Caco-2 we observed mostly nuclear staining in both Dex-treated and not treated cells, in IEC-6 the unliganded form of the receptor was located in the cytoplasm, incubation with Dex resulted its translocation to the nucleus. IL-1 β could repress this process.

The two cell lines were different regarding their receptor amount; we detected significant more specific binding sites in IEC-6 than in Caco-2.

Dexamethasone induced GR mediated transcriptional activity of the reporter gene in dose-dependent manner, and this process could be repressed by IL-1 β in both cell lines. We detected only functional inhibition of GR, IL-1 β did not change GR quantity apparently.

The proinflammatory cytokine IL-1 β could increase NF- κ B mediated transcription in each cell line. Glucocorticoids could inhibit IL-1 β induced NF- κ B activation only in IEC-6.

IL-1 β increased TNF α -mRNA expression, too. Dex had repressive effect only in case of IEC-6 but not in Caco-2.

Overexpressing GR using pRShGR α vector, both transactivation and transrepression by Dex could be restored in Caco-2. Blocking the NF- κ B-pathway by a dominant negative I κ B vector could not restore glucocorticoid receptor action disturbed by IL-1 β .

Conclusions: Our data suggest that proinflammatory cytokines may influence the effect of corticosteroids in intestinal epithelial cells.

The quantity of glucocorticoid receptor is crucial for responsiveness. The receptor number under a certain threshold may render cells unresponsive with respect to transrepressive effects, while transactivation is intact.

IL-1 β inhibits GR signalling. This inhibition seems to be independent on NF- κ B activation since blocking the NF- κ B pathway by I κ B overexpression does not counteract the IL-1 β effects on glucocorticoid receptor action.

2. INTRODUCTION

2.1 The mucosal immune system

Although the primary functions of the gastrointestinal mucosa are the digestion, absorption and transport of water, electrolytes and nutrients, it also serves as an important mechanical barrier to separate the host's internal milieu from the external environment and prevent systemic contamination by microbes that are normally contained within the lumen of the gastrointestinal tract. However the noninflamed mucosa also contains mononuclear cells. During intestinal infiltration large numbers of monocytes, macrophages and lymphocytes migrate into the lamina propria and produce proinflammatory cytokines (IL-1 β , IL-2, IL-6, TNF α) and chemokines (IL-8). Alone and in combination these mediators attract and activate further inflammatory cells. The process serves as an early signalling system to amplify the mucosal inflammatory response in the early stages of bacterial infection. The inflammatory mediator production can lead to local attack against microbes and systemic acute phase reaction. Moreover, some cytokines like IL-1 α , IL-1 β and IL-6 have effect on central nervous system modulating the release of various hormones, e.g. ACTH via activating the hypothalamic-pituitary-adrenocortical axis. Increased ACTH release results in glucocorticoid synthesis which causes decreased cytokine production. This negative feedback loop plays an important role in the regulation of inflammatory process.

It is supposed that intestinal epithelial cells are involved in immune response. Human colon epithelial cells *in vitro* can process and present antigens to T cells (Mayer, 1987), and the human colon epithelium *in vivo* and *in vitro* can be stimulated to express HLA class II (Mayer, 1991) and adhesion molecules (Kelly, 1992). In addition, human colon epithelial cells and cell lines produce neutrophil chemoattractant proinflammatory chemokines e.g. IL-8 (Eckmann, 1993) and monocyte-chemoattractant protein 1 (MCP-1) (Reinecker, 1995). Moreover, human colon epithelial cell lines express TGF β 1 (Eckmann, 1993; Anzano, 1989), complement proteins like C3, C4, Factor B (Andoh, 1993) and leukotrienes that might be involved in initiation and regulation of mucosal inflammatory process (Dias, 1992). Human colon epithelial cell lines can also respond to proinflammatory cytokine-stimulation e.g. IL-1 (Raitano, 1993), TNF α (Fantini, 1992), IFN γ (Phillips, 1990) and TGF β 1 (Mulder, 1990) with altered gene expression.

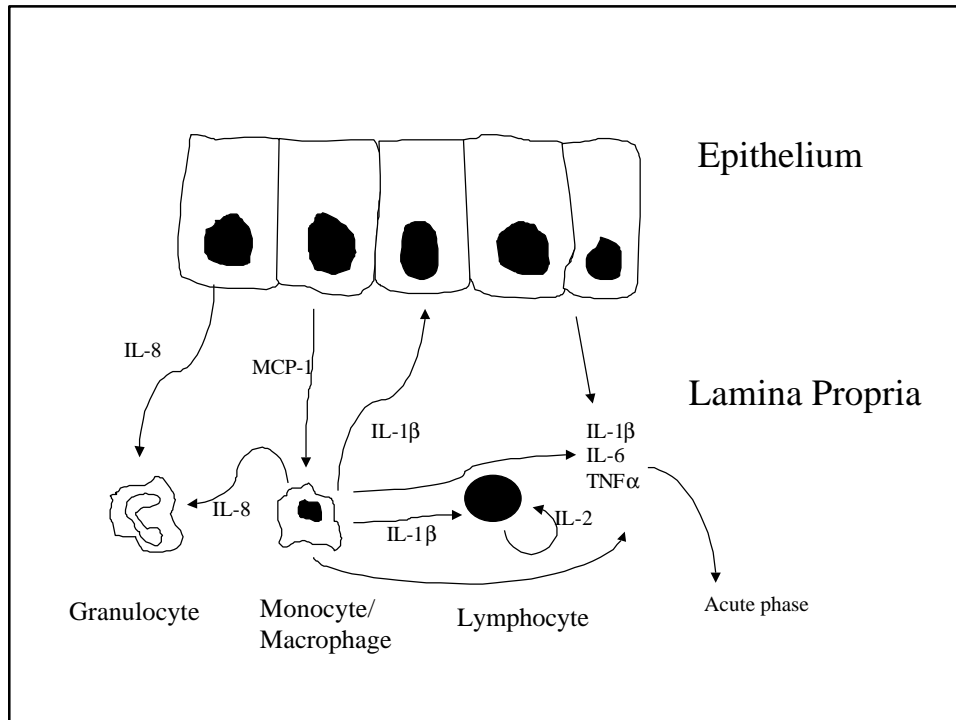


Figure 1. Interaction of inflammatory and epithelial cells during mucosal inflammation (simplified scheme).

Immune cells like granulocytes, monocytes, macrophages and lymphocytes infiltrate the inflamed mucosa. These cells produce various inflammatory mediators like **IL-1 β** , **IL-6**, **IL-2** and **TNF α** . This production has an effect on epithelial cells. These cells respond to the expressed cytokines by producing inflammatory chemokines like **IL-8** and **MCP-1** recruiting more inflammatory cells.

2.2 The inflammatory bowel disease

In the normal mucosa where the barrier function is intact the inflammatory mediator-production by either intestinal epithelial cells or lymphoid cells is in repose. In inflammatory bowel disease this balance is disturbed, the proinflammatory cytokines and chemokines are overexpressed. IL-1, IL-6, granulocyte-macrophage CSF and TNF α have been reported to be increased in serum (Gross, 1991), cultures of biopsy tissues (Breese, 1993; Isaacs, 1992; Stevens, 1992) or isolated lamina propria lymphocytes (Mahida, 1989; Simon, 1989) from patients suffering from inflammatory bowel disease. This process causes a damage of the gastrointestinal mucosa and loss of epithelial functions; e.g. the combination of TNF α and IFN γ is cytotoxic for the colon epithelial cell line, HT-29 (Deem, 1991). IL-1 β up-regulates IL-6 synthesis by colon epithelial cells (Panja, 1995). IL-1 β also is an important acute phase mediator while IL-6 is the main mediator of acute phase reaction (Heinrich, 1990).

Moreover, IL-1 β has particularly important role in the process, being one of the most abundantly secreted inflammatory mediators in IBD, produced by activated macrophages, epithelial cells, T- and B-cells.

The etiology of inflammatory bowel disease is less known, it is characterized by a culmination of a cascade of events and processes initiated by antigen. This antigen-driven response may be an appropriate one directed against a persistently abnormal stimulus (e.g. a structural alteration of the intestine or a causative agent in the environment), or an inappropriate response to otherwise innocuous antigen (i.e., aberrant regulation of the immune response) (Podolsky, 1991).

The inflammatory bowel disease has two forms: ulcerative colitis and Crohn's disease. Ulcerative colitis involves only the mucosal surface of the colon resulting in diffuse friability and erosions with bleeding. Crohn's disease can develop in any part of gastrointestinal tract, most frequently in the small intestine. In Crohn's disease the whole intestinal wall is involved unlike ulcerative colitis, resulting in mucosal inflammation and ulceration, stricturing, fistulous development and abscess formation. Both diseases can be characterised by periods of symptomatic flare-ups and remissions (Current Medical Diagnosis & Treatment, 1996).

Although all over the world many people suffer from inflammatory bowel disease, there are still no specific therapies for this illness. Glucocorticoids remain the most potent drugs. Despite the efficacy of glucocorticoids, almost 20% of the patients do not respond well to the therapy: 18% of the patients suffering from ulcerative colitis proved to be steroid resistant after the first, standard, five day long glucocorticoid therapy and 50% of them had recurrence of symptoms during the next 3 year long observation (Truelove, 1988). The statistics of Crohn's disease are very similar: 20% of

the patients were resistant during the standard 3 week long therapy and 55% of them became steroid dependent or resistant through the next year (Munkholm, 1994).

Steroid-dependency is the other serious problem of glucocorticoid therapy. In case of some patients only high dose of glucocorticoids can avert the flare-up of disease. But application of high dose of glucocorticoids can have strong side effects, e.g. diabetes, osteoporosis or Cushing-syndrome.

2.3 The effects of glucocorticoids

Glucocorticoids have long been used as effective immunosuppressive agents in the treatment of cell- or cytokine/chemokine mediated tissue damage. These drugs have been described to inhibit inflammation, suppress immune system activation and act as growth inhibitory agents both in vitro and in vivo (Cupps, 1982). There are many observations concerning their effects: they induce a rapid redistribution of lymphocytes from the circulation to other lymphoid compartments, lymphocyte apoptosis (Wyllie, 1980) the clonal expansion of T-cells and secretion of cytokines (Smith, 1980; Cupps, 1982). Interestingly, T-cells treated with exogenous IL-2 are able to proliferate in response to mitogenic stimulation in presence of glucocorticoid drugs (Gillis, 1979). Moreover glucocorticoids can repress the de novo transcription of many cytokine genes as IL-1, IL-6, TNF α and IFN γ (Taniguchi, 1988; Culpepper, 1987; Cupps, 1982). The expression of lipocortin-1, an inhibitor of phospholipase-A2 is up-regulated by glucocorticoids, resulting in a decrease of prostaglandin E2 synthesis in many kinds of cells, including epithelial cells (Croxtall, 1994). There are only some data concerning effect of glucocorticoids on gastrointestinal epithelium; they stabilize epithelial functions (Urayama, 1998) and restore Na⁺-absorption in chronically inflamed epithelium (Sundram, 1999). In addition glucocorticoids inhibit IL-1 β mediated MCP-1 expression (Reinecker, 1995) and IFN γ induced nitric oxide synthesis in the colon carcinoma cell line, Caco-2 (Chaves, 1999).

2.4 The mechanism of glucocorticoid receptor action

Glucocorticoid action is mediated by intracellular glucocorticoid receptors. These molecules are members of the steroid hormone receptor family, all of which contain a homologous DNA binding domain and divergent C-terminal ligand binding domains (Beato, 1989; Evans, 1988). The glucocorticoid receptor was first identified in rat thymocytes (Munck, 1968). During the last years its molecular properties have been characterised and the gene encoding the receptor has been cloned (Hollenberg, 1985; Miesfeld, 1986).

Two human receptor isoforms were identified (Hollenberg, 1985), hGR α and hGR β . They are synthesized by alternative splicing, the first 727 amino acids are common. The α isoform contains 777 amino acids, the β form 742 amino acids long. The hGR α binds hormone and seems to be the major physiological form (Hollenberg, 1985), hGR β is supposed to be an endogenous inhibitor of glucocorticoid action (Bamberger, 1995).

The inactive form of hGR α resides primarily in the cytoplasm associating with heat shock proteins (hsp90 and hsp70) and one p59 immunophilin molecule (Pratt, 1993; Guiochon-Mantel, 1996). When glucocorticoid binds to its receptor, the complex dissociates and the liganded receptor migrates into the nucleus. Here it interacts with promoter regions of different genes carrying glucocorticoid receptor responsive elements and modifies their expression. Interestingly, some researchers found both liganded and nonliganded forms to be present only in the nucleus (LaFond, 1988; Raddatz, 1996).

2.5 Immunosuppressive effects of glucocorticoids, the transrepression of NF- κ B

Glucocorticoids either induce gene transcription by interacting with specific DNA-elements (transactivation) or interfere with other transcription factors and inhibit their activity (transrepression).

Not many target genes carrying glucocorticoid receptor responsive elements are known until now. Increasing expression has been reported concerning I κ B α gene, encoding the inhibitory protein of the main transcription factor for response to inflammatory cytokines, nuclear factor kappa B.

NF- κ B, originally identified and named for its role in the regulation of immunoglobulin kappa chain gene expression in B-cells (Lenardo, 1989; Sen, 1986), is a heterodimer that typically consists of a p65 (RelA) and a p50 subunit (Baeuerle, 1989; Kawakami, 1988). The latent form resides in the cytoplasm forming a complex with its inhibitory I κ B proteins (Israel, 1995; Baldwin, 1996).

Activation of NF- κ B may occur through a variety of extracellular signals, which induce phosphorylation and ubiquitylation events on I κ B α , resulting in proteolytic degradation (Verma, 1995; Finco, 1995; Bauerle, 1996). Then NF- κ B is released from its inhibitor, translocates into the nucleus and activates target genes. Numerous genes have been identified which are regulated by NF- κ B: cytokine (IL-1 β , IL-2, TNF α) and chemokine (IL-8, MCP-1) encoding gene. I κ B α itself is expressed in autoregulatory fashion: resynthesized and restored in the cytoplasm trapping the transcriptionally active NF- κ B molecules.

The immunosuppressive effect of glucocorticoids on NF- κ B function may occur either via stimulation of I κ B α expression or via inhibition of migration of transcriptionally active NF- κ B into the nucleus. The latter effect is based on a direct protein-protein interaction between liganded hGR α and active NF- κ B because this complex can't translocate to the nucleus (Scheinman, 1995). However, recently it was reported that in the monocytic cell line THP-1 NF- κ B is activated by dexamethasone (Wang, 1997) suggesting a cell specificity for this phenomenon.

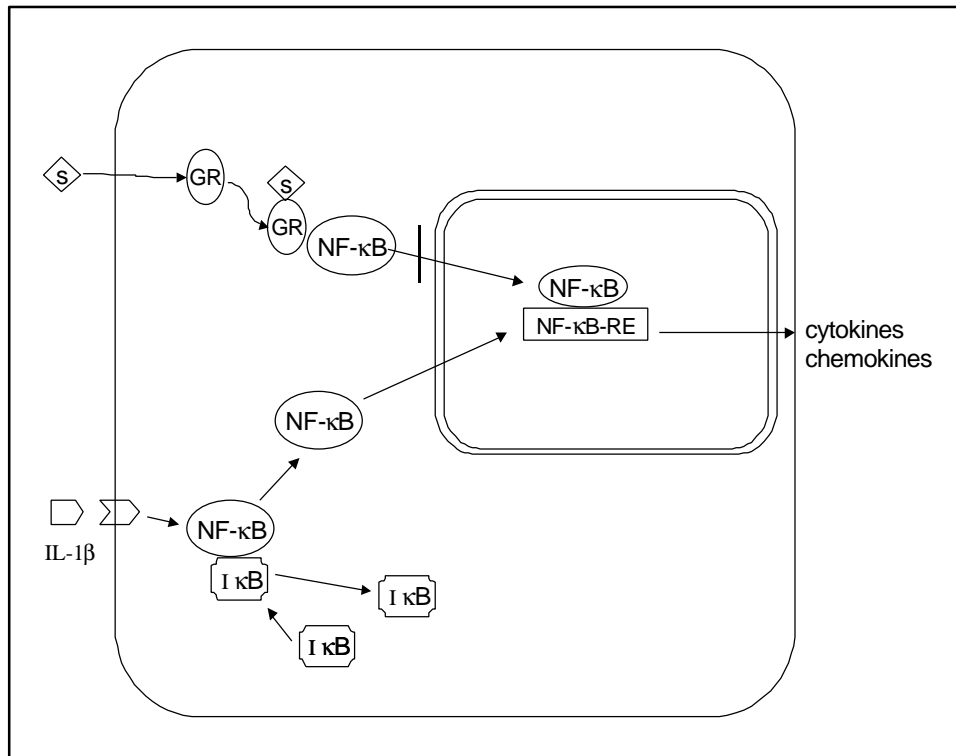


Figure 2. The mechanism of immunosuppression by glucocorticoids.

The inactive form of NF- κ B localises in the cytoplasm forming a complex with I κ B. Inflammatory cytokines like IL-1 β activate NF- κ B which translocates to the nucleus, binds to NF- κ B responsive element carrying genes and increases the expression of various cytokines and chemokines. The activated glucocorticoid receptor can bind to activated NF- κ B and trap it in the cytoplasm.

2.6 Influence of cytokines on GR expression and function

Cytokines can influence the expression and function of glucocorticoid receptors in a number of cells and tissues e.g. T-cells (Kam, 1993), monocytes and macrophages (Spahn, 1993; Falus, 1995), bronchial cells and lung (Liu, 1993; Verheggen, 1996) and liver (Falus, 1995). Some of these studies have reported that cytokine treatment induces a decrease in glucocorticoid receptor function (Kam, 1993; Liu 1993; Hill, 1986).

Pariante et al. described that in L929 mouse fibroblast cell line IL-1 α disrupts translocation of the glucocorticoid receptor from the cytoplasm to the nucleus (Pariante, 1999).

These findings show that not only glucocorticoid hormones can have effect on cytokine expression and function but, vice versa, inflammatory mediators also can modulate the effect of glucocorticoids, suggesting that a negative feedback loop reducing the efficacy of glucocorticoid hormones on cellular level.

These experimental evidences may have important clinical consequences concerning acute and chronic inflammatory diseases, e.g. asthma, sepsis, rheumatoid arthritis and the above mentioned inflammatory bowel disease where glucocorticoid resistance has been found.

Because of the important role epithelial cells have in the process of IBD and the potential therapeutic and physiological role glucocorticoids may have on epithelial cells, the aim of our work was to characterize their glucocorticoid receptor signalling pathway. Since primary cells cannot be cultivated for longer time, we studied two different epithelial cell lines: the human colon carcinoma cell line Caco-2 and rat intestinal cell line IEC-6. Both cell lines are well established cell culture models for the intestinal epithelium (Jumarie, 1991; Jung, 1995; Quaroni, 1999).

Since inflammatory mediators are supposed to disturb GR signalling, our further aim was to test the hypothesis that IL-1 β , a major proinflammatory cytokine in IBD may inhibit glucocorticoid receptor action.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory devices

Centrifuge Hettich Rotixa/K (Hettich Zentrifugen)

Centrifuge Sigma 3K30 (Sigma-Aldrich Chemie GmbH, Deisenhofen)

Centrifuge Sigma 5415D (Sigma-Aldrich Chemie GmbH, Deisenhofen)

Curix 60 film processor (Agfa-Geavert)

Environmental incubator shaker (New Brunswick Scientific Co. Inc. Edison)

GelCam digital control (Phase, Luebeck)

GeneQuant RNA/DNA Calculator (Pharmacia, Freiburg)

Heraeus Laminair® (Heraeus Instruments GmbH, Hanau)

Heraeus Thermostat (Heraeus Instruments GmbH, Hanau)

Horizontal Gel Electrophoresis Apparatus HORIZON® 11.14 (GIBCO BRL, Grand Island)

Laboratory microscope Axiovert 25 Zeiss (Zeiss, Oberkochen)

Microplate Reader MRX Dynatech (Dynatech Technologies GmbH, Denkendorf)

Minishaker MS1 (Rettberg, Goettingen)

Omnigene Temperature Cycling System (Hybaid Limited, Teddington)

Power supply PowerPac 300 (Bio-Rad, Hercules)

Power supply PowerPac 200 (Bio-Rad, Hercules)

Precisious scale Sartorius (Sartorius AG, Goettingen)

Ready Gel Cell (Bio-Rad, Hercules)

Shaking platform Heidolph Polimax 2040 (Heidolph)

Thermomixer comfort (Eppendorf-Netheler-Hinz GmbH, Hamburg)

Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules)

Transilluminator 2020E (Stratagene, La Jolla)

Wallac 1409 liquid scintillation counter (Wallac distribution GmbH, Freiburg)

3.1.2 Tools

6 well plates	(Nunc, Rushilde)
24 well plates	(Nunc, Rushilde)
75 cm culture flasks	(Nunc, Rushilde)
96 well plates	(Nunc, Rushilde)
Curix film cassette	(Eastman Kodak Company, Rochester)
Eppendorf tubes	(Eppendorf-Netheler-Hinz GmbH, Hamburg)
Lab-Tek Permanox eight-chamber slides	(Nunc, Rushilde)
Nitrocellulose membrane	(Novex, Frankfurt)
QIAshredder	(QUIAGEN GmbH, Hilden)
Tissue culture dishes (60/15 mm, Cellstar)	(Greiner GmbH, Frickenhausen)
Tris-Glycine Ready Gel for SDS PAGE, 7.5%	(Bio-Rad, Hercules)
X-OMAT autoradiography film	(Eastman Kodak Company, Rochester)

3.1.3 Cells

Caco-2 from human colon adenocarcinoma (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig)

IEC-6 from rat normal small intestine (American Type Culture Collection, Rockville)

3.1.4 Chemicals

[1,2,4- ³ H]Dexamethasone	(Amersham Pharmacia Biotech, Freiburg)
1,4-dithiothreitol /DTT/	(GIBCO BRL, Grand Island)
2'-deoxynucleoside 5'-triphosphates /dNTP Mix/	(Roche Molecular Biochemicals, Mannheim)
2-mercaptoethanol	(Merck KGaA, Darmstadt)
2-propanol	(Merck KGaA, Darmstadt)
7.5% SDS polyacrylamide gel	(Novex, Frankfurt)

Agarose	(GIBCO BRL, Grand Island)
Albumin / from bovine serum, fatty acid free/	(Roche Molecular Biochemicals, Mannheim)
Albumin /from bovine serum/	(Bio-Rad, Hercules)
Aminoacetic acid /glycin/	(Sigma-Aldrich, Deisenhofen)
Ampicillin	(Sigma-Aldrich, Deisenhofen)
Bacto-agar	(DIFCO, Detroit)
Bacto-tryptone	(DIFCO, Detroit)
Bacto-yeast extract	(DIFCO, Detroit)
Bio-Rad Protein Assay Dye Reagent Concentrate	(Bio-Rad, Hercules)
Dexamethasone	(Sigma-Aldrich, Deisenhofen)
Diazobicyclooctan /DABCO/	(Sigma-Aldrich, Deisenhofen)
Dilution buffer for PCR	(Roche Molecular Biochemicals, Mannheim)
Dimethyl Sulphoxide /DMSO/	(Sigma-Aldrich, Deisenhofen)
Di-Sodium hydrogen phosphate /Na ₂ HPO ₄ /	(Merck KGaA, Darmstadt)
Dulbecco's modified Eagles's medium /DMEM/	(Biochrom KG, Berlin)
Eagle's minimal essential medium /EMEM/	(BioWhittaker, Verniers)
Ethanol absolut	(Merck KGaA, Darmstadt)
Ethidium bromide	(Sigma-Aldrich, Deisenhofen)
Ethylenediaminetetraacetic acid /EDTA/	(Sigma-Aldrich, Deisenhofen)
Ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid /EGTA/	(Sigma-Aldrich, Deisenhofen)
Fetal calf serum	(GIBCO BRL Grand Island)
Fluorescein linked anti-mouse IgG	(Amersham Pharmacia Biotech, Freiburg)
Fluorescein linked anti-rabbit IgG	(Amersham Pharmacia Biotech, Freiburg)
Fluoromount-G	(Southern Biotechnology, Birmingham)
FuGENE™ 6 Transfection Reagent	(Roche Molecular Biochemicals, Mannheim)
Gelatine (from bovine skin)	(Sigma-Aldrich, Deisenhofen)
Glucose	(Sigma-Aldrich, Deisenhofen)
Glycerol	(Merck KGaA, Darmstadt)
Hydrochloric acid /HCl/	(Merck KGaA, Darmstadt)
Insulin /from porcine pancreas/	(Sigma-Aldrich, Deisenhofen)
Interleukin-1β /human/	(Roche Molecular Biochemicals, Mannheim)
Kanamycin	(Sigma-Aldrich, Deisenhofen)
Magnesium sulphate /MgSO ₄ /	(Merck KGaA, Darmstadt)

Methanol	(Merck KGaA, Darmstadt)
Mineral oil	(Sigma-Aldrich, Deisenhofen)
Moloney Murine Leukemia Virus Reverse Transcriptase /M-MLV-RT/	(Roche Molecular Biochemicals, Mannheim)
Mouse monoclonal antibody BuGR2	(Affinity BioReagents, Neshanic Station, NJ)
N-2 (hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) /HEPES/	(Sigma-Aldrich, Deisenhofen)
Non essential amino acids	(BioWhittaker, Verniers)
Nonidet NP-40	(Sigma-Aldrich, Deisenhofen)
Paraformaldehyde	(Sigma-Aldrich, Deisenhofen)
Penicillin/Streptomycin	(GIBCO BRL Grand Island)
Phenylmethansulfonyl fluoride /PMSF/	(Sigma-Aldrich, Deisenhofen)
Polyoxyethylenesorbitan monolaurate /Tween 20/	(Sigma-Aldrich, Deisenhofen)
Potassium chloride /KCl/	(Merck KGaA, Darmstadt)
Potassium dihydrogen phosphate /KH ₂ PO ₄ /	(Merck KGaA, Darmstadt)
Rabbit polyclonal antibody GR /P-20/	(Santa Cruz Biotechnology, Inc.)
Primer for cDNA Synthesis /p(dT) ₁₅ /	(GIBCO BRL Grand Island)
RPMI-1640	(BioWhittaker, Verniers)
RU 486 (Mifepristone)	(Exelgyn, Paris)
Scintillation fluid	(Hirschmann, Eberstadt)
Sodium chloride /NaCl/	(Merck KGaA, Darmstadt)
Sodium dodecyl sulphate /SDS/	(Sigma-Aldrich, Deisenhofen)
Sodium hydroxide solution /NaOH/	(Merck KGaA, Darmstadt)
Sodium pyruvate	(GIBCO BRL Grand Island)
Taq DNA Polimerase	(Roche Molecular Biochemicals, Mannheim)
t-Octylphenoxypolyethoxyethanol /Triton X-100/	(Sigma-Aldrich, Deisenhofen)
Trypsin/EDTA	(BioWhittaker, Verniers)

3.1.5 Solutions

1.5 % agarose gel:

agarose	1.5 % (w/v)
ethidium bromide	0.4 μ g/ml

in TBE buffer

Blotting buffer for Western blot:

Tris base	25 mM
Glycin	50 mM
Methanol	5M

in distilled water

Buffer "A" for protein isolation:

HEPES pH 7.9	10 mM
KCl	10 mM
EDTA	0.1 mM
EGTA	0.1 mM
DTT	1 mM
PMSF	1 mM

in distilled water

Buffer "B" for protein isolation:

HEPES pH 7.9	20 mM
NaCl	0.4 M
EDTA	1 mM
EGTA	1 mM
DTT	1 mM
PMSF	1 mM

in distilled water

Dilution buffer for PCR:

Tris-HCl	20 mM
Dithiotreitol	1 mM
EDTA	0.1 mM
KCl	0.1 mM
Nonidet P40	0.5 % (v/v)
Tween 20	0.5 % (v/v)
glycerol	50% (v/v)

pH 8.0

Luria-Bertani agar plates:

bacto-agar	1.5 % (w/v)
ampicillin/kanamycin	50 µg/ml

in LB medium

Luria-Bertani /LB/ medium:

bacto-tryptone	1.0 % (w/v)
bacto-yeast extract	0.5 % (w/v)
NaCl	1.0 % (w/v)

pH 7.0

in distilled water

Phosphate buffered saline /PBS/ solution:

NaCl	0.13 M
KCl	2.68 mM
Na ₂ HPO ₄	10.2 mM
KH ₂ PO ₄	1.5 Mm

in distilled water

Running buffer for Western blot:

Tris base	20 mM
Glycin	0.2 M
SDS	40 mM

in distilled water

SOC medium:

bacto-tryptone	2.0 %
bacto-yeast extract	0.5 %
NaCl	10 mM
KCl	2.5 mM
MgSO ₄	10 mM
glucose	20 mM

in distilled water

TBE-buffer:

Tris base	0.1 M
Boric acid	0.1 M
EDTA pH 8	1 mM

in distilled water

3.1.6 Kits

ECL Western blotting analysis system (Amersham Pharmacia Biotech, Freiburg)

EndoFree Plasmid Maxi Kit (QUIAGEN GmbH, Hilden)

Original TA Cloning Kit (Invitrogen, Groningen)

RNeasy Mini Kit (QUIAGEN GmbH, Hilden)

SEAP Reporter Gene Assay Kit (Roche Molecular Biochemicals, Mannheim)

3.1.7 Primers

human GR α (Hollenberg, 1985) (MWG-BIOTECH GmbH, Edersberg):

5`-ACC AAT CAG ATA CCA AA ATA-3` (sense, coding position 2272-2291 of hGR α cDNA)

5`-ATA CAC CAA CAG AAA GTC TA-3` (antisense, position 2716-2735)

human TNF α (Wang, 1985) (MWG-BIOTECH GmbH, Edersberg):

5`-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A-3` (sense, position 404-435)

5`-GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T-3` (antisense, position 847-816)

rat TNF α (Shirai, 1989) (CLONTECH Laboratories, Heidelberg):

5`-TAC TGA ACT TCG GGG TGA TTG GTC C-3` (sense, position 955-979)

5`-CAG CCT TGT CCC TTG AAG AGA AAC-3` (antisense, position 2180-2157)

3.1.8 Plasmid vectors

pBluescript® SK +/- phagemid Vector (Stratagene, La Jolla, CA)

pCMV-I κ B α M Vector (CLONTECH Laboratories, Heidelberg)

pGRE-SEAP Vector (CLONTECH Laboratories, Heidelberg)

pNF κ B-SEAP Vector (CLONTECH Laboratories, Heidelberg)

pRShGR α Vector (Hollenberg, 1985) (American Type Culture Collection, Rockville)

3.2 Methods

3.2.1 Cell culture conditions

Caco-2 cells were cultured in EMEM containing 20 % FCS, 1 % non-essential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C under an atmosphere of 5 % CO₂.

IEC-6 cells were cultured in combination of Dulbecco's MEM and RPMI 1640 (1:1) containing 10 % FCS, 1 % sodium pyruvate, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C under an atmosphere of 5 % CO₂. 10⁻⁸ M insulin was freshly added to the cells every day.

Medium was changed every third day, the confluent cells were separated via Trypsin/EDTA treatment.

3.2.2 Protein isolation for Western blot

Caco-2 and IEC-6 cells were grown in 75 cm culture flasks. 24 hours before drug treatment, 0.5 % bovine serum albumin containing medium was added to the cells than they were incubated with 1 µM concentration of dexamethasone and/or 500 U/ml concentration of IL-1β.

Proteins were isolated from cells as described previously by E. Schreiber et al (Schreiber, 1989):

The cells were trypsinised, washed with PBS and pelleted by centrifugation. The pellet was resuspended in 1 ml PBS, transferred into an Eppendorf tube and pelleted again by spinning for 15 sec in a microfuge. PBS was removed and the cell pellet was resuspended in 400 µl cold buffer "A" by gentle pipetting in a yellow tip. The cells were allowed to swell on ice for 15 min, then 25 µl of a 10 % solution of Nonidet P-40 was added and the tube was vigorously vortexed for 10 seconds. The homogenate was centrifuged for 30 sec. The cytoplasmic fraction containing supernatant was transferred into a fresh tube and stored in aliquots at -80 °C. The nuclear pellet was resuspended in 50 µl ice-cold buffer "B" and the tube was vigorously rocked at 4 °C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min and the supernatant was frozen in aliquots at -80 °C.

The concentration of protein extract was measured according to the Bio-Rad protein assay method: Increasing dilutions of bovine serum albumin standard containing from 20 to 500 µg/ml was prepared. 100 µl from each protein standard or probe was added to an Eppendorf tube containing 1.25 ml 20 % of Dye Reagent. The mixture was vortexed and incubated for 10 minutes. 300 µl from

each Eppendorf tube was transferred into a 96-plate and their absorbance was measured at 595 nm. The absorbance at 595 nm versus concentration of standards was plotted, the unknown concentrations were read from the curve.

3.2.3 Western blot

An aliquot containing 15 μg of protein was boiled in SDS and 2 β -mercaptoethanol containing loading buffer for 5 minutes and loaded into 7.5 % SDS polyacrylamide gel using 10 ng of recombinant human GR as positive control. SDS-polyacrylamide gel electrophoresis was performed using Tris/Glycine/SDS containing running buffer. Proteins were transferred onto nitrocellulose membrane for 30 minutes in blotting buffer in semidry transfer cell at 10 V. Filters were incubated for 1 hour with PBS/0.02 % Tween 20 containing 5 % non-fat dry milk at room temperature. After rinsing with PBS/Tween 20 the membrane was incubated overnight in case of Caco-2 with the rabbit polyclonal antibody GR (P-20) at 1 $\mu\text{g}/\text{ml}$ concentration, in case of IEC-6 with the mouse monoclonal antibody BuGR2 at 5 $\mu\text{g}/\text{ml}$ concentration at 4 °C. This step was followed by washes with PBS/Tween 20. After washing the membrane was incubated either with horseradish peroxidase-conjugated anti-rabbit IgG (Caco-2 cells) or horseradish peroxidase-conjugated anti-mouse IgG (IEC-6 cells) at 1:2000 dilution for 1 hour.

After further washes the immune complexes were visualised using ECL Western blotting analysis system:

Equal volumes of detection solution 1 and detection solution 2 were mixed. The membrane was covered by excess detection reagent and incubated without agitation for 1 minute at room temperature in dark room. The excess detection reagent was drained off and the membrane was transferred in nylon foil pocket removing air bubbles and the nylon foil was placed in film cassette. A sheet of autoradiography film was placed on top of the membrane, exposed for 1-5 minutes and developed immediately.

3.2.4 Immunostaining procedures

Caco-2 and IEC-6 cells were grown in Lab-Tek Permanox eight-chamber slides.

24 hours before drug treatment, 0.5 % bovine serum albumin containing medium was added to the cells. Four different treatments were performed: with PBS as vehicle, IL-1 β (500 U/ml) for 24 hours, dexamethasone (1 μ M) for 1 hour and IL-1 β (500 U/ml) for 24 hours followed by coincubation of IL-1 β (500 U/ml) plus Dex (1 μ M) for 1 hour.

After removal the medium the cells were washed twice in PBS, fixed in 2 % paraformaldehyde for 40 min at 37 °C and permeabilized in 0.3 % Triton X-100 dissolved in PBS for 10 min. Nonspecific antibody binding was blocked by 0.2 % gelatine/PBS solution. Caco-2 cells were then incubated with the rabbit polyclonal antibody GR (P-20) diluted in 0.2 % gelatine/PBS at 1 μ g/ml concentration, IEC-6 cells were incubated with the mouse monoclonal antibody BuGR2 at 5 μ g/ml concentration overnight at 4 °C. The following day Caco-2 cells were incubated with fluorescein linked anti-rabbit IgG, IEC-6 cells were incubated with fluorescein linked anti-mouse IgG diluted in 0.2 % gelatine/PBS at 1:200 dilution for 1 hour at 37 °C (in darkness). Two washes with PBS were performed between all steps. Thereafter the cells were washed with distilled water followed by mounting in Fluoromount.

3.2.5 Confocal laser scanning microscopy (CLSM)

Lab-Tek slides were viewed by fluorescence microscopy using a Zeiss LSM CLSM. One optical section with a thickness of 1.2 μ m was recorded through the centre of each visual field. Sections were stored as digital pictures with a resolution of 512 \times 512 pixels.

3.2.6 Transformation of *E. coli*

Transformation of competent bacteria was performed according to manufacturers of Original TA Cloning Kit.

2 μ l of 0.5 M β -mercaptoethanol was pipetted into a 50 μ l vial of melted One Shot INV α F' competent cells and mixed by stirring gently with the pipette tip. 500 ng of plasmid DNA was added directly to the competent cells, mixed than incubated on ice for 30 minutes. Heat shock was performed for 30 seconds in 42 °C water bath then the cells were placed on ice for 2 minutes. 450 μ l of SOC medium was added into the cells at room temperature and the vial was shaken horizontally at 37 °C for one hour at 225 rpm in a rotary-shaking incubator. Thereafter the vial with transformed bacteria was placed on ice.

100 μ l of transformed bacteria was spread on LB agar plate containing 50 μ g/ml of ampicillin or kanamycin. The agar plate was incubated at 37 °C for 18 hours.

3.2.7 Purification of plasmid DNA

Purification of plasmid DNA from transformed bacteria was performed according to manufacturers of EndoFree Plasmid Maxi Kit.

A single colony of transformed bacteria was picked from the streaked LB agar plate and a 2 ml starter culture of ampicillin containing LB medium was inoculated. The bacterial culture was incubated for 12 hours at 37 °C with vigorous shaking at 300 rpm.

Thereafter the starter culture was diluted to 1:1000 and bacteria were grown at 37 °C for 16 hours with shaking at 300 rpm.

The bacterial cells were harvested by centrifugation at 6000 g for 15 minutes at 4 °C. The medium was removed and the pellet was resuspended in 10 ml Buffer P1. The cells were lysed by adding 10 ml Buffer P2 and the lysate was incubated at room temperature for 5 minutes. Genomic DNA, proteins and cell debris was precipitated by 10 ml chilled Buffer P3 and filtered by QIAfilter Cartridge. The filtered lysate first was incubated on ice for 30 minutes after adding 2.5 Buffer ER then applied to the anion-exchange resin containing QIAGEN-tip followed by two washed with 30 ml Buffer QC. Plasmid DNA was eluted with 15 ml Buffer QN and precipitated by adding 10.5 ml room-temperature isopropanol. The mixture was centrifuged immediately at 15000 g for 30 minutes at 4 °C. After centrifugation the DNA pellet was washed with 70 % ethanol, pelleted at 15000 g for

10 minutes. Thereafter the supernatant was disturbed and the pellet was air-dried. Finally DNA was redissolved in 200-500 μ l Buffer TE and its concentration was determined by spectrophotometry measuring the absorbance at 260 and 280 nm.

3.2.8 Transient transfection of Caco-2 and IEC-6 cells

Caco-2 and IEC-6 cells were grown in 24 well plates at a density of 30000/well in 1 ml medium. 24 hours before the transfection 0.5 % bovine serum albumin containing medium was added to the cells. The transfection was performed by non-liposomal formulation FuGENE according to manufacturer's protocol. FuGENE was prediluted in 100 μ l serum free medium (3 μ l FuGENE/ μ g DNA), then the concentrated plasmid DNA was added to the mixture. It was incubated for 15 minutes then pipetted into the wells containing 900 μ l serumfree medium.

In transactivation experiments 200 ng of pGRE-SEAP/well, in transrepression studies 200 ng of pNF κ B-SEAP/well was added to the cells. In cotransfection examinations the cells were transfected either with 1 μ g pRShGR α expression vector or with 200 ng pCMV-I κ B α M and 200 ng of appropriate reporter plasmid. In the control groups DNA content was equilibrated by pBluescript vector.

In transactivation experiments different concentrations of dexamethasone (10^{-6} M – 10^{-9} M) and/or 10^{-6} M of RU 486 were added to the cells in presence or absence of IL-1 β (5-500 U/ml) 24 hours post transfection.

In transrepression studies the cells were treated with different doses of IL-1 β (5-500 U/ml) in presence or absence of 1 μ M concentration of dexamethasone 24 hours post transfection.

In both studies control cells were treated with appropriate amount of vehicles (PBS or ethanol). In transactivation experiments the cells were incubated for 24 hours, in transrepression assays for 6 hours. After this time media were collected to determinate the reporter gene activity.

3.2.9 SEAP Reporter Gene Assay

The quantification of alkaline phosphatase secreted by transfected cells is based on the phosphorylation of CSPD [3-(4-metoxyspiro[1,2-dioxetane-3,2'(5'-chloro)-tricyclo(3.3.1.1)decane]-4-yl)phenyl-phosphate] by alkaline phosphatase. The resulting unstable dioxetane anion decomposes and emits light with its maximum activity at 477 nm.

The alkaline phosphatase activity was determined according to protocol of SEAP Reporter Gene Assay Kit.

50 μ l aliquot of culture supernatant was centrifuged to pellet any debris then diluted 1:4 with Dilution Buffer followed by incubation for 30 minutes at 65 °C to heat-inactivate the endogenous alkaline phosphatase activity. Thereafter samples were pelleted again, then 100 μ l of supernatant was transferred to a tube and the same amount of Inactivation Buffer was added. After 5 minutes incubation 100 μ l of Substrate Reagent was pipetted into the tube. The sample was rocked gently for 10 minutes. The chemiluminescent signal was counted in a liquid scintillation counter.

3.2.10. ³H-dexamethasone binding assay

Caco-2 and IEC-6 cells were grown in 6 well plates at a density of 500.000/well covered by serum-free medium. The cells were treated with 50 nM concentration of ³H-dexamethasone in absence or presence of 1000 fold molar excess of unlabeled steroid for two hours at 37 °C to determine total and unspecific dexamethasone binding. After dexamethasone treatment the supernatant was removed. The cells were first washed three times with ice cold PBS then harvested by 1ml 0.1 normal NaOH. 1 ml 0.1 normal HCl was added to the cells to neutralize the pH. The 2 ml solutions were transferred to scintillation tubes containing 8 ml of scintillation fluid and measured in a liquid scintillation counter.

3.2.11 RNA isolation

Caco-2 and IEC-6 cells were cultured in tissue culture dishes (60/15 mm, CellStar) at a density of 500 000/well in 3 ml medium. 24 hours before drug treatment, 0.5 % bovine serum albumin containing medium was added to the cells than they were incubated with dexamethasone and/or IL-1 β . Total RNA isolation was performed according to RNeasy Mini Protocol.

The cells were first washed with 3 ml PBS than lysed in 500 μ l highly denaturing guanidinium isothiocyanate-containing buffer (Buffer RLT). RNases were inactivated by adding β -mercaptoethanol to the buffer previously (10 μ l/1ml Buffer RLT). The lysate was pipetted directly onto a QIAshredder column sitting in the 2 ml collection tube and centrifuged for 2 min at maximum speed to homogenise. 500 μ l 70% ethanol was added to the homogenised lysate to provide appropriate binding conditions and mixed well by pipetting. The lysate was applied to an RNeasy spin column in 500 μ l aliquots and centrifuged for 1 min at 10000 g. The membrane-bound RNA was washed first by pipetting 500 μ l washing buffer (Buffer RW1) than by adding 500 μ l ethanol containing Buffer RPE onto the column and centrifuging for 1 min at 10000 g. The RNeasy membrane was dried by 500 μ l Buffer RPE centrifuging for 2 min at maximum speed and the column was transferred to a 1.5 ml Eppendorf tube. 30 μ l RNase-free water was added to the membrane and the RNA was eluted by centrifuging for 1 min at 10000 g. The total RNA was stored at -80°C .

The concentration of total RNA was determined by spectrophotometry measuring the absorbance at 260 and 280 nm. (An absorbance of 1 unit at 260 nm corresponds to 40 μ g RNA per ml. This relationship is valid for measurements of water-diluted RNA.)

3.2.12 RT-PCR

1 µg of total RNA was reverse transcribed to copy-DNA (cDNA) in a final volume of 40 µl containing 200 U M-MLV reverse transcriptase, 50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 10 µg/ml (dT)₁₅-primer and 2 mM dNTP for 1 hour at 37°C.

RT-products were amplified by polymerase chain reaction in 25 µl PCR-mixture containing 1 µl aliquot of cDNA, 0.2 µM each of sense and antisense primer, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 0.01 % gelatine, 0.8 mM dNTP, and 1 U Taq polymerase.

Using specific primer sequences for human GRα /5'-ACC AAT CAG ATA CCA AA ATA-3' (sense, coding position 2272-2291 of hGRα cDNA) and 5'-ATA CAC CAA CAG AAA GTC TA-3' (antisense, position 2716-2735)/, amplification was carried out by 38 cycles at 95 °C for 45 seconds, 54 °C for 90 seconds and 72 C° for 90 seconds.

PCR reactions with human TNFα primer /5'-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A-3' (sense, position 404-435) and 5'-GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T-3' (antisense, position 847-816)/ and with rat TNFα primer /5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3' (sense, position 955-979) and 3'-CAG CCT TGT CCC TTG AAG AGA AAC-3' (antisense, position 2180-2157)/ were carried out by 40 cycles at 95 C° for 45 seconds, 60 C° for 45 seconds and 72 C° for 90 seconds.

PCR-products were electrophoresed in a 1.5 % agarose gel and visualised by ethidium bromide staining.

4. RESULTS

4.1 Glucocorticoid receptor expression in Caco-2 and IEC-6

To characterize the glucocorticoid receptor expression in Caco-2 and IEC-6 cells, Western blot analysis was performed.

The results revealed that GR protein was expressed in both cell lines. In Caco-2 the specific band comigrated with a recombinant human glucocorticoid receptor expressed in a baculovirus system using as positive control (Figure 3.).

Both cytosolic and nuclear protein extracts were examined. In case of the human cell line Caco-2 the majority of GRs were detected in the nuclear extract, in the rat cells obvious cytosolic localisation was observed (Figure 4.).

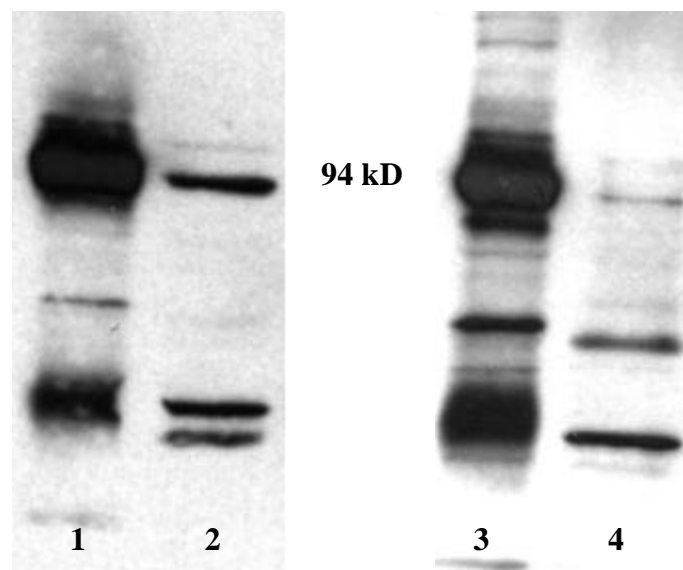


Figure 3. Human GR α protein in Caco-2. Western blot analysis.

The presence of GR α was detected in cytosolic and nuclear extracts prepared from Caco-2 cells using the polyclonal antibody P-20. Lane 1 and lane 3 show the recombinant GR, lane 2 demonstrates the nuclear extract, lane 4 the cytoplasmic fraction. The additional bands may be degradation products.

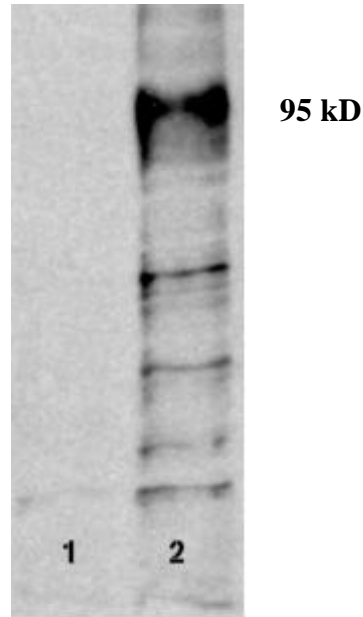


Figure 4. Rat GR protein in IEC-6. Western blot analysis.

The presence of GR was detected in cytosolic and nuclear extracts prepared from IEC-6 cells using the monoclonal antibody BuGR2. Lane 1 shows the nuclear extract, lane 2 demonstrates the cytoplasmic fraction.

To characterize the functional activity of GR, radioligand-binding assay was performed using ^3H -dexamethasone. We detected 66400 ± 6700 specific binding sites/cell in Caco-2 and 99600 ± 15300 sites/cell in IEC-6. However, the unspecific binding in Caco-2 was very high, only 10 % of the total binding was specific. So that the number of specific binding sites is probably overestimated.

4.2 The gene regulatory effects of glucocorticoids can be measured by transfection experiments in intestinal epithelial cells

The transactivation effect of dexamethasone can be monitored by transfection with a reporter gene construct carrying glucocorticoid receptor responsive (GRE) element. Glucocorticoid treatment after transfection results in glucocorticoid receptor activation and increased transcription of the responsive element containing reporter gene.

In Caco-2 cells glucocorticoid receptor activation in presence of 1 μ M dose of dexamethasone resulted in a 3.9 ± 0.5 fold (mean of fold induction \pm SEM), in IEC-6 cells a 5.2 ± 0.4 fold induction of reporter gene activity relative to the basal transcription. In both cell lines decreasing concentrations of dexamethasone resulted in a dose dependent induction of SEAP activity, which was inhibited by the competitive glucocorticoid receptor antagonist, RU-486. The antagonist alone had no effect on SEAP activity (Figure 5. and Figure 6.).

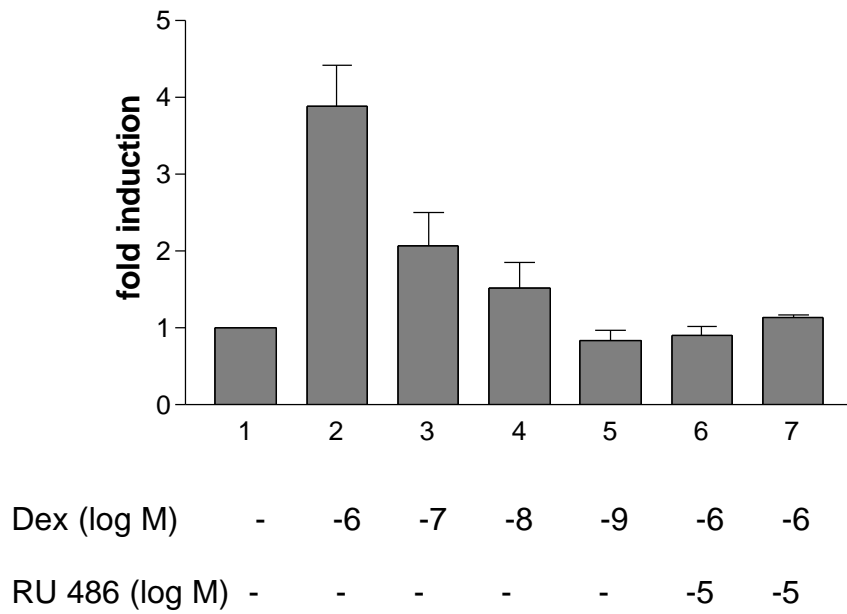


Figure 5. Effect of dexamethasone on transcriptional transactivation in Caco-2 cells.

Caco-2 cells were transiently transfected with pGRE-SEAP (200 ng/well) then incubated with decreasing concentrations of dexamethasone (10^{-6} M - 10^{-9} M) in absence or presence of RU 486 (10^{-5} M) for 24 hours. Alkaline phosphatase activities are shown relative to the level of SEAP activity of the control group treated with the vehicle substance ethanol alone.

Relative SEAP activities are demonstrated as means of fold induction over controls \pm SEM for $n=3$. The experiments were performed in duplicate.

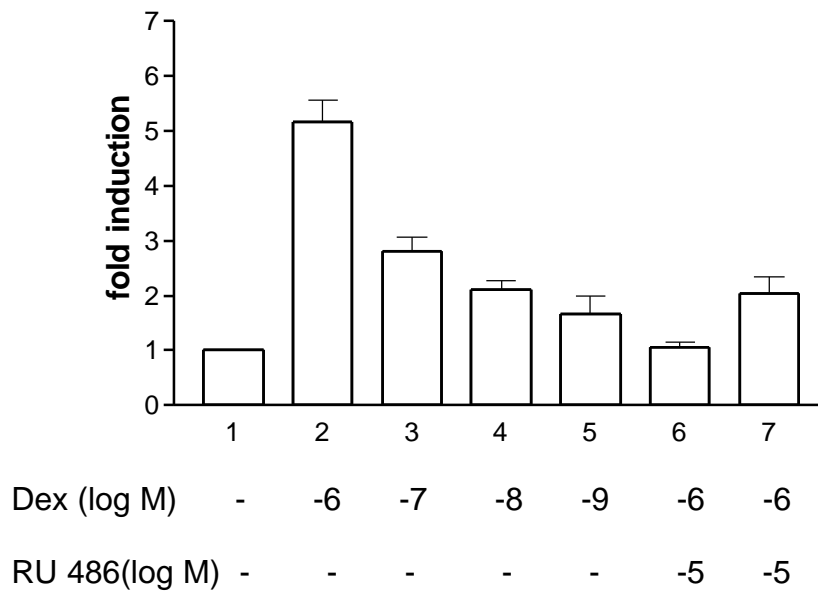


Figure 6. Effect of dexamethasone on transcriptional transactivation in IEC-6 cells.

IEC-6 cells were transfected with pGRE-SEAP (200 ng/well) then incubated with decreasing concentrations of dexamethasone (10^{-6} M - 10^{-9} M) in absence or presence of RU 486 (10^{-5} M) for 24 hours. Reporter gene activities are shown as compared to the level of SEAP activity of control group treated with the vehicle substance ethanol alone.

Relative SEAP activities are demonstrated as means of fold induction over controls \pm SEM for $n=3$. Each experiment was performed in duplicate.

The transrepressive effect of dexamethasone was measured by transfection with the NF- κ B responsive element carrying reporter gene construct pNF- κ B-SEAP.

As it is demonstrated in the figures below, IL-1 β , a known NF- κ B activating agent induced a dose dependent increase in NF- κ B mediated reporter gene activity in both cell lines. In Caco-2 cells 5 U/ml concentration resulted in a 4.6 ± 0.8 fold, 50 U/ml caused 7.65 ± 0.3 fold, 500 U/ml 8.6 ± 0.2 fold increase of SEAP-activity (Figure 7.). In IEC-6 cells we found 2.8 ± 0.8 fold increase at 5 U/ml, 5.1 ± 0.3 fold increase at 50 U/ml, and 7.3 ± 0.3 fold increase at 500 U/ml concentration of IL-1 β (Figure 8.).

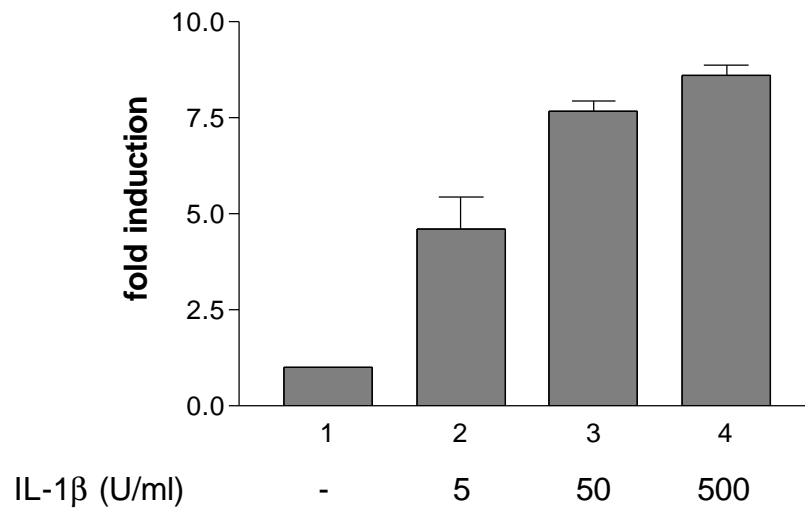


Figure 7. IL-1 β mediated NF- κ B activation in Caco-2 cells.

Caco-2 cells were transfected with NF- κ B responsive element carrying reporter gene construct (pNF- κ B-SEAP, 200 ng/well). 24 hours post transfection the cells were stimulated with increasing concentrations of IL-1 β (5-500 U/ml) for 6 hours. Then media were collected and the reporter gene activities were measured. Transcriptional activities of SEAP are shown as relative to controls (PBS treated cells). Relative SEAP activities represent means of fold induction \pm SEM for n=3. The experiments were performed in duplicate.

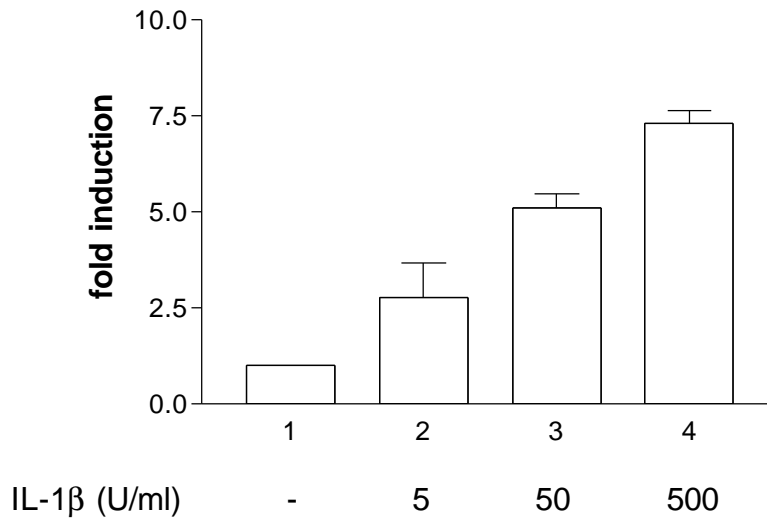


Figure 8. IL-1b mediated NF- κ B activation in IEC-6 cells.

IEC-6 cells were transfected with pNF- κ B-SEAP (200 ng/well). 24 hours post transfection the cells were stimulated with increasing concentrations of IL-1b (5-500 U/ml) for 6 hours. Thereafter media were collected and SEAP activities were measured. SEAP activities are shown compared to control cells, which received PBS. Relative SEAP activities represent means of fold induction \pm SEM for $n=3$. The experiments were performed in duplicate.

4.3 The transrepressive effect of dexamethasone on IL-1b induced, NF- κ B mediated reporter gene activity in Caco-2 and IEC-6 cells

Surprisingly, in Caco-2 cells 1 μ M dexamethasone had no influence on IL-1 β induced, NF- κ B mediated reporter gene activity. In this experiment the cells were coincubated with 1 μ M dexamethasone and increasing concentrations of IL-1 β (5-500 U/ml) for 6 hours. We found that Dex could not decrease the IL-1 β induced reporter gene activity at all. (Figure 9.).

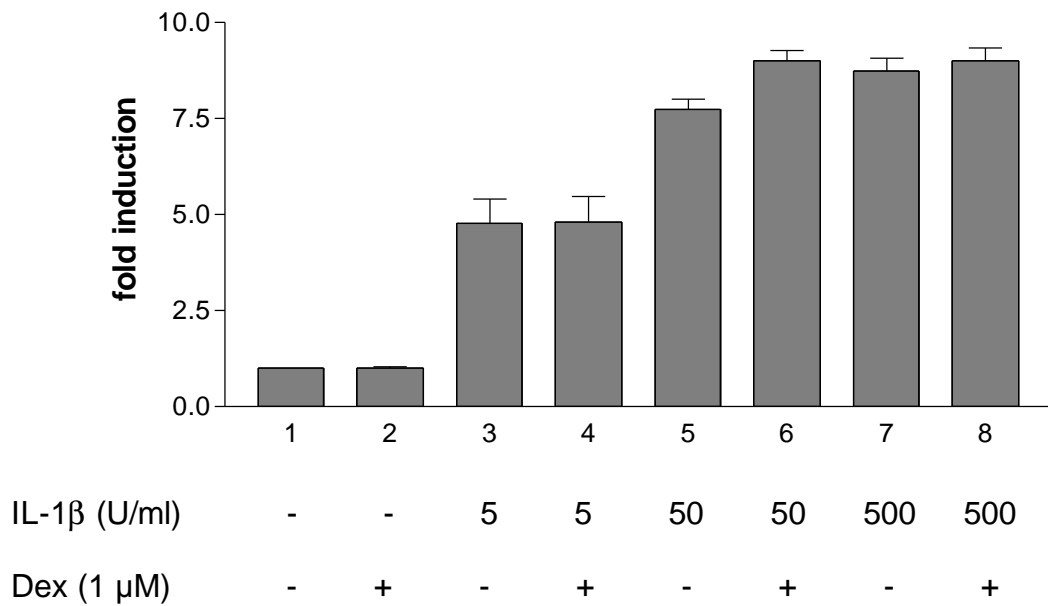


Figure 9. Effect of dexamethasone on IL-1 β induced, NF- κ B mediated SEAP activity in Caco-2 cells.

Caco-2 cells were transfected with pNF- κ B-SEAP (200 ng/well) and treated with increasing concentrations of IL-1 β (5-500 U/ml) in presence or absence of Dex (1 μ M) for 6 hours. After the various treatments media were collected and reporter gene activities were measured. They are shown as relative activities compared control, which did not receive IL-1 β . Relative SEAP activities are presented as means of fold induction \pm SEM for $n=3$. The experiments were performed in duplicate.

In contrast to Caco-2, in IEC-6 cells 1 μ M dexamethasone could inhibit the NF- κ B mediated SEAP activity at each doses of IL-1 β (5, 50 and 500 U/ml). In presence of dexamethasone the reporter gene activity was 80 % \pm 20 % at 5 U/ml of IL-1 β , 60 % \pm 6 % at 50 U/ml and 50 % \pm 8.5 % at 500 U/ml compared to cells incubated without Dex.

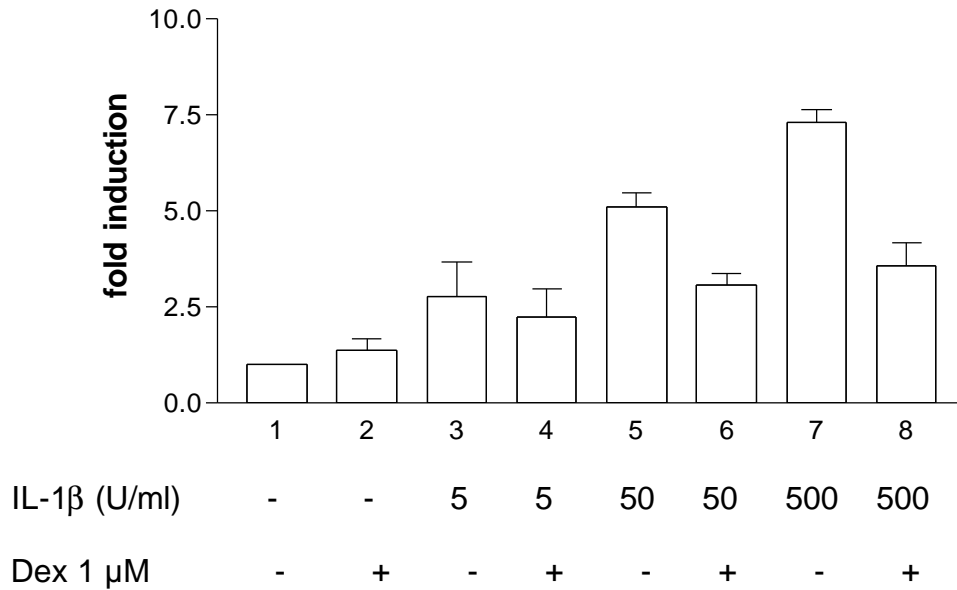


Figure 10. Effect of dexamethasone on IL-1b induced, NF-kB mediated SEAP activity in IEC-6 cells.

IEC-6 cells were transfected with pNF-kB-SEAP (200 ng/well) then incubated with increasing concentrations of IL-1b (5-500 U/ml) in presence or absence of Dex (1 μM) for 6 hours. After the various treatments media were collected and SEAP activities were measured. The activities are shown relative to PBS treated cells (control group). Relative SEAP activities are demonstrated as means of fold induction ± SEM for n=3. The experiments were carried out in duplicate.

4.4 IL-1b repressed the stimulating effect of dexamethasone on transactivation in both of Caco-2 and IEC-6 cells

To test the influence of IL-1β on GR mediated reporter gene activity, Caco-2 and IEC-6 cells were transfected with GRE carrying reporter gene construct pGRE-SEAP and stimulated with 1 μM dexamethasone. Coincubation with increasing concentrations of IL-1β resulted in a reduced reporter gene activity in both cell lines. IL-1β alone had no effect on SEAP activity (data not shown).

In Caco-2 cells 5 U/ml dose of IL-1 β resulted 57 % \pm 6 % GR mediated reporter gene activity, at 50 U/ml 33 % \pm 10 %, at 500 U/ml 19 % \pm 3 % activity compared to SEAP activity of cells incubated in absence of IL-1 β (Figure 11.).

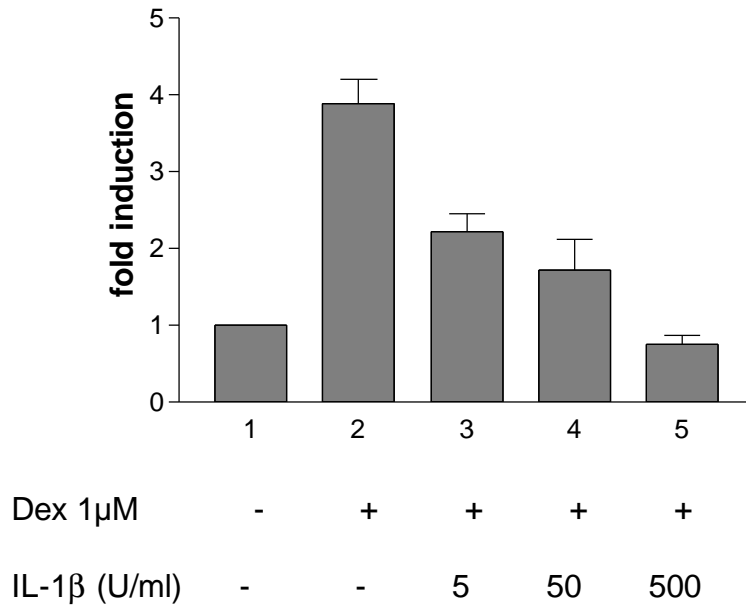


Figure 11. Effect of IL-1 β on dexamethasone induced, GR mediated reporter gene activity in Caco-2 cells.

Caco-2 cells were transfected with pGRE-SEAP reporter gene construct (200 ng/well), then stimulated with increasing concentrations of IL-1 β (5-500 U/ml) in presence of 1 μ M dose of dexamethasone. After 24 hours media were collected and SEAP activities were measured. Reporter gene activities of IL-1 β - and dexamethasone-treated groups were compared to the control. Relative SEAP activities are demonstrated as means of fold induction \pm SEM for n=3. The experiments were performed in duplicate.

In IEC-6 cells the lowest dose of IL-1 β (5 U/ml) resulted in a 53 % \pm 6 % reporter gene activity, 50 U/ml of IL-1 β caused a reduction to 50 % \pm 5 %, 500 U/ml dose resulted in a decrease to 40 % \pm 4 % activity compared to the cells treated with Dex alone (Figure 12.).

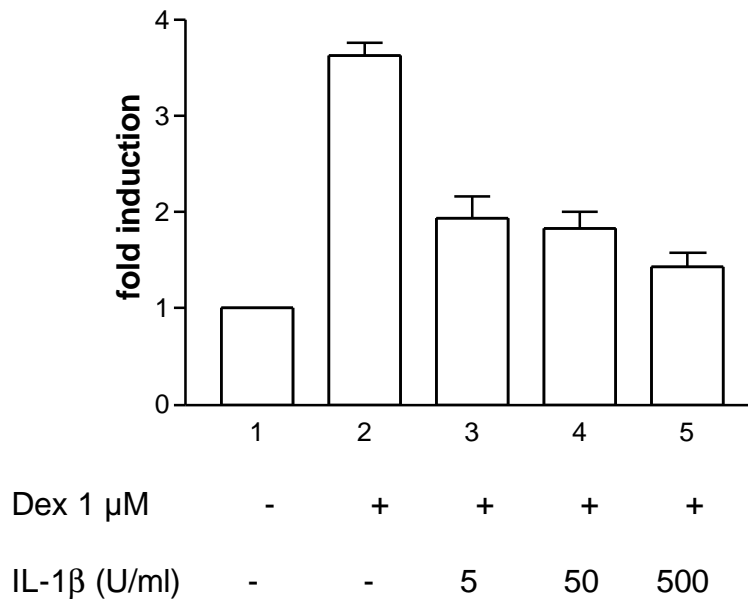


Figure 12. Effect of IL-1 β on dexamethasone induced, GR mediated reporter gene activity in IEC-6 cells.

IEC-6 cells were transfected with pGRE-SEAP reporter gene construct (200 ng/well), then stimulated with increasing concentrations of IL-1 β (5-500 U/ml) in presence of 1 μ M dexamethasone for 24 hours. After incubation media were collected and SEAP activities were measured. Reporter gene activities of IL-1 β - and dexamethasone-treated groups were compared to the control. Relative SEAP activities are demonstrated as means of fold induction \pm SEM for n=3. Each experiment was performed in duplicate.

4.5 IL-1b inhibits glucocorticoid receptor translocation

The insufficiency of dexamethasone in presence of IL-1 β regarding both transactivation and trans-repression in Caco-2 and IEC-6 cells supposed that the glucocorticoid receptor action is disturbed in presence of the cytokine.

To confirm our findings, GR translocation from cytoplasm to nucleus was examined using GR immunostaining.

As it is demonstrated on the figures below, mainly nuclear staining could be observed in the vehicle treated Caco-2 cells, neither IL-1 β nor dexamethasone treatment changed the pattern of GR staining (Figure 13).

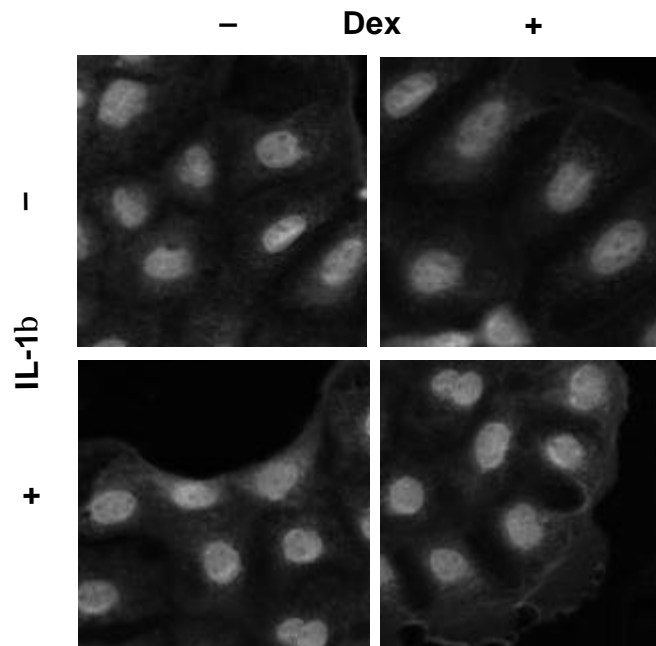


Figure 13. GR immunostaining after treatment with Dex and/or IL-1b in Caco-2 cells.

Caco-2 cells were treated with vehicle, Dex (1 μ M) for 1 hour, IL-1b (500 U/ml) for 24 h followed by coincubation with IL-1b (500 U/ml) and Dex (1 μ M) for 1 h and IL-1b (500 U/ml) for 24 h. GRs were immunostained with rabbit anti-GR polyclonal antibody P-20 (1 μ g/ml). Optical sections (1.2 μ m) were recorded by CLSM.

In vehicle treated control IEC-6 cells the fluorescent signal was more intense in the cytoplasm than in the nucleus, a ring-shaped perinuclear localisation could be observed.

After Dex treatment cells presented more intense nuclear staining, confirming that incubation with dexamethasone induced GR translocation from the cytoplasm to the nucleus.

Coincubation with Dex and IL-1 β resulted in a complete inhibition of GR translocation.

Finally, treatment with IL-1 β alone had no apparent effect on the pattern of GR staining (Figure 14.).

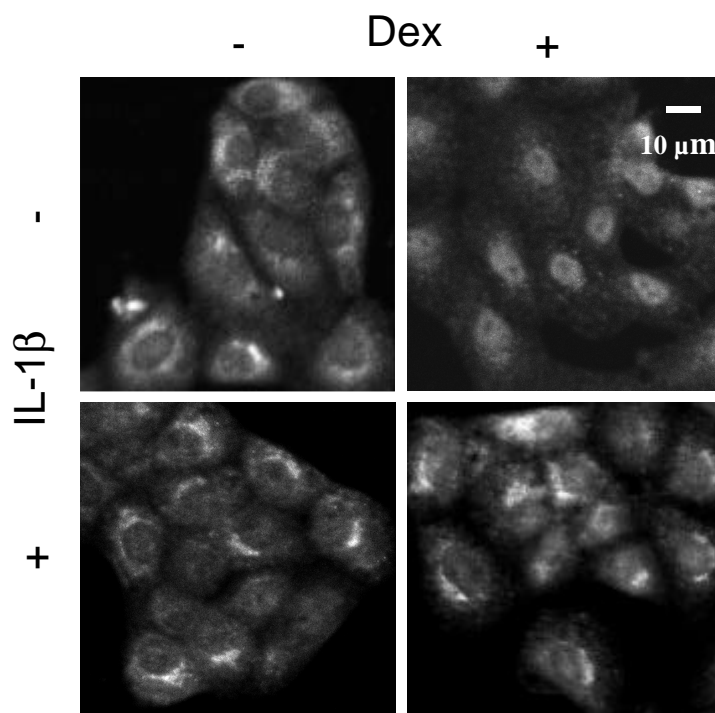


Figure 14. Immunostaining of GR after treatment with Dex and/or IL-1 β in IEC-6 cells.

IEC-6 cells were treated with vehicle, Dex (1 μ M) for 1 hour, IL-1 β (500 U/ml) for 24 h followed by coincubation with IL-1 β (500 U/ml) and Dex (1 μ M) for 1 h and IL-1 β (500 U/ml) for 24 h. GR protein was stained with mouse monoclonal antibody BuGR2 (5 μ g/ml). Optical sections (1.2 μ m) were recorded by CLSM.

4.6 IL-1b does not reduce dexamethasone binding in Caco-2 and IEC-6

Our findings presented above confirm the observation that the proinflammatory cytokine, IL-1 β reduces glucocorticoid receptor function.

To answer the question whether IL-1 β downregulates glucocorticoid receptor quantity, radiobinding assays were performed. GR-levels were measured in Caco-2 and IEC-6 cells treated with IL-1 β (500 U/ml) or vehicle.

As it is demonstrated in the figures below, there was no apparent change in the glucocorticoid receptor numbers.

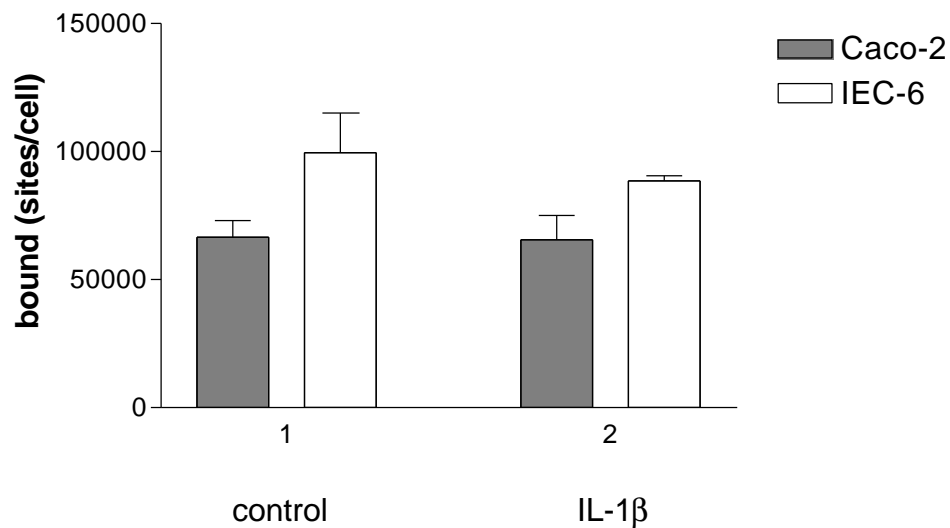


Figure 15. Specific binding of ³H- dexamethasone to Caco-2 and IEC-6 cells. Influence of IL-1b treatment.

Caco-2 and IEC-6 cells were incubated with IL-1 β (500 U/ml) or PBS (vehicle substance) at 37 °C for 24 hours, then treated with 50 nM concentration of ³H-dexamethasone in absence or presence of 1000 fold molar excess of unlabeled steroid for two hours. The total and unspecific Dex binding was measured in a liquid scintillation counter.

4.7 Dexamethasone can reduce the IL-1 β -induced TNF α expression only in IEC-6 cells but not in Caco-2

Based on the observation that LPS significantly increased the production of TNF α in Caco-2 cells (Ogle, 1997), we asked the question whether IL-1 β can induce TNF α expression and this cytokine production can be inhibited by dexamethasone in Caco-2 and IEC-6 cells.

To answer the question RT-PCR experiments were performed using human and rat TNF α primers. The cells were incubated with vehicle (1), Dex (1 μ M) (2), IL-1 β (500 U/ml) (3) and combination of IL-1 β and Dex (4) for 4 hours. After the various treatments total RNAs were isolated and RT-PCR was performed (Figure 16.).

Nonstimulated Caco-2 cells did not express TNF α , IEC-6 cell did. 500 U/ml concentration of IL-1 β stimulated the cytokine expression in both cell lines, in Caco-2 the effect was more intense than in IEC-6. 1 μ M dose of dexamethasone had effect only on TNF α expression produced by IEC-6, in Caco-2 Dex could not inhibit the production of TNF α mRNA.

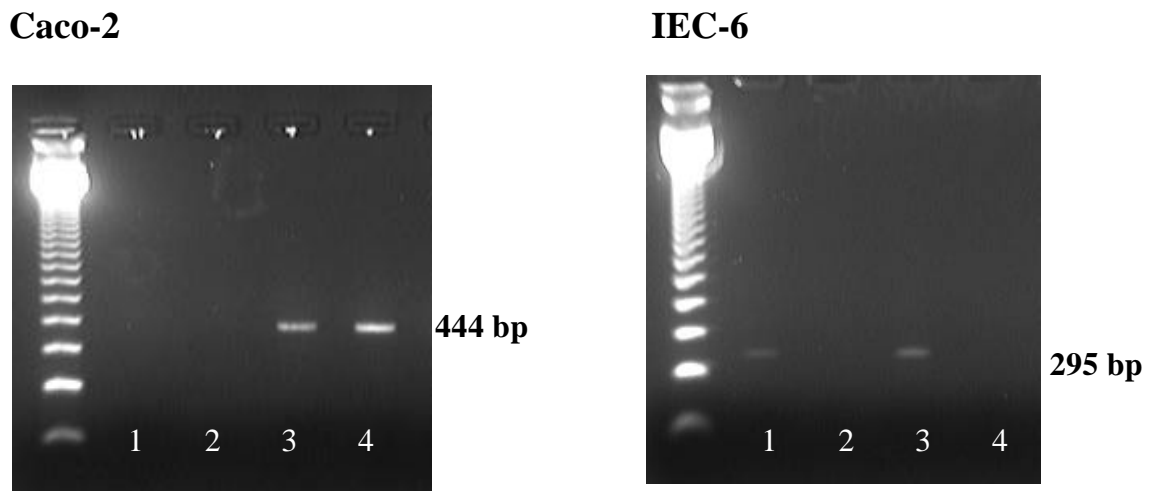


Figure 16. Effect of dexamethasone on TNF α mRNA expression induced by IL-1 β in Caco-2 and IEC-6 cells.

Caco-2 and IEC-6 cells were incubated with vehicle (1), Dex (1 μ M) (2), IL-1 β (500 U/ml) (3) and combination of IL-1 β and Dex (4) for 4 hours. Thereafter total RNA was isolated and copy DNA was prepared. RT-PCR was performed using specific TNF α primers. PCR products were run on 1.5 % agarose gel and visualised with ethidium bromide. The figures show one representative result out of three independent experiments.

4.8 Glucocorticoid receptor action could be restored by increasing the receptor content by GR-overexpression in Caco-2

As Caco-2 cells were apparently endowed with less GRs than IEC-6 cells we hypothesised that increase of the receptor content may result more sufficient glucocorticoid receptor action.

To increase the receptor quantity, Caco-2 cells were transfected with glucocorticoid receptor-overexpression vector (pRShGR α) (Hollenberg, 1985). To examine the effect of pRShGR α on quantity of glucocorticoid receptor mRNA, Caco-2 cells were grown in 6 cm tissue culture dishes at a density of 600000/well in 3 ml medium, transfected with 9 μ g pRShGR α vector then total RNA was isolated. The GR mRNA expression was determined by RT-PCR. As it is demonstrated in Figure 17., glucocorticoid receptor mRNA was increased after transfection with overexpression vector.

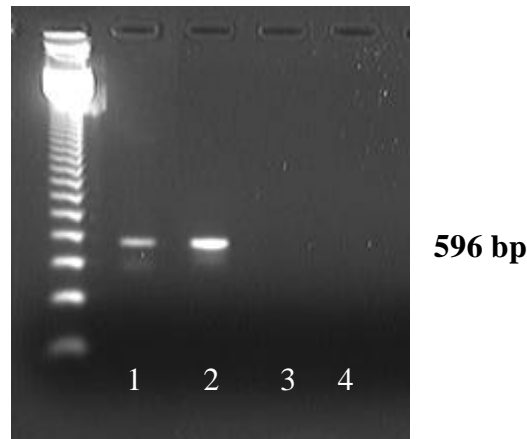


Figure 17. RT-PCR of hGR α mRNA: increased glucocorticoid receptor mRNA after transfection with pRShGR α vector in Caco-2 cells.

Caco-2 cells were transfected with glucocorticoid receptor overexpression vector (pRShGR α , 9 μ g), then total RNA was isolated. 1 μ g of total RNA was reverse transcribed to RT, then RT-PCR was performed. The first band (1) shows hGR mRNA content in untreated cells, the second band (2) shows the hGR mRNA production in pRShGR α transfected cells. Copy DNA of negative controls (3 and 4) were prepared in absence of M-MLV reverse transcriptase to exclude DNA contamination. PCR products were run on 1.5 % agarose gel and visualised with ethidium bromide. On the figure one result out of three independent experiments is demonstrated.

To confirm that pRShGR α really increased the receptor content in Caco-2 cells, the following step was to test the transactivating effect of dexamethasone on GR-overexpressing cells.

Cotransfection with pRShGR α and pGRE-SEAP followed by treatment with decreasing concentrations of dexamethasone resulted a significant, dose dependent increase in reporter gene activity (1 μ M Dex caused 16.3 ± 0.6 fold induction) (Figure 18.). In absence of dexamethasone GR overexpression had no effect on SEAP activity.

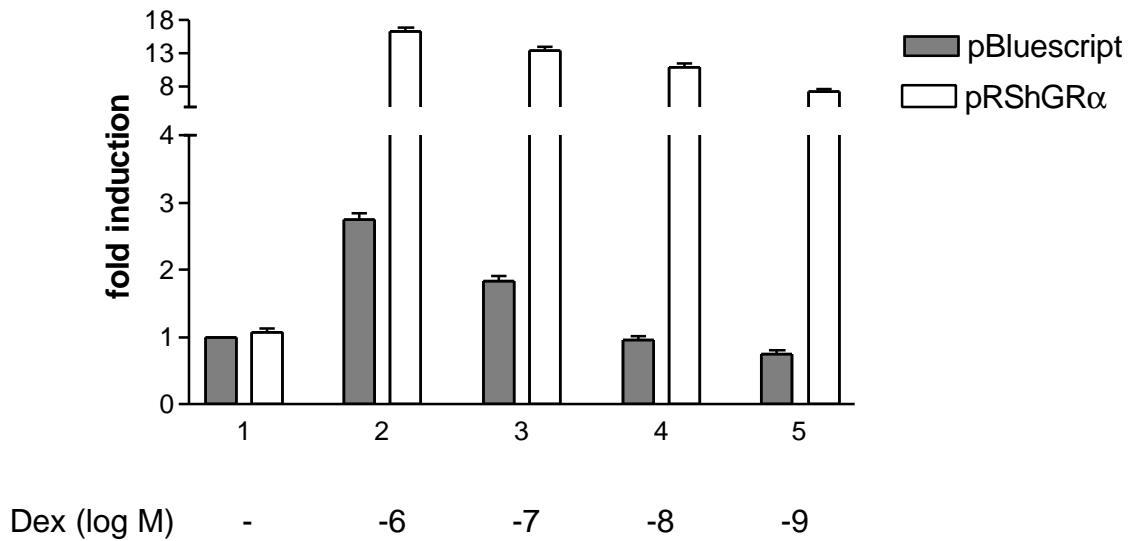


Figure 18. Effect of dexamethasone on transactivation in Caco-2 cells overexpressing glucocorticoid receptor.

Caco-2 cells were cotransfected with pRShGR α overexpression vector (1 μ g/well) and pGRE-SEAP (200 ng/well) then incubated with decreasing concentrations (10^{-6} M - 10^{-9} M) of dexamethasone for 24 hours. In controls DNA amount was equilibrated by pBluescript vector (1 μ g/well).

Reporter gene activities are shown as compared to the level of SEAP activity of the control group transfected with pBluescript and treated with ethanol as vehicle substance.

Relative SEAP activities are presented as means of fold induction over controls \pm SEM for n=3. The experiments were performed in duplicate.

Coincubation with IL-1 β (500 U/ml) and Dex (1 μ M) could repress the transactivating effect in receptor overexpressing Caco-2 compared to cells treated with 1 μ M Dex alone, but the decrease was not as dramatic as in cells containing the normal receptor level. In cells with basal GR content 20 % \pm 9,5 % reporter gene activity was observed after coincubation with Dex and IL-1 β , in GR overexpressing cells 75 % \pm 2 % activity was detected compared to cell treated with Dex. IL-1 β alone had no effect on GR mediated transactivation. (Figure 19.).

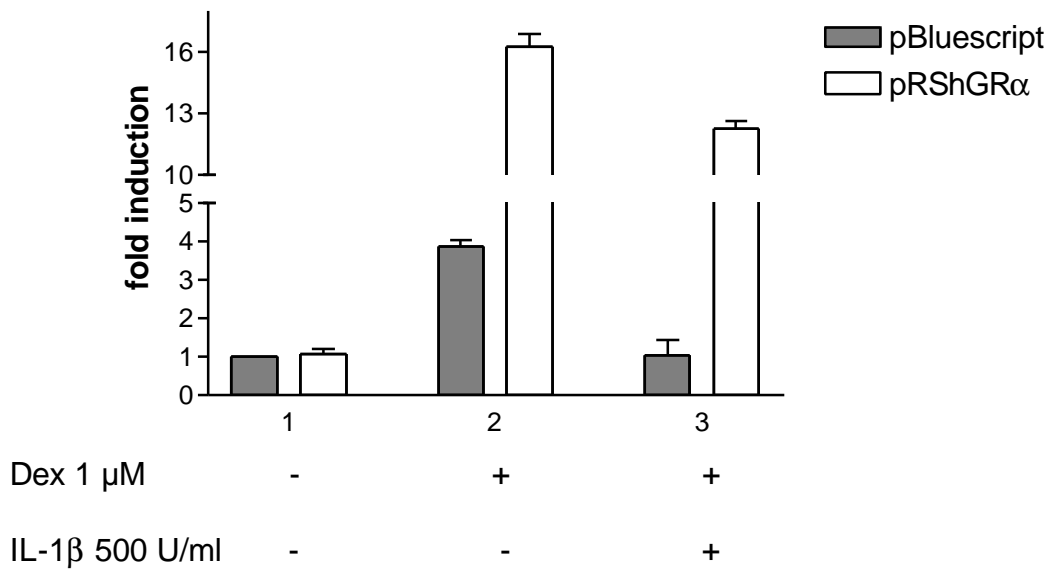


Figure 19. Effect of IL-1 β on dexamethasone induced, GR mediated reporter gene activity in GR overexpressing Caco-2 cells.

Caco-2 cells were cotransfected with pRShGR α (1 μ g/well) and pGRE-SEAP (200 ng/well), then incubated with 1 μ M dexamethasone for 24 hours in presence or absence of 500 U/ml concentration of IL-1 β . DNA amount was equilibrated by pBluescript vector (1 μ g/well). Reporter gene activities were compared to the control. Relative SEAP activities are demonstrated as means of fold induction \pm SEM for $n=3$, the experiments were performed in duplicate.

The transrepressive regulatory effect of Dex also could be restored by GR overexpression.

Caco-2 cells were cotransfected with pRShGR α (1 μ g/well) and pNF κ B-SEAP (200 ng/well) then incubated with IL-1 β (500 U/ml) for 6 hours.

We found that spontaneous NF κ B mediated reporter gene activity was reduced to 60% \pm 3 % in GR-overexpressing cells compared to controls transfected with pBluescript. Incubation with 1 μ M dexamethasone resulted in a further decrease (44 % \pm 4 %).

500 U/ml IL-1 β stimulated the reporter gene activity. In Caco-2 cells transfected with pBluescript dexamethasone could not repress the IL-1 β mediated NF- κ B activity at all, but in GR overexpressing cells Dex reduced NF- κ B activity compared to cells treated with IL-1 β alone (Figure 20.).

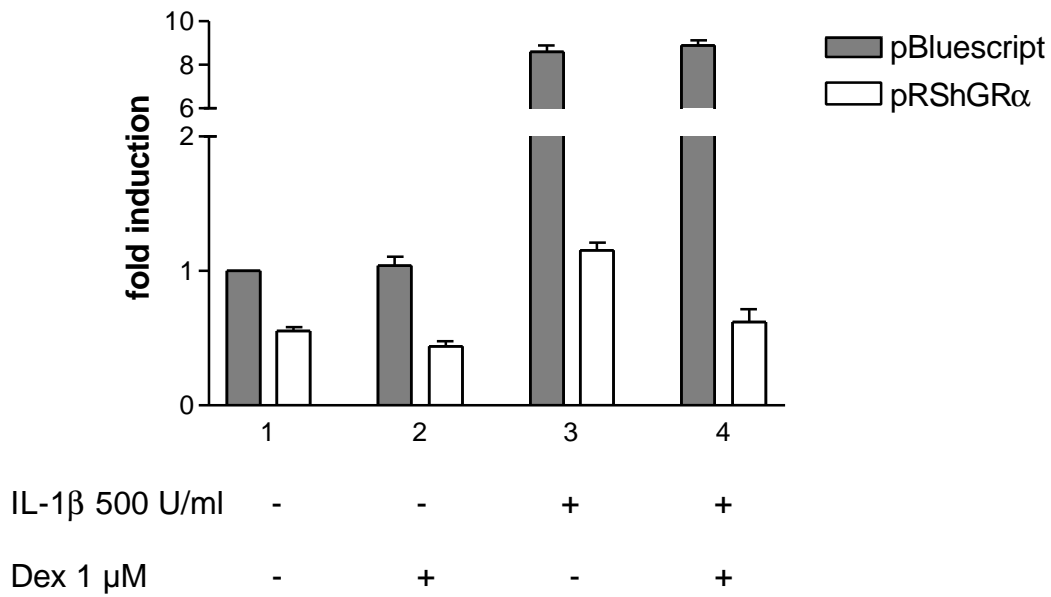


Figure 20. Effect of dexamethasone on IL-1 β induced, NF- κ B mediated SEAP activity in GR overexpressing Caco-2 cells.

Caco-2 cells were cotransfected with pRShGR α (1 μ g/well) and pNF- κ B-SEAP (200 ng/well) and coincubated with 1 μ M Dex and 500 U/ml concentration of IL-1 β for 6 hours. In control groups DNA amount was equilibrated by pBluescript vector (1 μ g/well). Reporter gene activities were compared to the activity of control cells transfected with pBluescript. Relative reporter gene activities are shown as means of fold induction \pm SEM for n=3. The experiments were performed in duplicate.

4.10 NF- κ B is not responsible for the impairment of glucocorticoid receptor action in Caco-2

As a direct protein-protein interaction between liganded hGR α and active NF- κ B was observed in HeLa cells (Scheinman, 1995), our conception was that this physical connection can be involved in the impairment of GR action in Caco-2. Under normal conditions glucocorticoid receptors can inhibit the NF- κ B activity via protein-protein binding, but in presence of a high amount of transcriptionally active NF- κ B all the glucocorticoid receptors may be blocked.

To test the hypothesis that NF- κ B really plays a role in this process, Caco-2 cells were transfected with the pCMV-I κ B α M vector. The plasmid construct encodes a mutant form of I κ B α with a serine-to-alanine mutation at residues 32 and 36. This variant is not capable to dissociate from NF- κ B-I κ B complex so that in cells overexpressing the mutant I κ B form the NF κ B-pathway can be blocked.

Caco-2 cells were cotransfected with pCMV-I κ B α M (200 ng/well) and pNF- κ B-SEAP (200 ng/well) then incubated with 500 U/ml dose of IL-1 β . Figure 21. demonstrates that in presence of pCMV-I κ B α M IL-1 β could not increase the NF- κ B mediated reporter gene activity at all showing, that the NF- κ B-pathway is successfully blocked.

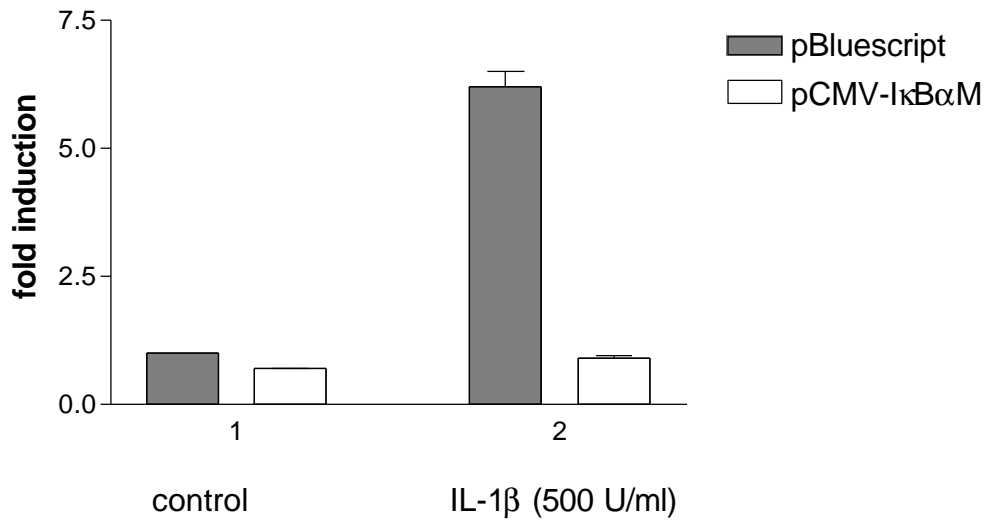


Figure 21. IL-1 β induced, NF- κ B mediated reporter gene activity in I κ B α overexpressing Caco-2 cells.

Caco-2 cells were cotransfected with pCMV-I κ B α M (200 ng/well) and pNF- κ B-SEAP (200 ng/well), then incubated with 500 U/ml of IL-1 β for 6 hours. In control groups DNA amount was equilibrated by pBluescript vector (200 ng/well). Reporter gene activities were compared to the activity of control cells transfected with pBluescript. Relative reporter gene activities are presented as means of fold induction \pm SEM for $n=3$, the experiments were performed in duplicate.

To answer the question if glucocorticoid receptor action can be restored by blocking the NF- κ B activity via I κ B overexpression, Caco-2 cells were cotransfected with pCMV-I κ B α M and pGRE-SEAP then incubated with 1 μ M dexamethasone in presence or absence of IL-1 β (500 U/ml).

As it is shown in Figure 22., the glucocorticoid receptor action was not restored by blocking the NF- κ B-pathway in Caco-2 cells since I κ B overexpression was not capable of reversing the Dex induced transactivation disturbed by IL-1 β .

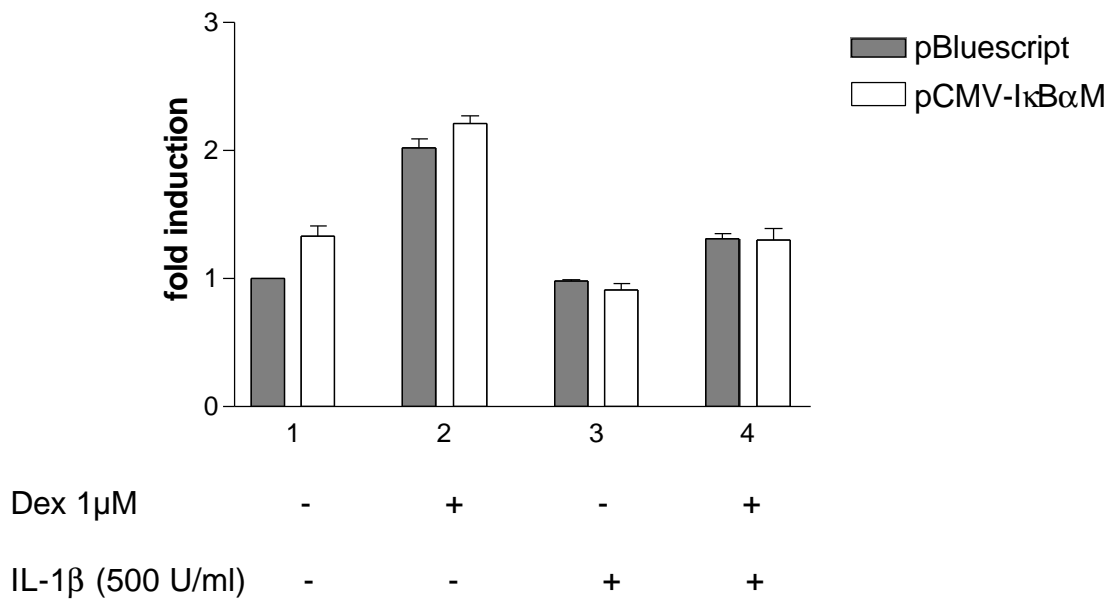


Figure 22. Dexamethasone induced, GR mediated transactivation in presence of IL-1b in IκBα-overexpressing Caco-2 cells.

Caco-2 cells were cotransfected with pCMV-IκBαM (200 ng/well) and pNF-κB-SEAP (200 ng/well) and incubated with 1 μM Dex in presence or absence of IL-1b (500 U/ml) for 24 hours. In control groups DNA amount was equilibrated by pBluescript vector (200 ng/well). Reporter gene activities were compared to the activity of control cells transfected with pBluescript. Relative reporter gene activities are presented as means of fold induction ± SEM for n=2, the experiments were performed in duplicate.

5. DISCUSSION

In the present study GR signalling and the effect of IL-1 β on glucocorticoid receptor function was investigated in two different intestinal epithelial cell lines: in human colonic adenocarcinoma cell line Caco-2, and rat intestinal cell line IEC-6.

Caco-2 was previously described to possess characteristics of normal small intestinal epithelium (Jumarie, 1991); in vitro these cells express a similar array of cytokines as cultured primary epithelial cells do (Jung, 1995).

For these reasons the cell line Caco-2 supposed be a well suited model system to investigate the molecular mechanisms of glucocorticoid action within the intestinal epithelium in human.

IEC-6 is an adult rat small intestinal epithelial cell line. It is widely used model for normal crypt cells in cultures. Rat cell lines (IEC-6, IEC-17, IEC-18) are frequently chosen instead of primary crypt cells because the survival time of isolated primary cells is short (about three days) (Fonty, 1993; Quaroni, 1999).

The glucocorticoid receptor is an ubiquitously expressed transcription factor. Its distribution in the colon is well characterised in rat model (Whorwood, 1993) and there is functional evidence for the presence of the receptor in the human intestine since glucocorticoids increase sodium absorption in normal and inflamed mucosa (Sandle, 1986).

In the present study we could show by Western blot analysis that glucocorticoid receptor protein is expressed in both Caco-2 and IEC-6 cells.

We found that in Caco-2 cells the majority of glucocorticoid receptors reside in the nucleus, in case of IEC-6 we detected GR mostly in the cytoplasm.

Immunostaining experiments confirmed these findings and, moreover, showed that dexamethasone treatment resulted in GR translocation from the cytoplasm to the nucleus in IEC-6 but not in Caco-2 cells. In the human cell line Dex had no effect on the pattern of glucocorticoid receptor staining.

The latter result coincides with the observation of LaFond et al. that both liganded and nonliganded forms of GR can be present only in the nucleus (LaFond, 1988). Raddatz et al came to the same conclusion examining human hepatoma cell lines HepG2 and Hep3B (Raddatz, 1996), Brink et al. observed nuclear localisation of the receptor both in presence and absence of hormone in rat hepatoma cells (Brink, 1992).

Although the unliganded form of GR has been reported to be cytoplasmic, many data suggest that its distribution is not unique, it can be cell line specific.

By transfection experiments both transactivation and transrepression by dexamethasone could be analysed. Using a GR responsive element carrying reporter gene construct (pGRE-SEAP) the transactivation, by using a NF- κ B responsive element carrying vector (pNF- κ B-SEAP) the transrepression could be observed.

We found that in both cell lines treatment with dexamethasone resulted in a dose dependent transcriptional activation of the reporter gene SEAP, and that this effect could be antagonised by the specific GR antagonist RU 486. These findings demonstrate that glucocorticoid receptor is functionally intact in Caco-2 and IEC-6 cells.

Numerous genes are known to be NF- κ B regulated, e.g. IL-1 β , IL-2, TNF α , IL-8, MCP-1.

It has been described that glucocorticoids can reduce the expression of some inflammatory mediators like TNF α or IL-8 in human peripheral blood mononuclear cells (Mukaida, 1989). Neurath et al. found that inhibition of NF- κ B activity using antisense oligonucleotide against NF- κ B mRNA improved experimental colitis in mice (Neurath, 1996), Rogler et al. reported that NF- κ B is activated in intestinal epithelial cells and macrophages in biopsy specimens taken from IBD patients (Rogler, 1998).

These experiences show that NF- κ B is involved in the process of inflammatory bowel disease. Moreover Su et al. have described a possible new, alternative treatment for patients suffering from IBD. Their concept is that NF- κ B can be inhibited by peroxisome proliferator-activated receptor- γ ligands as a new class of antiinflammatory substances (Su, 1999).

To monitor the transrepression of NF- κ B by dexamethasone, the cell lines Caco-2 and IEC-6 were transfected with NF- κ B responsive element containing construct.

We found that IL-1 β really caused increased reporter gene activity concentration dependently in both cell lines but – surprisingly – Dex could reduce it only in the rat cells.

There are several reports describing altered GR expression and function in inflammatory processes in vivo, like asthma (Spahn, 1995) or rheumatoid arthritis (DiBattista, 1993). Leung et al. observed that cytokines may modulate the glucocorticoid sensitivity in glucocorticoid resistant asthma (Leung, 1998).

Accordingly we hypothesised that IL-1 β itself can have an influence on the glucocorticoid receptor action. To answer this question we performed transfection experiments with pGRE-SEAP and examined the activating effect of Dex in presence of IL-1 β .

We found that treatment with IL-1 β led to significant reduction in Dex induced GR mediated gene transcription in case of both cell lines.

These results were confirmed by immunostaining experiments. By this method the shuttling of glucocorticoid receptor could be examined only in IEC-6 cells since in case of Caco-2 we could not detect any apparent change regarding receptor localization upon stimulation by glucocorticoids. Anyway, in Dex-treated IEC-6 cells we found GR translocation from the cytoplasm to the nucleus, but in presence of IL-1 β the shuttling of GR could not be observed.

Simultaneous with our study Pariante et al. found that in the mouse fibroblast cell line, L929 the proinflammatory cytokine, IL-1 α reduced the glucocorticoid receptor translocation and function and caused its up-regulation. (Pariante, 1999).

In contrast to Pariante's observation, we did not find change in the receptor number in neither of our cell lines.

Anyway, the effect of cytokine treatment on GR quantity is not clear, some researchers found down-regulation, e.g. human recombinant IL-1 caused reduced receptor number in Reuber hepatoma cells (Hill, 1988), others observed up-regulation: Costas et al. described that TNF α increased GR number in L-929 mouse fibroblasts (Costas, 1996). This process seems to be cell line and cytokine specific.

The inefficacy of dexamethasone on inhibition of TNF α mRNA expression in Caco-2 cells can be a further evidence for the impaired glucocorticoid receptor action regarding transrepression. We found that IL-1 β can stimulate TNF α mRNA expression in both cell lines. We showed that dexamethasone could inhibit the IL-1 β induced TNF α mRNA expression only in IEC-6 cells, in Caco-2 Dex was inefficient.

We found that IEC-6 cells contain significant more GR binding sites than Caco-2. The GR content in Caco-2 is relatively low compared to other human epithelial cell lines e.g. HeLa cells (Scheinman, 1995). Most certainly in Caco-2 cells the relative lack of glucocorticoid receptors play a role in the inefficacy of dexamethasone to inhibit TNF α expression and NF- κ B activation.

The glucocorticoid receptor quantity could be effectively increased by glucocorticoid receptor overexpression. Thereafter we could present increased reporter gene activity after Dex treatment in pGRE-SEAP transfected, GR overexpressing cells. This dramatically enhanced reporter gene activity could be observed only in Dex-treated cells, indicating that the transactivation is strictly hormone dependent.

An effective inhibition by dexamethasone was found regarding IL-1 β induced, NF- κ B mediated SEAP activity in GR-overexpressing cells. Interestingly, unstimulated glucocorticoid receptor was also capable of inhibiting NF- κ B, suggesting, that the transrepressive effect of GR is not completely hormone dependent.

Similar observations were described by Inoue et al. They found that in bovine arterial endothelial cells Dex could not inhibit LPS stimulated prostaglandin E synthesis and LPS induced promoter activity of the Cox-2 gene. Transfection with GR expression vector could restore the inhibitory effect of dexamethasone on the promoter activity of Cox-2 (Inoue, 1999).

These findings provide a cellular model for the process of glucocorticoid resistance. Our data suggest that IL-1 β does not only counteract the inhibitory effect of dexamethasone on NF- κ B activity, but also represses the GR mediated transactivation and the translocation of the receptor in Caco-2 cells.

A further question was how IL-1 β could impair the glucocorticoid receptor action. One possible explanation may be a protein-protein interaction between GR and other transcription factors. A cross-talk has been described between GR and NF- κ B in Hela cells by Scheinman et al. (Scheinman, 1995) and in the monkey kidney cell line, COS-1 by McKay and Cidlowski (McKay, 1998).

Concerning Caco-2 we hypothesised that a physical interaction between these two transcription factors can cause the impairment of the GR action: if a high amount of IL-1 β stimulated NF- κ B molecules are active, many GRs can be trapped, and prevents glucocorticoid receptor from binding glucocorticoid receptor responsive elements. The intracellularly available receptor content can be a limiting factor for NF- κ B inhibition.

Another possible interaction between NF- κ B and GR was reported by Sheppard et al. They found that the p65 component of NF- κ B and the glucocorticoid receptor mutually repress each other's ability to activate transcription: the maximal activity of both these transcription factors depends on availability of the coactivators CREB-binding protein and steroid receptor coactivator 1 (SRC-1). The cross-talk between p65 and GR was supposed to be due to a nuclear competition for limiting amounts of the same coactivators (Sheppard, 1998).

To answer the question if NF- κ B is the candidate transcription factor, that blocks GR action we examined the effect of dexamethasone on Caco-2 cells in which NF- κ B pathway was blocked by overexpression of a mutant form of I κ B, that irreversibly blocks NF- κ B.

Although we could show that in fact NF- κ B activity was blocked, we found that glucocorticoid receptor action could not be restored by this way in Caco-2.

This finding suggests that in Caco-2 cells NF- κ B is not responsible for the negative effects of IL-1 β on GR mediated transactivation.

The signalling cascade of IL-1 β involves numerous transcription factors, which can be candidate for impairing glucocorticoid receptor action either by protein-protein interaction or competition for cofactors.

The function of these transcription factors is modulated via activation of protein kinase cascades by IL-1 β . One of these cascades involves a NF- κ B inducing kinase. The activation of this kinase leads to phosphorylation of I κ B, degradation of NF- κ B/I κ B complex and activation of NF- κ B. The signalling cascade also involves three distinct mitogen-activated kinase cascades, which modulate a number of other transcription factors. The activating transcription factor-2 (ATF-2) and c-Jun, which is one of the components of activating protein 1 (AP-1) is activated by the c-Jun-N-terminal or stress-activated protein kinase. This pathway is activated in all responsive cells in tissue in vivo. Cyclic AMP response element binding protein (CREB) is also activated by MAP kinase. Furthermore, IL-1 β activates the transcription factor NF-IL6, which is one of the main factors for the transcriptional activation of IL-6 and IL-8 genes (Stylianou, 1998; O'Neill, 1995).

Although the interaction between most of these transcription factors and glucocorticoid receptor action is unclear, protein-protein interaction has been described between GR and c-Jun (Yang-Yen, 1990). So that AP-1 may be involved in the inhibition of GR pathway. Experiments using other dominant negative expression vectors or antisense oligos are required to answer this question in the future.

Furthermore, it has been reported that cytokines may act on heat shock proteins, which are one of the components of the glucocorticoid receptor complex. D'Souza et al. described that interleukin 1 induced heat shock protein expression in human oligodendrocytes (D'Souza, 1994). IL-1 β -induced overexpression of heat shock proteins may keep glucocorticoid receptor anchored in the cytoplasm and inhibit its translocation.

The intracellular cAMP level is an important regulator of glucocorticoid responsiveness. Rangarajan et al. described that both adenylyl cyclase and protein kinase A activators have been found to influence glucocorticoid receptor function (Rangarajan, 1992). PKA up-regulated GR expression and enhanced the affinity of glucocorticoids to GR in F9 embryonal carcinoma cells. Dong et al. de-

scribed that cAMP enlarged the amount of GR by increasing the stability of its mRNA in rat hepatoma cells. (Dong, 1989). However, regulating the glucocorticoid sensitivity of the cell, defective cAMP-dependent protein kinase may be involved in the process of glucocorticoid resistance.

The 11 β -hydroxysteroid dehydrogenase (OHSD) is another important factor in the regulation of glucocorticoid efficacy. This enzyme protects the non-selective mineralocorticoid receptor from occupation by glucocorticoids by converting the active cortisone to inactive 11-dehydrocorticosterone and maintains a normal in vivo specificity of mineralocorticoids for their receptor. (Edwards, 1988; Funder, 1988). The enzyme was found to localize in the distal nephron, where mineralocorticoids stimulate the transepithelial sodium transport (Stanton, 1986) and significant levels of OHSD have been reported in the human rectum (Burton, 1983). Moreover, Whorwood et al. described its expression and activity in the rat distal colon, where adrenocortical steroids also regulate the sodium transport (Whorwood, 1993). Although there is clinical evidence only for compromised OHSD-function (Stewart, 1989, Ulick, 1979), increased enzyme activity can modify the efficacy of glucocorticoid drugs in IBD patients. Therefore the question if cytokines like IL-1 β may up-regulate the activity of OHSD should be investigated in the future.

In conclusion our data suggest that the proinflammatory cytokine IL-1 β , which is produced during intestinal inflammation, induces glucocorticoid resistance in the epithelial cell lines Caco-2 and IEC-6. The mechanism of its inhibitory effect is unclear, NF- κ B is not involved in the process in Caco-2 cells on cellular level. The glucocorticoid receptor quantity of the cells is essential in the efficacy of glucocorticoids. New, alternative treatment forms of the inflammatory bowel disease may include gene therapy strategies utilizing glucocorticoid receptor overexpression.

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