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Chapter 7

Molecular Mechanisms Conferring Resistance/Sensitivity to Glucocorticoid-Induced Apoptosis

Ilhem Berrou, Marija Krstic-Demonacos and Constantinos Demonacos

Additional information is available at the end of the chapter

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1. Introduction

1.1. Glucocorticoids therapeutic effects

Synthetic glucocorticoids (GCs) as effective anti-inflammatory therapeutics are the most widely prescribed drugs in the clinic for the treatment of various conditions including asthma, ulcerative colitis, rheumatoid arthritis and hay fever [1-4]. Glucocorticoids in some cases exert effective anti-neoplastic effects in cancers of blood origin such as acute lymphocytic leukaemia (ALL) as a result of the ability of these hormones to induce cell death in blood cells [1-3]. Resistance to glucocorticoid mediated cell death remains one of the main reasons for inefficient therapy [1] and usually occurs upon prolonged glucocorticoid treatment [2] compromising significantly the success of therapy [3]. The molecular mechanisms mediating glucocorticoid dependent initiation of programmed cell death have been extensively investigated but there are several aspects of these pathways that have not yet been clearly defined. Since understanding of these mechanisms would be beneficial towards improving the glucocorticoids therapeutic efficacy further research exemplifying resistance to glucocrticoid treatment is necessary.

Glucocorticoids (GCs) exert their anti-inflammatory and immunosuppressive effects through either genomic or non-genomic mechanisms. Non-genomic early effects of glucocorticoids are induced in tissues bearing high concentrations of this hormone by interfering with the physicochemical properties of plasma and mitochondrial membranes [4]. In particular, glucocorticoids intercalate into these membranes altering lipid peroxidation and membrane permeability [5]. In addition, non-genomic effects of glucocorticoids include early suppression of the mitogen-activated protein kinase (MAPK) and hence inflammatory signal transduction cascades such as calcium influx, phagocytosis,

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neutrophil degranulation and cellular adhesion [6, 7, 8, 9, 10, 11]. The non genomic effects of GCs are very important in delivering short-term therapeutic benefits to asthma and rheumatoid arthritis which are diseases characterized by high inflammatory state [12].

2. Mechanisms of GC-mediated cell death

At the molecular level GCs exert their function by interacting with their intracellular GC receptor (GR), which is a hormone responsive transcription factor that modulates gene expression of its target genes [13]. Glucocorticoids activate the cellular death machinery through transcriptional and non-transcriptional pathways by means of either the extrinsic or intrinsic pathway of apoptosis [3, 14, 15, 16].

The extrinsic pathway is induced upon activation of the membrane death receptors such as the tumour necrosis factor (TNF) receptor superfamily, member 6, Fas-Ligand (Fas-L) [17]. The binding of Fas-L leads to the activation of effector caspases (caspases 3, 6 and 7) via the activation of inducer caspases, particularly caspase 8 [18]. Evidence that glucocorticoids are involved in the regulation of the extrinsic pathway of apoptosis has been provided by observations suggesting that glucocorticoids inhibit the induction of Fas-L (but not Fas) signalling in T-cell hybridomas [19, 20]. On the contrary inhibition of the extrinsic pathway using the caspase 8 inhibitor cytokine response modifier A (crmA) in pre-B leukemic cells treated with glucocorticoids indicated that GR does not initiate apoptosis in these cells through the extrinsic pathway [21, 22]. The involvement of the intrinsic pathway, on the other hand, in the glucocorticoid mediated cell death and in particular the regulation of the balance between pro- and anti-apoptotic members of the bcl-2 family has been shown in hepatocytes, small cell lung cancer, primary ALL lymphoblasts and animal systems [23, 24].

The intrinsic pathway of cell death is stimulated in response to intracellular signals, and involves mitochondria releasing pro-apoptotic molecules, formation of the apoptosome and activation of the effector caspases via the initiator caspase 9 [25]. The balance between pro- and anti-apoptotic members of the B cell leukaemia/ lymphoma 2-like (Bcl-2) family plays a crucial role in the execution of apoptosis by glucocorticoids through the intrinsic pathway [18]. In particular, glucocorticoids regulate the expression of various genes involved in the initiation of apoptosis, including the pro-apoptotic Bcl-2 family member BCL2-like 11 (Bim) [22, 26, 27, 28]. Transactivation of Bim results in the activation of the Bcl-2–associated X protein (Bax) and the Bcl2-antagonist/killer 1 (Bak), which mediate the disruption of mitochondrial membrane potential and the release of cytochrome c into the cytosol [29]. Cytochrome c then binds to its adaptor apoptotic protease activating factor (Apaf-1), thereby activating caspase 9 and several effector caspases [30]. Furthermore, mitochondria mediate the generation of reactive oxygen species (ROS), which may potentially have an add-on effect on glucocorticoid-induced apoptosis [31].

In some cases glucocorticoids induce apoptosis by using both the intrinsic and the extrinsic pathways. This is mediated by the cleaved caspase-8 which subsequently leads to activation of the pro-apoptotic member of the Bcl-2 family, Bcl-2 homology 3 (BH3) interacting domain death agonist (Bid) [32]. The C-terminal truncated Bid (t-Bid) activates caspase 9 and the

effector caspases 3, 6 and 7 [33]. The intrinsic and extrinsic pathways through which glucocorticoids induce apoptosis in cells responsive to these hormones are illustrated in Figure 1.

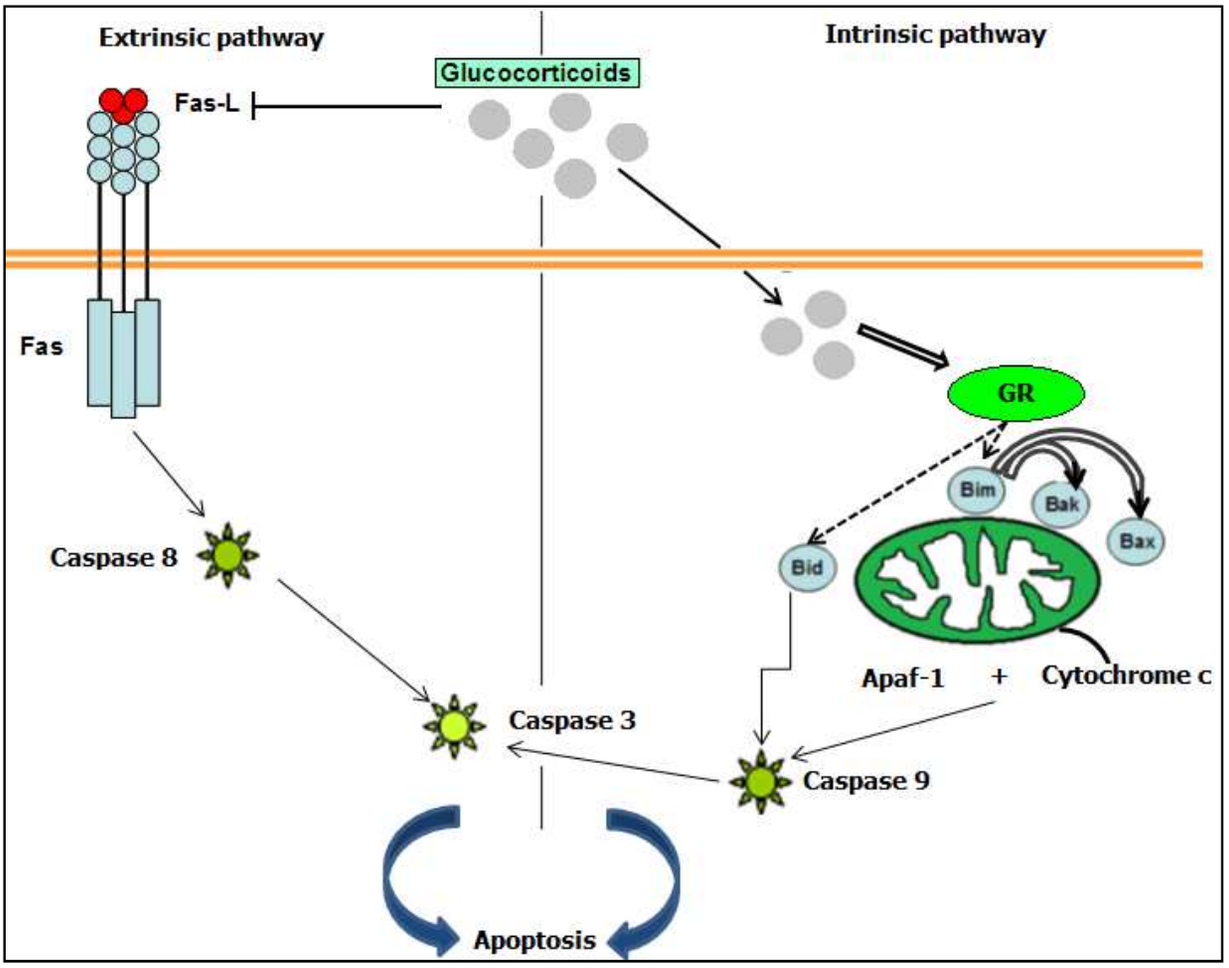

Figure 1. Schematic diagram indicating the extrinsic and intrinsic pathways through which glucocorticoids regulate apoptosis.

Several mechanisms have been proposed to explain the evasion of glucocorticoid mediated apoptosis in resistant cells [34]. These include alterations in the activity of the glucocorticoid receptor [35, 36] either due to changes in GR protein levels, presence of multiple GR variants, or post-translational modifications. GR induces apoptosis by directly modulating the expression of genes involved in cell survival/apoptosis [18, 37], or affecting gene networks involved in stress signalling resulting in an apoptotic stimulus [36, 38].

3. GR transcriptional activity is necessary for its pro-apoptotic function

Glucocorticoid induced apoptosis depends on the presence of adequate amounts of transcriptionally active GR [34, 39, 40, 41, 42]. It has also been shown that the presence of specific GR splicing variants is necessary for the stimulation of GCs dependent apoptosis [3]. Alternative hGR splicing produces two receptor variants called GRα and GRβ, which

are highly homologous differing by 50 additional amino acids present in the carboxy terminal region of GRα [43]. GRα is mainly cytoplasmic, and exhibits the typical glucocorticoid receptor function in terms of ligand-dependent transcriptional regulation and as such it is the major functional GR isoform therefore its expression is crucial for cellular sensitivity to GCs [3]. GRβ on the other hand, does not bind ligands, and exerts a dominant negative effect on GRα transcriptional activity [43, 44, 45]. In clinical studies reduced GRα protein levels correlate with resistance to GCs induced apoptosis and disadvantageous prognosis in ALL [3]. Additional indication that GR transcriptional activity is necessary for the initiation of the GCs mediated apoptosis has been provided by the observation that various mutations affecting GR transcriptional activity have been detected in patients exhibiting resistance to glucocorticoids treatment [2, 46]. Furthermore, prolonged glucocorticoid treatment significantly reduces GRα expression [47], whereas GRβ expression is not affected [48].

4. GR dependent regulation of the balance between pro- and anti-apoptotic Bcl-2 family members

B cell leukaemia/ lymphoma 2-like (Bcl-2) family members are categorised into pro- and anti-apoptotic and the balance between the levels of these two types of proteins determines the cellular fate (survival or death) [23]. The involvement of the Bcl-2 family members in the glucocorticoid-induced apoptosis has been shown in cellular and animal studies [49]. For example, the expression of the anti-apoptotic Bcl-2 family member in glucocorticoid-sensitive thymocytes is lower compared to that in glucocorticoid-resistant ones [2], and overexpression of the anti-apoptotic Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) in human ALL prevents glucocorticoids induced apoptosis [50, 51, 52, 53] whereas knock down of the pro-apoptotic member Bim confers resistance to dexamethasone mediated apoptosis [54]. Furthermore, over-expression of the pro-apoptotic Bax [55] and knock down of the myeloid cell leukemia sequence 1 (Mcl-1) sensitises ALL cells to glucocorticoid treatment [56]. We have shown recently that the balance between Mcl-1 and phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) is a determinant of resistance / sensitivity of ALL cells to glucocorticoid-induced apoptosis [57, 58]. Several studies have shown that the pro-apoptotic Bcl-2 family member Bim plays crucial role in the glucocorticoid-induced apoptosis but further investigation is required to define the detailed molecular mechanisms of this process. Up-regulated Bim has been observed in various cell lines upon glucocorticoid treatment including ALL cells [59], primary chronic lymphocytic leukaemia (CLL) cells [27], and some patients with ALL [60]. Moreover knockout of Bim, p53 up-regulated modulator of apoptosis (Puma) or Noxa, or double knockouts of Bax and Bak confer resistance to glucocorticoid-mediated apoptosis in thymocytes [61, 62, 63]. Overall the balance of the levels between pro- and anti-apoptotic Bcl-2 family members has been recognised as a crucial factor in the determination of lymphocytes survival or death and induction of the glucocorticoid dependent programmed cell death.

Gene	Protein	Function	Role in glucocorticoid-induced apoptosis
Bcl2	B-cell Lymphoma 2 (Bcl-2)	Anti-apoptotic	Over-expression of Bcl-2 in human ALL cells prevents glucocorticoids induced apoptosis [41, 64]
BCL-xL	B-cell lymphoma-extra large (Bcl-xL)	Anti-apoptotic	Over-expression of Bcl-xL in human ALL cells prevents glucocorticoids induced apoptosis [23]
BCL2L11	BCL2 Like 11 (Bim)	Pro-apoptotic	Knock down of Bim confers resistance of ALL cells to glucocorticoid-induced apoptosis [65]. Upregulation of Bim sensitizes cells to glucocorticoid-induced apoptosis [27, 66].
Mcl1	Myeloid cell leukemia 1 (Mcl-1)	Anti-apoptotic	Knock down of Mcl-1 sensitises ALL cells to glucocorticoids apoptotic effect [56].
Phorbol-12-myristate-13-acetate-induced protein 1	Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa)	Pro-apoptotic	Noxa regulates the Mcl-1 protein stability and Noxa/Mcl-1 balance determines cell survival or death [57].
p53 up-regulated modulator of apoptosis	p53 upregulated modulator of apoptosis (Puma)	Pro-apoptotic	Puma facilitates glucocorticoid-induced apoptosis of lymphocytes [62, 65].
Bax	Bcl-2–associated X (Bax)	Pro-apoptotic	Bax protein regulates glucocorticoid induced apoptosis in thymocytes [67]. Double knockouts of Bax and Bak confer resistance to glucocorticoid-induced apoptosis in thymocytes [68].
Bak	Bcl-2 homologous antagonist/killer (Bak)	Pro-apoptotic	Double knockouts of Bax and Bak confer resistance to glucocorticoid-mediated apoptosis in thymocytes [68].

Table 1. Pro- and anti-apoptotic Bcl-2 family members implicated in the glucocorticoids induced apoptosis

5. Autophagy

Autophagy is a several steps process leading to cellular degradation of unfolded or aggregated proteins and organelles in response to diverse types of stress such as starvation or metabolic stress and is an essential mechanism contributing to survival, differentiation, development, and homeostasis. Autophagy protects against chronic inflammatory conditions occurring in a variety of pathological situations such as infections, cardiovascular disease, neurodegenaration, inflammatory bowel diseases, aging and cancer [69]. Autophagy has been shown to be involved in prosurvival processes facilitating resistance of cancer cells to chemotherapy as well as under certain conditions in apoptosis [70]. Taking into account the fact that autophagy is prosurvival in addition to reports indicating that

inhibitors of autophagy re-sensitise cancer cells to anticancer therapeutics [70, 71] as well as that autophagy is associated with inflammation and resistance to cancer therapy investigators were prompted to study the role of autophagy in the resistance to glucocorticoid induced apoptosis in ALL [72, 73]. These studies have indicated that dexamethasone induces autophagy before the initiation of apoptosis in ALL cells and in actual fact autophagy is a prerequisite for the efficient execution of apoptosis mediated by dexamethasone [72, 73]. More recently the molecular mechanism by which activation of autophagy overturns glucocorticoid resistance has been elucidated [74] signifying the important role of the autophagy inducer beclin-1 and the anti-apoptotic Bcl-2 family member Mcl-1 [74, 75]. Furthermore the inhibition of caspase by selective degradation of catalase and consequent generation of high concentrations of reactive oxygen species might be an alternative mechanism explaining the role of autophagy in dexamethasone induced apoptosis [76].

6. Post-translational modifications

Post-translational modifications of GR are important in regulating its transcriptional activity, protein stability, binding of GR with other transcription factors or co-modulators and subcellular localisation, and for these reasons post-translational modifications are highly relevant to glucocorticoids therapeutic efficacy [77, 78, 79, 80, 81]. GR acetylation is cell type dependent and it is suggested to suppress the receptor's transcriptional activity by reducing its ability to bind to the Glucocorticoid Responsive Elements (GREs) present in its target genes [82], or inhibit the ability of GR to translocate into the nucleus [83]. Regulation of GR transcriptional activity also takes place through ubiquitination and proteasomal degradation of the receptor upon ligand binding [84, 85]. Ubiquitination promotes interaction of GR with E2 conjugating and E3 ligase proteins causing turnover of the receptor and thus down-regulation of its transcriptional activity [85]. In support of these conclusions the proteasome inhibitor MG-132 enhances the transcriptional activity of GR [84]. Ligand-independent sumoylation has been shown to both inhibit [86, 87] as well as to stabilise and potentiate GR transcriptional activity [88]. Three sumoylation sites have been identified within the GR protein conferring GR transcriptional target selectivity [87]. We have recently reported that GR sumoylation is assisted by its phosphorylation at particular sites under certain conditions [89].

GR phosphorylation has been extensively investigated and several different kinases have been identified to induce phosphorylation of the receptor at distinct serine and threonine residues located within the N-terminal AF-1 transactivation domain of the receptor, either in the presence or in the absence of glucocorticoid hormone [90, 91, 92, 93, 94, 95]. S203 residue in human GR is targeted by cyclin/cyclin dependent kinase (CDK) complexes and is located mostly in the cytoplasm thus it is thought to be transcriptionally inactive [89, 95]. GR phosphorylated at S211 is transcriptionally active due to conformational changes which facilitate increased recruitment of the receptor to GRE-containing promoters and is a target for phosphorylation by both cyclin/CDK kinases and MAPK families depending on the cell

type [91, 96]. GR phosphorylation at S226 results in inhibition of the GR function, possibly due to increased GR nuclear export and is a result of JNK activation [80, 89, 97, 98]. Finally, phosphorylation of GR at S404 attenuates GR signalling and is due to GSK3 kinase activation [99]. We have recently reported that differential GR phosphorylation in the resistant CEM-C1-15 versus sensitive CEM-C7-14 ALL cells modulates GR transcriptional activity and target selectivity resulting in diverse pro- or anti-apoptotic Bcl-2 family members' gene expression in the two cell lines [58]. In particular we have shown that GR phosphorylation at S211 is predominant in the glucocorticoid-sensitive CEM-C7-14 whereas GR phosphorylation at S226 by c-Jun N-terminal Kinase (JNK) occurs more frequently in the glucocorticoid-resistant CEM-C1-15 cells [58]. These observations lend support to the suggestion that different kinase pathways are responsible for GR phosphorylation in resistant versus sensitive cells to glucocorticoid induced apoptosis, thereby causing GR transcriptional inactivity in the resistant cell lines [58] concomitant Mcl-1 overexpression and hence resistance to GC treatment [56].

Apart from the differential expression of pro- and anti-apoptotic Bcl-2 family members [58] we have recently reported that GR isoforms localised in mitochondria are predominantly phosphorylated at serine 232 compared to serine 246 of the rat GR (corresponding to human GR Ser211 and Ser226 respectively) [96] possibly due to differential conformation of the two phosphoisoforms or diverse interaction patterns with components of the mitochondrial import machinery of the GR phosphorylated at serine 211 versus the GR phosphorylated at serine 226. These observations provide an additional potential explanation for the resistance of the CEM-C1-15 to glucocorticoids induced apoptosis in accord with recent reports indicating differential GR mitochondrial localisation in resistant compared to sensitive ALL cells to glucocorticoid induced apoptosis [93, 94, 95, 97].

7. Mitochondrial GR, glucocorticoids and cellular energy metabolism

Glucocorticoid hormones directly affect mitochondrial membranes inducing loss of mitochondrial transmembrane potential, thereby affecting vital cellular processes mediated by the function of this organelle such as ATP generation via oxidative phosphorylation, regulation of calcium flux and apoptosis [98]. In addition, glucocorticoids exert their effects on mitochondrial biogenesis through the mitochondrial GR [97, 99, 100, 101]. The intracellular trafficking of the glucocorticoid receptor has been shown to play important role in glucocorticoid-induced apoptosis [97, 102]. The glucocorticoid receptor translocates to mitochondria [97, 100, 101, 102], in various cells including rat brain and various other tissues' mitochondria [99], as well as lymphoma cells [97]. GR translocation to the nucleus occurs in both glucocorticoid-sensitive and glucocorticoid-resistant cells, whereas in contrast, GR translocation into mitochondria occurs only in the glucocorticoid-sensitive and not the resistant cells [97, 103]. The mechanisms by which GR translocates to mitochondria and its effects on the regulation of the expression of mitochondrially encoded genes have been partially elucidated [97, 100, 101, 102] and require further investigation. However, it is noteworthy that glucocorticoids modulate mitochondrial biogenesis and mitochondrial

energy production pathways by regulating the transcription of the mitochondrial genome [99, 100].

The process of programmed cell death is energy dependent requiring the precise coordination of several biochemical processes including the transcriptional regulation of the expression of genes encoding enzymatic components of the energy production pathways (oxidative phosphorylation (OXPHOS) and glycolysis) [29] and mutations affecting cellular energy production lead to defects in apoptosis and tumourigenesis [104, 105, 106, 107, 108, 109]. In humans the OXPHOS pathway consists of five multi-subunit complexes whose components are encoded by genes located in both the nuclear and the mitochondrial genomes [110] supporting the notion that a transcription factor operating in both subcellular compartments could coordinate the expression of nuclear and mitochondrial genes ensuring appropriate stoichiometry and timely gene expression of the components of the respiratory chain [111]. In addition to the availability of the components encoded in the nuclear and the mitochondrial compartments an appropriate assembly mechanism of these subunits is essential for the functionality of the oxidative phosphorylation system [110]. One possible candidate transcription factor able to orchestrate nuclear and mitochondrial gene expression is the glucocorticoid receptor which mediates gene expression of both nuclear and mitochondrial encoded genes [98, 99, 100, 102, 112, 113, 114, 115, 116].

In fact, dexamethasone has been shown to affect energy metabolism and the balance between OXPHOS and glycolysis [117, 118]. Also, evidence has been recently presented indicating that changes in metabolic patterns and cellular proliferation are key aspects of resistance to GC mediated apoptosis in ALL [39]. Elevated glycolytic rate due to increased expression of genes involved in glucose metabolism are associated with resistance to glucocorticoid induced apoptosis and this resistance can be reversed by inhibitors of glycolysis in ALL [119, 120]. Moreover, glucocorticoid induced apoptosis is regulated by genes involved in cellular energy metabolism [121, 122, 123, 124] suggesting that dexamethasone contributes to the apoptosis / survival decisions in ALL cells indirectly by modulating the balance between OXPHOS and glycolysis [15, 120]. Indeed coordination of oxidative phosphorylation and glycolysis by mechanisms involving glucocorticoid receptor mediated transcriptional regulation of genes encoding enzymes implicated in both pathways has been extensively reported in the literature [99, 125, 126]. Enzymes participating in the tricarboxylic acid cycle encoded by the nuclear genome such as malate dehydrogenase 1 (Mdh1) and succinyl coenzyme A synthetase (Suclg1) are GR transcriptional target genes [127]. In addition, several mitochondrial genes encoding subunits of the OXPHOS pathway possess one or more functional glucocorticoid responsive elements [99, 100, 102, 112, 113] implying that glucocorticoids exert direct effects on mitochondrial biogenesis and respiration. Key enzymes involved in glycolysis including 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase [126] lactate dehydrogenase B (LdhB) and aldolase A (AldoA) [128] are under GR transcriptional control [120].

To shed light on the molecular mechanisms involved in GR mediated regulation of the OXPHOS pathway bioinformatic analysis using the TRED or CCTFSP software [129, 130, 131] was performed to identify potential glucocorticoid responsive elements (GREs) in the promoters of genes encoding Surf-1, and SCO2 enzymes which are essential for the assembly of the Cytochrome *c* Oxidase (COX) wholoenzyme [132, 133, 134]. Similar approaches were used to detect possible existence of potential GREs in the regulatory region of the promoter of the nuclear gene encoding COX-Va. Putative GREs were identified in the regulatory regions of the promoters of Surf-1, SCO2, and COX-Va using this approach. To test whether these GREs conferred glucocorticoid responsiveness to the expression of these genes, qRT-PCR experiments were performed to quantify their mRNA levels in untreated or dexamethasone treated CEM-C1-15 (resistant to glucocorticoid induced apoptosis) and CEM-C7-14 (sensitive to glucocorticoid induced apoptosis) ALL cells [135] (Figure 2).

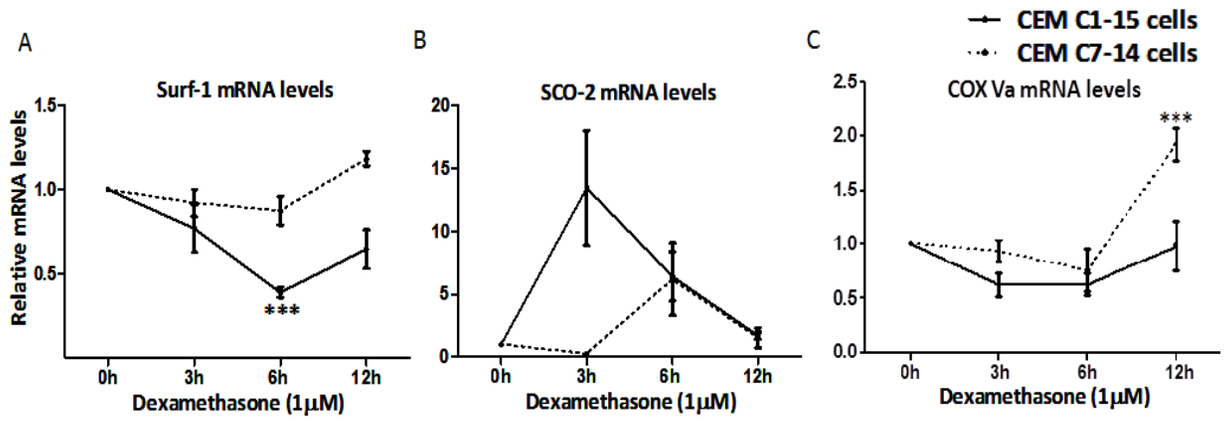

Figure 2. Relative mRNA levels of the COX assembly enzymes Surf-1 (A), SCO-2 (B), and the nuclear COX-Va (C) OXPHOS subunit were followed in the resistant CEM-C1-15 and the sensitive CEM-C7-14 to glucocorticoid induced apoptosis ALL cells treated with dexamethasone for the indicated time points by quantitative real-time PCR. Solid lines represent mRNA levels in CEM-C1-15 and dotted lines correspond to mRNA levels determined in CEM-C7-14 cells. Error bars represent standard error of the mean of five independent experiments and asterisks indicate statistical significance of $p<0.05$ compared to the untreated sample.

A marked reduction of Surf-1 mRNA levels in the resistant CEM-C1-15 cells was observed after 6 and 12 hours of dexamethasone treatment compared to the non-treated cells (Figure 2A, solid line). In contrast, in the sensitive to glucocorticoid-induced apoptosis CEM-C7-14 cells, Surf-1 mRNA levels remained constant during the first 6 hours of dexamethasone treatment and a moderate increase of Surf-1 mRNA levels was observed only 12h after the addition of the hormone (Figure 2A, dotted line). SCO-2 mRNA levels initially increased after 3h and 6h of dexamethasone treatment in CEM-C1-15 cells and later after 12h of dexamethasone treatment decreased to the level of that exhibited in the untreated cells (Figure 2B, solid line).

The fact that COX-Va expression is altered in various tumours and its association with Surf-1 in the formation of sub-complexes consisting of variable COX subunits such as

COX-I, COX-II, COX-III, and COX-IV [136] triggered our interest to investigate the regulation of the expression of the COX-Va gene. This investigation aimed to test whether differential COX-Va cellular levels in the resistant versus the sensitive CEM cells in response to glucocorticoid treatment was taking place in a similar way as that observed for Surf-1. COX-Va mRNA levels were higher in CEM-C7-14 compared to those detected in the CEM-C1-15 cells in all time points of dexamethasone treatment investigated in this study and the longer the incubation with the hormone the higher the COX-Va mRNA levels in the CEM-C7-14 cells (Figure 2C, compare dotted to solid line). Results shown in Figure 2A, 2B, and 2C support the notion that the Surf-1 and COX-Va genes encoded by the nuclear genome are under GR transcriptional control and their expression is higher in the sensitive to glucocorticoid induced apoptosis cells compared to the resistant CEM-C1-15 cells.

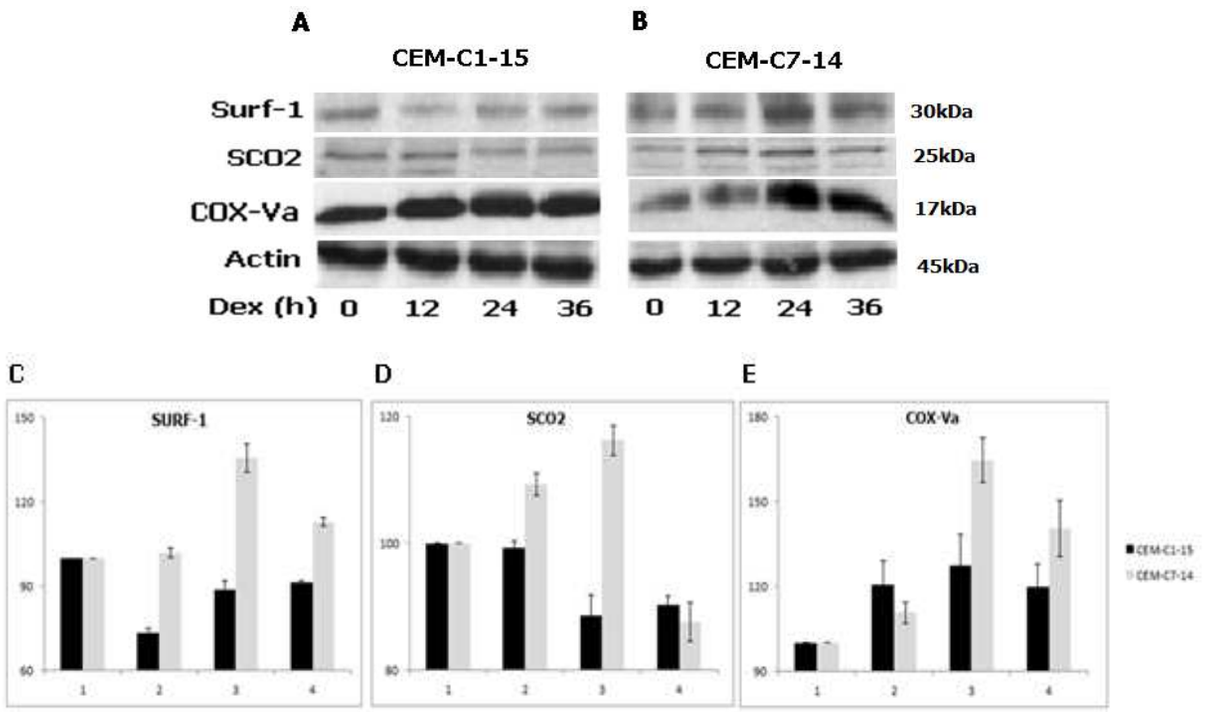

Figure 3. Western blot analysis of Surf-1, SCO-2, and COX-Va in CEM-C1-15 (A) and CEM-C7-14 (B) cells treated with dexamethasone for the indicated times. Actin was used as a loading control. Densitometric analysis of the western blots presented in A and B was carried out using Image J software. The intensity of the bands of Surf-1 (C), SCO2 (D) and COX-Va (E) at each time point was normalised to the intensity of the actin band at the respective time point and the obtained values were plotted. The intensity of the bands in the untreated cells normalised to the intensity of the actin band in the untreated cells was arbitrarily set to 100. The values representing the intensities of the bands in the treated cells were calculated as follows: Intensity of band in treated cells/intensity of actin band in treated cells x 100 / intensity of band in untreated cells/intensity of actin in untreated cells. Black bars represent the CEM-C1-15 cells and the grey bars the CEM-C7-14 cells.

The protein levels of Surf-1, SCO2 and COX-Va were also followed in CEM-C7-14 and CEM-C1-15 cells under the same conditions (Figure 3). Higher Surf-1 protein levels were observed

in dexamethasone treated CEM-C7-14 cells compared to the non-treated cells for all time points tested (Figure 3A, B and C compare grey bars 2, 3 and 4 to grey bar 1). In contrast lower Surf-1 protein levels were observed in the dexamethasone treated CEM-C1-15 cells irrespectively of the length of the dexamethasone treatment compared to the untreated cells (Figure 3A, B and C compare black bars 2, 3 and 4 to black bar 1). In a similar manner to that observed for Surf-1, increasing SCO2 protein levels were observed in CEM-C7-14 cells treated with dexamethasone for 12h and 24h compared to the untreated cells (Figure 3A, B and D compare grey bars 2 and 3 to bar 1). On the contrary lower SCO2 protein levels were observed in CEM-C7-14 cells 36h after the addition of dexamethasone compared to the untreated cells (Figure 3A, B and D compare grey bar 4 to grey bar 1). On the other side the SCO2 protein levels in CEM-C1-15 cells were lower than those measured in the untreated cells for all the time points of dexamethasone treatment examined in this study (Figure 3A, B and D compare black bars 2, 3 and 4 to black bar 1). Finally the COX-Va protein levels were higher in both CEM-C1-15 and CEM-C7-14 cells treated with dexamethasone compared to the non-treated cells (Figure 3A, B and E compare black and grey bars 2, 3, and 4 to grey and black bars 1).

The expression of COX subunits and oxidative phosphorylation are affected by glucocorticoid treatment [137] possibly through the GR transcriptional activity. Given that some OXPHOS subunits are encoded by the nuclear genome, and others by the mitochondrial DNA, it was thought that glucocorticoids exert indirect effects on the regulation of transcription of mitochondrial encoded OXPHOS subunits by modulating the activity of nuclear factors involved in the gene expression of mitochondrial genes [101]. However, the identification of mitochondrial GR, and the existence of GRE-like domains in the mitochondrial genome [100, 101, 138] suggest that glucocorticoids exert direct effects on mitochondrial transcription. Taking into consideration the fact that glucocorticoid receptor potentially regulates the expression of several COX assembly factors and nuclear COX subunits (Figure 2A, 2B and 2C) it was interesting to investigate whether GR fine tunes the expression of OXPHOS genes in both nuclear and mitochondrial genomes. In order to assess this hypothesis, and in parallel, to strengthen the perception that energy metabolism is a possible pathway by which CEM cells determine resistance / sensitivity to glucocorticoid induced apoptosis we followed the mRNA levels of the mitochondrial OXPHOS subunits COX-I, COX-II and COX-III in CEM C1-15 and CEM C7-14 cells treated with dexamethasone (Figures 4A, 4B and 4C).

A similar picture to that observed for the nuclear OXPHOS components was observed for the mitochondrial OXPHOS subunits. Specifically higher cellular levels of the mitochondrial COX-I, COX-II and COX-III OXPHOS subunits were observed in CEM-C7-14 compared to CEM-C1-15 cells (Figure 4A-4C). Increased levels of COX-I mRNA in both cell lines after 12 hours of dexamethasone treatment were observed in both CEM-C1-15 and CEM-C7-14 cells (Figure 4A, compare solid with dotted line). COX-II mRNA expression initially decreased in both cell lines 3h after the addition of dexamethasone

(Figure 4B, compare solid and dotted lines 3h point) and then started to increase steadily at 6 and 12h after the treatment with the hormone but it reached higher levels in the CEM-C7-14 cells (Figure 4B, compare solid line with dotted line). COX-III gene expression remains unaffected by glucocorticoid treatment during the first 3 hours, in CEM-C1-15 cells, followed by two fold increase 6h and 12h after hormone addition (Figure 4C, solid line compare 0 time point with 6 and 12h time points). A similar pattern was evident in CEM-C7-14 cells where the induction of COX-III mRNA after 6h and 12h of dexamethasone treatment was four and five fold higher respectively compared to those detected in the untreated cells (Figure 4C, dotted line compare 0 time point with 6 and 12h time points).

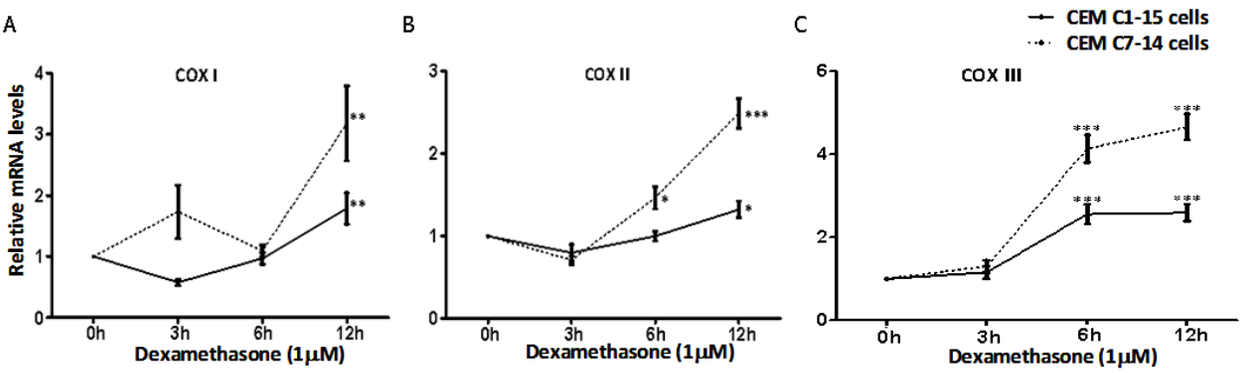

Figure 4. Relative mRNA levels of the mitochondrial OXPHOS subunits COX-I (A), COX-II (B), and COX-III (C) were followed in the resistant CEM-C1-15 and the sensitive CEM-C7-14 to glucocorticoid induced apoptosis ALL cells treated with dexamethasone for the indicated time points by quantitative real-time PCR. Solid lines represent mRNA levels in CEM-C1-15 and dotted lines correspond to mRNA levels determined in CEM-C7-14 cells. Error bars represent standard error of the mean of five independent experiments and asterisks indicate statistical significance of $p<0.05$ compared to the untreated sample.

Taken together, results presented in this manuscript endorse the hypothesis that the glucocorticoid resistance / sensitivity in CEM cells is determined by differences in the efficacy of the OXPHOS system to produce energy in the two cell lines. Support to this conclusion is lent by observations shown in Figure 2A, 2B and 2C indicating that the mRNA levels of nuclear encoded components of the OXPHOS system were higher in the sensitive CEM-C7-14 than in the resistant CEM-C1-15 cells. Higher levels of COX assembly factors in CEM-C7-14 cells imply a more efficient energy metabolism in these cells. This could be a result of distinct signalling pathways operating in the two cell lines giving rise to increased transactivation function of GR in CEM-C7-14 cells, where the receptor could be phosphorylated at S211 [58] and stimulate the expression of its potential transcription targets Surf-1 and COX-Va, whereas on the contrary, it is phosphorylated at S226 in CEM-C1-15 cells, thereby targeted to different subset of its transcriptional targets in these cells [58]. The link between altered metabolism in cancer cells and varying expression of COX subunits has been well established [139, 140, 141]. The presence of mitochondrial GR and of GRE-like domains in the mitochondrial genome [100, 102, 138, 142, 143, 144] and regulation

of the mitochondrial COX subunits (COX-I, COX-II and COX-III) provide a probable mechanism explaining the dexamethasone dependent regulation of the mitochondrial COX subunits gene expression (Figure 4). In addition, differences in post translational modifications of the receptor, which in the case of CEM-C7-14 cells, allow activation [58] and possibly translocation of GR into mitochondria [96] while in CEM-C1-15 cells these events are not permissible could be an additional justification for these observations.

8. Conclusions and future perspectives

Resistance to glucocorticoid induced apoptosis in ALL has been attributed to several different processes and pathways including predominance of certain GR isoforms with reduced transcriptional activity [34, 145, 146], either due to altered binding capacity of the receptor to other transcription factors [147, 148, 149, 150] or co-regulators [81, 151], disparate expression and consequent dissimilar balance between pro- versus anti-apoptotic members of the Bcl-2 family [18], GR mitochondrial localisation [97], autophagy [72, 73, 74, 75], and post-translational modifications which affect GR transcription target selectivity and protein stability [22, 38, 89, 152, 153].

We have recently presented evidence to suggest that differential GR phosphorylation in resistant versus sensitive to glucocorticoid induced apoptosis ALL cells results in selective induction of anti-apoptotic and inhibition of pro-apoptotic Bcl-2 family members gene expression in the resistant cells [58]. Several reports have indicated the potential role of differential kinase activity in glucocorticoid resistant and sensitive cells in determining the GR subcellular localisation [96, 98, 154] and diverse effects on the induction of autophagy [72, 73, 74].

In this study we provide new evidence signifying the differential expression of OXPHOS components in glucocorticoid resistant versus sensitive ALL cells and we propose that resistant and sensitive CEM cells use different pathways to produce energy. These results are in accord with reports showing that resistance to GC in ALL is associated with increased glucose consumption [155] with concomitant induction of Mcl-1 expression and resistance to apoptosis [39]. In our anticipated model (Figure 5) in resistant lymphocytes the predominantly phosphorylated at S226 GR is possibly transcriptionally inactive and thus incapable to significantly induce OXPHOS assembly enzymes and COX subunits gene expression or translocate into mitochondria thereby exhibiting reduced oxidative phosphorylation consistent with a proliferative phenotype, and this is probably one of the mechanisms employed by the glucocorticoid-resistant CEM-C1-15 cells to survive GC treatment.

The results presented in this study endorse the hypothesis that differential GR phosphorylation affects components of cellular energy production pathways in distinct ways in resistant versus sensitive cells altering energy production and possibly ROS generation in unique ways in the two cell lines, suggesting that combination of kinase inhibitors, and glycolytic modulators together with dexamethasone could be a possible mean by which resistance to glucocorticoid induced apoptosis could be circumvented in ALL.

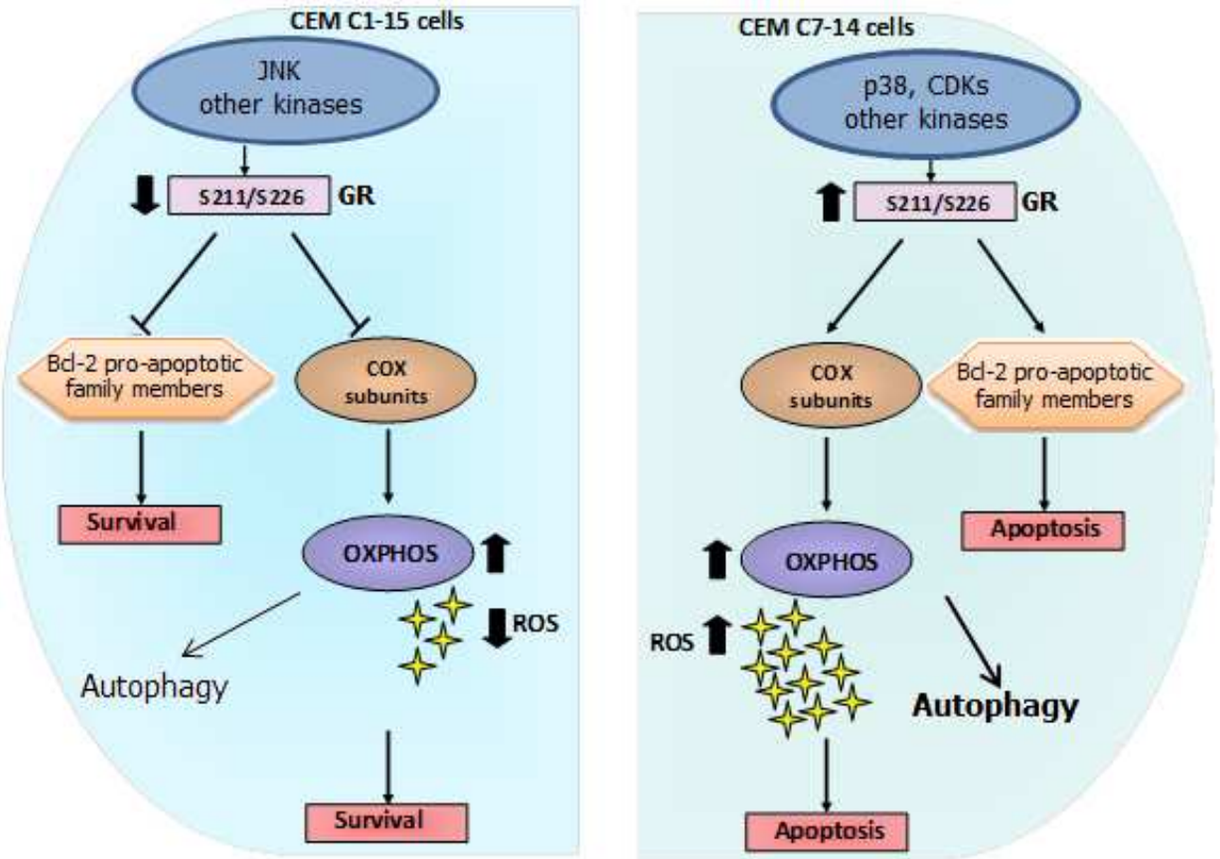

Figure 5. Model summarising the proposed mechanism determining resistance / sensitivity of CEM cells to glucocorticoid-induced apoptosis. Differential phosphorylation of GR in glucocorticoid resistant versus sensitive cells leads to the reduction of gene expression of OXPHOS subunits and pro-apoptotic Bcl-2 family members in the resistant cells (left panel) whereas the opposite is the case for the sensitive cells (right panel).

Author details

Ilhem Berrou, Marija Krstic-Demonacos, Constantinos Demonacos
University of Manchester, School of Pharmacy and Faculty of Life Sciences, Manchester, UK

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