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NON-GENOMIC EFFECTS OF GLUCOCORTICOIDS: AN UPDATED VIEW

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Abstract

Glucocorticoid (GC) anti-inflammatory effects generally require a prolonged onset of action and involve genomic processes. Because of the rapidity of some of GC effects, however, the concept that non-genomic actions may contribute to GC mechanisms of action has arisen. While the mechanisms have not been completely elucidated, the non-genomic effects may play a role in the management of inflammatory diseases. For instance, we recently reported that GC “rapidly”

27 enhanced the effects of bronchodilators, agents used in the treatment of allergic asthma. In this
28 review, we will discuss i) the non-genomic effects of GCs on pathways relevant to the pathogenesis
29 of inflammatory diseases and ii) the putative role of membrane GC receptor. Since GC side effects
30 are often considered to be generated through its genomic actions, understanding GC non-genomic
31 effects will help design GCs with a better therapeutic index.

32 **Mechanism of action of glucocorticoids (GC).**

33 GCs primarily mediate their effects by activating the ubiquitously expressed intracellular **GC**
34 **receptor (GR)** (see Glossary) [1]. In its inactive state, the GR resides in the cytoplasm and, upon
35 ligand activation, translocates to the cell nucleus to interact with GC response elements (GREs)
36 thereby producing **genomic effects** that alter protein expression. Interestingly, evidence suggests
37 that GCs also manifest almost immediate **non-genomic actions** on several signaling processes [2].
38 GC non-genomic effects involve non-specific interactions with the cell membrane, or specific
39 interactions with cytosolic GRs (cGR) or membrane-bound GRs (mGR) (Table 1). This report
40 summarizes the current knowledge on non-genomic effects of GCs, with a focus on GR-mediated
41 events and GR-associated signaling pathways. Where appropriate, potential links to inflammatory
42 diseases will be highlighted in the main text and their potential impact will be discussed in Box 1
43 and 2.

44
45 **GCs exert rapid effects on levels of intracellular calcium.**

46 Studies suggest that GC rapidly (within seconds) modulates basal intracellular calcium levels
47 and agonist-induced **calcium mobilization** (Tables 2 & 3).

48 *Effects of GCs on intracellular calcium homeostasis.* GCs can increase or decrease cytosolic
49 calcium depending on the cell type. Evidence from non-immune cells, such as primary or
50 immortalized human bronchial epithelial cells, consistently demonstrate that acute exposure to
51 GC, and to a lesser extent to the mineralocorticoid (MC), aldosterone, reduces basal $[Ca^{2+}]_i$ [3, 4].
52 Similarly, in rat thymocytes [5] and mouse neuroblastoma cells [6] $[Ca^{2+}]_i$ decreased following
53 acute exposure to GC, and in cichlid fish pituitary cells cortisol inhibited $[Ca^{2+}]_i$ and reduced
54 prolactin secretion [7]. However, in immune cells, it is unclear if GCs genuinely exert non-
55 genomic effects on basal $[Ca^{2+}]_i$. For example, while acute exposure to GC was reported to
56 decrease $[Ca^{2+}]_i$ in leukocytes, these leukocytes were obtained from donors who were treated with
57 oral prednisolone for 7 days [8], potentially confounding the results of the study. Similarly, studies
58 in human lymphoblasts show that cortisol markedly reduced basal $[Ca^{2+}]_i$ only after 48 hrs of
59 treatment [9]. These data argue against a role for non-genomic effects of GC in altering basal
60 $[Ca^{2+}]_i$ in immune cells.

61 With regard to the lungs, evidence supports that a variety of GCs differentially modulate basal
62 $[Ca^{2+}]_i$ upon immediate exposure. For instance, the acute inhibitory effects of dexamethasone
63 (within 30 seconds) on basal $[Ca^{2+}]_i$ in bronchial epithelial cells were comparable to triamcinolone
64 acetonide and hydrocortisone but not to budesonide [10]. Interestingly, the GR antagonist RU486
65 and the protein synthesis inhibitor cycloheximide failed to prevent these acute GC effects,
66 suggesting the involvement of GR-independent and non-genomic pathways. These observed
67 effects could be due to the various degrees of lipophilicity among GCs, as well as direct
68 interactions of GCs with the cell membrane [10]. Non-genomic mechanisms have been proposed
69 mostly based on the use of pharmacological inhibitors. Urbach and colleagues found that the rapid
70 GC effects involved pathways regulated by the SERCA type Ca^{2+} -ATPase pump, adenylyl cyclase
71 and protein kinase A (PKA) but not protein kinase C (PKC) [10]. Collectively, these studies show
72 the complexity of mechanisms involved in the rapid, GR-independent effect of GCs on $[Ca^{2+}]_i$,
73 which likely occurs through an adenylyl cyclase/PKA mediated stimulation of a thapsigargin
74 sensitive Ca^{2+} -ATPase [10].

75 Conversely, acute stimulatory effects of GC on basal calcium levels have been documented. A
76 brief exposure to GC can increase $[Ca^{2+}]_i$ in several cell types. For example, in mouse cortical
77 collecting duct cells, dexamethasone and aldosterone increased $[Ca^{2+}]_i$. Interestingly, the effect of
78 aldosterone was mediated by a non-genomic activation of PKC pathway as evidenced by the
79 abolishment of its effect on basal $[Ca^{2+}]_i$ in the presence of the PKC inhibitor, chelerythrine
80 chloride, but not the mRNA synthesis inhibitor, actinomycin D [11]. Similarly, in rat vascular
81 smooth muscle cells, GCs rapidly increased $[Ca^{2+}]_i$ [12] potentially through GC-mediated
82 increases in inositol 1,4,5-triphosphate (IP3) levels associated with the translocation of the
83 calcium- and lipid-dependent PKC from the cytosolic to the membranous compartment [13]. In
84 these cells, while the administration of epinephrine by itself had little effect on IP3 levels,
85 epinephrine potentiated the rapid response induced by cortisol [13]. Collectively, these findings
86 highlight a role of PKC in the rapid increase of basal $[Ca^{2+}]_i$ by GCs.

87
88 *Effects of GCs on agonist-induced calcium mobilization.* The effects of GCs on agonist-
89 induced calcium mobilization are variable depending on the agonist, the extra-cellular stimuli and
90 the cell type. Evidence suggests that GCs rapidly inhibit, at least partially, the ability of adenosine
91 triphosphate (ATP) to increase $[Ca^{2+}]_i$ in some cell types. In human bronchial epithelial cells for

92 example, 15 min exposure to dexamethasone (1 nM) markedly reduced ATP-induced increases in
93 $[Ca^{2+}]_i$. The ATP-induced Ca^{2+} response was independent of extracellular calcium but did involve
94 a Ca^{2+} -mobilization from thapsigargin-sensitive intracellular stores [10]. Similarly, in murine HT4
95 neuroblastoma cells, acute (5 min) pre-incubation with corticosterone dose-dependently inhibited
96 $[Ca^{2+}]_i$ signals induced by ATP [6]. Unlike in human bronchial epithelial cells, the Ca^{2+} -response
97 induced by ATP in these cells relies on Ca^{2+} -influx across the plasma membrane and Ca^{2+} -release
98 from intracellular stores [6]. Inhibition of PKA abrogated the inhibitory action of corticosterone
99 on ATP-induced Ca^{2+} -elevation, whereas little influence was observed with respect to PKC
100 inhibition. Additional studies demonstrated that these GC inhibitory effects were unaffected by
101 GR blockade. These key findings obtained from studies in HT4 cells suggest that GC activates
102 membrane-initiated, non-genomic, PKA-dependent, PKC-independent pathways [6, 14]. In
103 contrast, in rat B103 neuroblastoma cells, the inhibitory effects of corticosterone on serotonin-
104 induced peak $[Ca^{2+}]_i$ were found to be PKC-dependent [15]. Together, these studies suggest that
105 the mechanisms mediating the acute non-genomic effects of GC on agonist-evoked calcium
106 mobilization are stimuli and cell type-dependent.

107 In contrast to human bronchial epithelial and murine HT4 neuroblastoma cells,
108 pretreatment of guinea pig cochlear spiral ganglion neurons (SGN) with dexamethasone (10 min)
109 enhanced ATP-induced Ca^{2+} -mobilization [16]. This effect was prevented in the presence of a GR
110 antagonist and mediated by rapid Ca^{2+} -influx through activation of ionotropic purinergic P2X
111 receptors [16]. Of note, all P2X subtypes are expressed in SGN albeit to different extents [17, 18].
112 Similarly, in rat hippocampal neurons, pretreatment with corticosterone or dexamethasone for 10-
113 20 min prolonged N-methyl-D-aspartate (NMDA)-induced transient elevation in $[Ca^{2+}]_i$ [19].
114 Importantly, the steroid effect was reversed by the removal of corticosterone indicating that the
115 steroid effect was not due to irreversible impairment of Ca^{2+} -extrusion from the neurons.
116 Thapsigargin and cyclohexamide had little effect on the potentiating effect of corticosterone,
117 excluding the involvement of a thapsigargin sensitive Ca^{2+} -ATPase or *de novo* protein synthesis,
118 respectively. Interestingly, the GC effect was reproduced by the use of a membrane impermeable
119 BSA-conjugated cortisol, suggesting that mGR likely underlies the rapid non-genomic effects of
120 GC [19]. However, canonical genomic actions of GC can also alter Ca^{2+} mobilization. In human
121 lymphoblasts, while cortisol reduced basal $[Ca^{2+}]_i$ (as indicated above), Ca^{2+} -mobilization induced

122 by platelet activating factor (PAF) is enhanced only by chronic treatment (48 hrs) with cortisol [9]
123 (Figure 1).

124

125 **GCs rapidly modulate skeletal and smooth muscle function.**

126 Several studies have reported variable acute effects of GCs on **muscle reactivity** and tone.
127 The specific example of airway smooth muscle cells in the pathogenesis of inflammatory diseases
128 is highlighted in **Box 1**. In mouse skeletal myotubes (C2C12 immortalized myoblasts), treatment
129 with dexamethasone (for less than 20 min) reduced glucose uptake induced by electrical pulse
130 stimulation (EPS)-mediated contraction, in a Ca^{2+} /calmodulin protein kinase II (CaMKII) and
131 AMP activated protein kinase (AMPK) dependent fashion [20]. The effects were unaffected by
132 blockade of GR (RU486) or inhibition of protein synthesis (cyclohexamide), indicating a rapid
133 non-genomic and GR-independent effect. In another study, cortisol synergized with isoprenaline
134 in reducing tracheal spasms in response to histamine [21]. The spasmolytic effect was fully
135 prevented in the presence of RU486 (implicating a GR-dependent pathway), partially reduced by
136 PKC inhibition, but was unaffected by actinomycin D (excluding *de novo* RNA synthesis) again
137 suggesting a non-genomic, GR-mediated signaling pathway involving PKC [21].

138 Other studies support a role for GCs in rapidly reducing airway smooth muscle (ASM)
139 tone. Pretreatment with budesonide (within 15 min) suppressed histamine-induced isometric
140 tension in guinea pig tracheal rings and shrinkage in individual tracheal ASM cells; effects that
141 were unaffected by cycloheximide (suggesting non-genomic actions by budesonide) [22]. Unlike
142 the findings by Wang and colleagues [21], these budesonide effects were insensitive to RU486,
143 excluding classic GR involvement [22]. Similarly, in murine ASM cells, exposure to
144 dexamethasone for 10 min decreased basal $[Ca^{2+}]_i$ and reduced peak elevations in $[Ca^{2+}]_i$ induced
145 by acetylcholine, effects that were insensitive to GR blockade and cycloheximide [23].
146 Consistently, studies using an *in vivo* guinea pig model of asthma, an established model to study
147 allergen-induced asthmatic reactions and airway hyperresponsiveness [24], revealed a beneficial
148 effect on ovalbumin-induced changes in lung resistance and compliance by acutely inhaled
149 budesonide. The protective effects of budesonide were evident within 10 minutes, suggesting a
150 non-genomic course of action [25]. In summary, GCs have acute spasmolytic actions in ASM that

151 can require both GR-dependent and -independent pathways, and potentially PKC-mediated
152 signaling.

153 A recent study in rat vascular smooth muscle cells under conditions of lipopolysaccharide
154 (LPS)-induced septic shock showed that dexamethasone treatment for 10 min promotes
155 norepinephrine (NE)-induced phosphorylation of key proteins associated with contraction [26].
156 While no significant effect on myosin light chain 20 (MLC20) phosphorylation was observed after
157 exposure to either dexamethasone or NE alone, the combined treatment markedly enhanced
158 phospho-MLC20, an effect that was unaltered by GR blockade with RU486. Interestingly,
159 inhibition of Rho-kinase with Y-27632 completely reversed the potentiating effects of
160 dexamethasone on NE-induced phospho-MLC20. Together, these findings could be of clinical
161 significance and indicate that the impaired vascular response to NE observed in septic shock may
162 be restored by short-term exposure to dexamethasone through non-genomic activation of Rho-
163 kinase activity [26].

164

165 **GCs exert rapid effects on Reactive Oxygen Species (ROS)/Reactive Nitrogen Species**
166 **(RNS).**

167 Studies demonstrated a rapid effect of GCs on ROS generation and the involvement of
168 nitric oxide (NO) in mediating some GC effects. An example of the role NO/ROS in the
169 pathogenesis of inflammatory disease is highlighted **in Box 2**. In breast cancer cells, cortisol
170 rapidly increased levels of ROS and RNS (as early as 15 min) and induced DNA damage. The GR
171 antagonist (RU486) blocked the cortisol effect while L-NAME and 1400 W dihydrochloride
172 demonstrated the involvement of nitric oxide synthase (NOS) and inducible (i)NOS, respectively.
173 The pharmacological inhibition of Src by PP2 prevented GC-induced RNS elevation, suggesting
174 the ability of GC to rapidly stimulate Src- and iNOS-dependent release of damaging RNS levels
175 [27].

176 Rapid effects of GCs on endothelial NOS (eNOS), an important mediator of vascular
177 integrity with anti-inflammatory, anti-ischemic, and anti-atherogenic properties, have been
178 described as well [28-30]. Indeed, the treatment of human vascular endothelial cells with
179 dexamethasone rapidly enhanced (as early as 10 min), in a concentration-dependent manner, eNOS
180 activity, NO-production and NO-dependent vasorelaxation [31]. These GC effects were abrogated

181 by RU486, PI3-kinase inhibitors wortmannin and LY292002, or L-NAME, but not by the
182 transcriptional inhibitor actinomycin D.

183 Additional evidence supporting rapid effects of GCs on NOS/NO showed an augmented
184 ATP-induced, NOS-dependent NO release in guinea pig type I spiral ganglion neurons by
185 dexamethasone that was thought to be a consequence of ATP-induced $[Ca^{2+}]_i$ [16]. Similarly, GR-
186 mediated increases in $[Ca^{2+}]_i$, eNOS phosphorylation, and NO production, were observed in
187 human umbilical vein endothelial cells [32]. Interestingly, NO production increased $[Ca^{2+}]_i$
188 originating from intracellular and extracellular Ca^{2+} sources [32].

189 The PI3K/Akt pathway is critical in the activation of NO signaling, e.g. phosphorylation
190 of eNOS [33], and the involvement of this pathway in the rapid effects of GCs has been
191 documented [33]. For example, dexamethasone rapidly increased (within 20 min), in a dose-
192 dependent manner, GR-dependent phosphorylation and activation of PI3K as demonstrated by
193 phosphorylation of Akt and glycogen synthase kinase (GSK)-3, indicating that GCs can
194 functionally activate PI3K and downstream targets in human endothelial cells [31]. The potential
195 clinical relevance of these observations was confirmed in two different mouse models of ischemic
196 injury (i.e. transient myocardial ischemia and transient focal cerebral ischemia) where GC exerted
197 rapid protective effects (within 30 min) via GR-dependent activation of PI3K and eNOS pathways
198 as evidenced by the administration of RU486, wortmannin and L-NAME, respectively [31, 32].
199 Additional studies in COS-7 cells demonstrated a key role for GR in GC-induced activation of the
200 PI3K/Akt pathway. When cells were transfected with a dimerization-defective GR mutant (A458T,
201 a construct that is unable to bind DNA and transactivate GC target genes), acute dexamethasone
202 stimulation still activated the PI3K/Akt pathway [34]. Together, these findings suggest the
203 involvement of a non-transcriptional/non-genomic mechanism in the GR-dependent activation of
204 PI3K/Akt by GCs.

205 Since NO signaling plays a key role in chronic airway inflammatory diseases, such as
206 asthma and COPD [35], we believe that the cross-talk between GC and NO signaling warrants
207 further investigation to determine whether the rapid effects of GC on NO signaling would be
208 beneficial or detrimental in disease pathogenesis.

209

210 **GCs exert acute effects on inflammatory and apoptotic pathways.**

211 Evidence shows rapid non-transcriptional actions of GCs on inflammation both in
212 transformed cells and immune cells. In transformed cells, such as A549 adenocarcinoma cells,
213 acute exposure (as early as 1 min) to dexamethasone rapidly inhibited epidermal growth factor
214 (EGF)-induced arachidonic acid (AA) release, an important mediator of inflammation [36]. This
215 inhibitory effect was due to hindering the recruitment of Grb2, p21ras and Raf to the EGF receptor
216 (EGFR) through a GR-dependent (RU486-sensitive) and transcription-independent (actinomycin
217 D-insensitive) mechanism. The inhibition of Grb2 recruitment was accompanied by lipocortin-1
218 recruitment to EGFR in the cell membrane. Subsequently, lipocortin-1 competitively inhibited
219 Grb2 binding to EGFR, thereby blocking the recruitment of critical signaling molecules necessary
220 for EGF actions [36].

221 The acute effects of GCs on inflammatory pathways were also observed in immune cells,
222 such as human neutrophils, where acute exposure (5 min) to methylprednisolone or hydrocortisone
223 significantly inhibited N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil
224 degranulation, effects that were not prevented by RU486 or cycloheximide treatments, suggesting
225 the involvement of GR-independent and non-genomic pathways [37]. Also, in murine
226 macrophages acutely treated with dexamethasone (30 min), toll like receptor 9 (TLR9)-induced
227 activation of different inflammatory signaling pathways, such as those involving NF- κ B and
228 mitogen-activated protein kinases (MAPKs), was dramatically suppressed [38]. Following TLR-9
229 engagement, IL-1R-associated kinase 1 (IRAK1) is recruited to the cell membrane. A critical step
230 in activating the TLR signaling cascade is the ubiquitination of IRAK1 through its physical
231 interaction with the E3 ligase, β -TrCP. Such ubiquitination and degradation of IRAK1 promotes
232 the trafficking of the “TNFR-associated factor 6 (TRAF6)– TAK1 adaptor proteins (TAB)–
233 Transforming growth factor beta-activated kinase 1 (TAK1)” complex to the cytosol to
234 subsequently induce MAPK and NF- κ B activation. Dexamethasone inhibition of IRAK1
235 ubiquitination did not occur in the presence of RU486 suggesting the involvement of GR-
236 dependent mechanisms [38]. Further investigation of the molecular mechanisms revealed that by
237 physically interacting with IRAK1, GR interferes with the interaction between β -TrCP and IRAK1
238 thereby impeding its ubiquitination, a critical step in the activation of the TLR9-dependent
239 inflammatory cascade [38].

240 Rapid GC treatment can also exert pro-inflammatory action in other cell types. For
241 example, in PC12 cells (cell line derived from rat adrenal gland), corticosterone induced rapid

242 activation (within 15 min) of ERK1/2, p38, and JNK in a PKC-dependent manner [39, 40]. The
243 activation of MAPK pathways following GC treatment appears to be mediated by the putative
244 mGR, since corticosterone-BSA can rapidly (with 15 min) activate all MAPKs [39, 40]. Similarly,
245 in rat vascular smooth muscle cells, dexamethasone either alone or in combination with NE,
246 rapidly (within 10 min) induces ERK1/2 and p38 MAPK activities [26]. Thus, in certain cells, GCs
247 can activate MAPK in a non-genomic manner.

248 In CCRF-CEM cells, cell line derived from human T-cells (from pediatric ALL patients),
249 sensitivity to acute dexamethasone induced cell death was determined in the presence and absence
250 of phosphodiesterase (PDE) inhibitors [41]. Non-specific PDE and specific PDE4 inhibition
251 reversed steroid resistance and markedly increased sensitivity to dexamethasone. This effect is
252 likely due to increased cAMP levels, consistent with abundant documentation on interactions
253 between GR and cAMP pathways in the induction of apoptosis in lymphoid cells by both [42, 43].
254 To date, the mechanisms of cAMP-induced apoptosis are unclear, but the presence of GR appears
255 to be required, even in the absence of GCs. For instance, in parental T-cells, elevation of cAMP,
256 with either forskolin or dibutyryl cAMP, induced apoptotic cells death, whereas GR deficient cells
257 were insensitive to the apoptotic effects of cAMP elevation. When GR expression was
258 reconstituted by transfection, not only was GC sensitivity restored, but the sensitivity to cytolytic
259 effects induced by cAMP was promoted as well [42].

260 The effects of GCs on the mitochondrial control of cell metabolism and apoptosis have
261 been extensively reviewed elsewhere [44-47]. For instance, Sekeris and colleagues were the first
262 to discover the presence of GR in mitochondria [48]. Through its acute non-genomic effects, GCs
263 promote mitochondrial apoptotic pathways resulting in the disruption of the mitochondrial
264 membrane-potential and the release of pro-apoptotic factors such as Cytochrome C [49].
265 Importantly, the translocation of GR from the cytoplasm to the mitochondria correlates with the
266 sensitivity of a given cell type to GC-induced apoptosis [50, 51]. In line with this, recent studies
267 in mouse thymocytes showed that short term treatment with GC induces a direct interaction of GR
268 with the pro-apoptotic Bcl2 family member associated proteins such as Bim [52]. Such interaction
269 subsequently activates Bax decreasing thereby the mitochondrial membrane potential,
270 Cytochrome C release, and Caspase-9 activation. However, it important to note that the effects of
271 GC on the mitochondria control of apoptosis involve also genomic pathways. For example, in
272 murine neuronal stem cells, dexamethasone was able to augment 2,3-methoxy-1,4-

273 naphthoquinone-induced apoptosis where a large percentage of studied genes involved in the
274 mitochondrial respiratory chain and some encoding for anti-oxidant enzymes were downregulated
275 by long-term treatment with GC [53]. These events allowed GCs to increase cellular sensitivity to
276 oxidative stress promoting thereby neurotoxicity. This is clinically relevant as it can occur during
277 prenatal exposure of the fetal brain to excess GCs [53].

278 **Potential role of a putative mGR in mediating the rapid effects of GCs.**

279 As previously described, the rapid non-genomic effects can, at least in part, be mediated
280 through a putative mGR. Over the years, caveolin-1 (Cav-1), the major protein component of
281 caveolae, has been implicated as a scaffold for the organization of several cytoplasmic signal
282 complexes at the plasma membrane [54, 55]. In lung epithelial cells (A549), dexamethasone
283 treatment leads to a rapid (within 2 min) phosphorylation of Cav-1 and protein kinase B (PKB)/Akt
284 in a Src-dependent fashion [56]. Subcellular fractionation revealed co-localization of GR and Src
285 to caveolin-containing membrane fractions [56]. Interfering with caveolae/caveolin (by disruption
286 of lipid raft formation, impairment of function using dominant negative caveolin, down regulation
287 of Cav-1 using shRNA, or genetic ablation of Cav-1) prevented acute (within 2 min) GC-induced
288 PKB phosphorylation. Of note, caveolin down-regulation had little effect on GC-mediated
289 transactivation, supporting the existence of a putative mGR. Further functional studies in caveolin
290 knockout cells revealed considerable inhibition of GC-mediated cell growth arrest, suggesting that
291 membrane-proximal signals acutely initiated by GC are required to mediate delayed effects (anti-
292 proliferative effects) previously ascribed exclusively to the nuclear actions of GR [56]. Further
293 evidence supporting a role for caveolae in mGR function stems from studies of membrane nuclear
294 receptors such as estrogen receptor (ER) [57] showing requirement of Cav-1 in mediating acute
295 cellular actions. Indeed, using epitope proximity ligation assays, Watson and colleagues
296 demonstrated interactions of ER α with Cav-1. Interestingly, the use of nystatin, which binds to
297 cholesterol and disrupts caveolar structures, blocked estrogen-induced rapid (5 min) ERK
298 activation in pituitary tumor cells [57]. Together these findings indicate a critical role of Cav-1 in
299 acute nuclear receptor/steroid signaling.

300 While the expression of mGR has been demonstrated in a myriad of cell types [58], the co-
301 localization and cross-talk between mGR and Cav-1 is variable and highly cell-specific. Indeed,
302 in U2-OS and MCF-7 cells, double recognition proximity ligation assays demonstrated the
303 physical association of Cav-1 with the mGR [58]. However, studies in human CD14⁺ monocytes

304 showed that mGR and Cav-1 are not co-localized and overexpression of the recombinant Cav-1
305 transcript in human K562 chronic myelogenous leukemia cells did not affect mGR
306 expression/appearance suggesting that in these specific cell lines Cav-1 is not the limiting factor
307 for mGR expression/appearance, without ruling out the possibility that it is a component of the
308 transport machinery of GR from the cytosol to the membrane [59]. Palmitoylation, a critical post-
309 translational modification occurring through the addition of fatty acid (e.g. palmitic acid) on amino
310 acid residues of membrane proteins, plays a major role in the subcellular trafficking of proteins
311 between membrane compartments [60]. Interestingly, the involvement of palmitoylation in the
312 recruitment of other nuclear receptors, such as ER, to the plasma membrane has been reported
313 [61]. Recent studies investigated whether this process is necessary for the recruitment of GR to the
314 membrane and its co-localization with Cav-1 in COS-7 cells. Treatment of cells with the
315 palmitoylation inhibitor, 2-bromopalmitate, had little effect on membrane localization of GR and
316 its co-localization with Cav-1, and little influence on the acute effects of GC on MAPK signaling
317 pathways. In addition, human GR α did not undergo S-palmitoylation, rendering this process
318 unlikely to modulate membrane recruitment of GR [62]. Future studies on the mechanisms
319 underlying GR recruitment to caveolae rich parts and its potential association with Cav-1 are
320 warranted, specifically in airway cells.

321 Several studies have reported an interaction of mGR with other membrane receptors,
322 particularly GPCRs [63]. Zhang and colleagues demonstrated the involvement of mGR and GPCR-
323 dependent mechanisms in the rapid effect (as early as 1 min) of corticosterone on NMDA-evoked
324 currents in hippocampal neurons [63] and further suggested that mGR may couple to multiple G
325 proteins, including G_s and G_{q/11}. Other studies indicate that mGR directly elicits the activation of
326 downstream intracellular signaling pathways. For instance, corticosterone might act via mGR to
327 rapidly elicit PKC-dependent activation of ERK1/2 MAPK pathway (with 15 min) in PC12 cells
328 [39]. Interestingly, proteomic analysis of the lymphoma cell line CCRF-CEM identified 128
329 proteins that were differentially regulated by the specific activation of mGR using BSA-conjugated
330 cortisol for a short-term period (5 and 15 min) [58]. These actions were unique to mGR, as no
331 activation of cGR target genes, such as GILZ, were observed. The majority of networks rapidly
332 activated by mGR were mainly involved in cellular growth and cancer (after 5 min treatment with
333 cortisol-BSA), cellular development, or hematological system development and function (after 15
334 min treatment with cortisol-BSA). Ingenuity pathway analysis provided strong evidence that mGR

335 is involved in pro-apoptotic, immune-modulatory, and metabolic pathways that are also regulated
336 by GCs through cGR, suggesting that acute mGR stimulation can trigger rapid early priming
337 events, ultimately paving the way for the slower genomic activities by GCs [58].

338

339 **Concluding Remarks and Future Perspectives.**

340 Although we have some insight in how GCs regulate different signaling pathways in a non-
341 genomic fashion, future in depth investigations are warranted to further unravel details of these
342 complex interactions. Indeed, key questions (see Outstanding Questions) still need careful
343 consideration and additional research must address several important issues: i) the differential
344 nature of non-genomic effects of GC in immune cells versus non-immune/structural cells; ii)
345 differences between non-genomic effects of various steroids based on their lipophilicity [10]; iii)
346 the fact that not all non-genomic effects are GR-mediated (RU486 insensitive) and may be due to
347 non-specific interactions of GC with the cell membrane [2]; iv) the possibility that non-genomic
348 and genomic effects are interconnected, where the acute non-genomic effects pave the way for the
349 slower genomic activities of GCs [58]; and v) the significant role of Cav-1, and possibly other
350 scaffolding/anchoring proteins, as a modulator of mGR activation, where the relative numbers of
351 mGR associated with Cav-1 are critical in mediating non-genomic effects of GC [58, 64, 65]. Since
352 **side effects** associated with GC therapy are often generated through its genomic actions [66],
353 uncovering the non-genomic actions of GC with beneficial effects will likely lead to the
354 development of compounds that selectively activate non-genomic signaling and thus have
355 improved therapeutic profiles.

356

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359

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505

506

507 **Text boxes.**

508

509 **Box 1: Calcium regulation, ASM tone, and asthma pathogenesis.**

510 Because airway smooth muscle (ASM) serves as the pivotal tissue regulating the bronchomotor
511 tone, changes in the pathways regulating ASM contractile properties may play an important role
512 in the development of abnormal lung function in asthma. Abnormal G-protein-coupled receptor
513 (GPCR)-associated calcium homeostasis and ASM shortening may represent one of such
514 mechanisms. Changes could occur that at different levels of the contraction cascade including i)
515 $[Ca^{2+}]_i$ release from internal stores, ii) myosin light-chain kinase (MLCK) activity, iii) myosin
516 light chain phosphorylation (pMLC), and iv) actin-myosin crossbridge cycling leading to cell
517 shortening. Changes in ASM shortening could also be due to changes in sensitivity of the
518 contractile apparatus to $[Ca^{2+}]_i$ initiated by the small GTPase, RhoA, which activates Rho kinase
519 (ROCK) to inactivate myosin light chain phosphatase (MLCP). Decreased MLCP activity results
520 in an increase in pMLC levels for a given level of $[Ca^{2+}]_i$, and thus enhancing ASM contractility.
521 It is important to note that there are other parallel pathways where actin polymerization also
522 mediates agonist-induced ASM shortening independently from Ca^{2+} and pMLC but potentially
523 through the phosphorylation of other proteins such as vinculin. Collectively, this evidence suggests
524 that ASM contractile function can mediate airway hyperresponsiveness in chronic airway
525 inflammatory diseases by involving, at least partially, changes in Ca^{2+} -regulatory pathways.

526

527 **Box 2. Role of NOS/NO signaling in asthma pathogenesis.**

528 Altered NO production has been implicated in the development of both acute and chronic allergen-
529 induced AHR. Production of NO occurs through the action of nitric oxide synthase (NOS), of
530 which 3 isoforms have been identified thus far: two constitutive (c)NOS isoforms referred as
531 neuronal (n), endothelial (e) NOS, and one inducible isoform called (i) NOS. Upon activation,
532 cNOS isoforms produce relatively low amounts of NO, whereas iNOS can produce high and
533 potentially damaging levels of NO. Whereas NO generated by eNOS is associated with beneficial
534 bronchodilatory effects in allergic asthma, iNOS-derived NO is generally considered detrimental,
535 as it has been linked to for instance epithelial damage, inflammatory cell infiltration, and mucus
536 hypersecretion. These detrimental effects are largely due to the accumulation of Reactive Nitrogen

537 Species (RNS), including peroxynitrite, which are reaction products of NO and superoxide anions.
538 Since NOS/NO signaling and RNS play key roles in chronic airway inflammatory diseases,
539 including asthma and COPD, an acute role for GC/GR signaling and (inducible and/or endothelial)
540 NOS activity can be envisioned. In depth studies are warranted to determine whether such
541 functional interaction exists and whether or not targeting it would provide any therapeutic benefit
542 for asthma.

543

544

545

546 **Tables.**

GC effects	Acute (simultaneous or within 30 min)	Chronic (delayed)
Genomic effects	-	+
Inhibitory effects of CHX or Actinomycin D	-	+
GR involvement	- or +	+
Inhibitory effects of RU486	- or +	+
Type of GR involved	None, membrane GR or cytosolic GR	Cytosolic GR
GR-independent mechanisms	GC interaction with membrane	None

547

548 **Table 1:** Various criteria (either alone or in combination) used to distinguish genomic effects from
549 non-genomic effects of glucocorticoids.

550

551

Cell types	GCs	References
• Human bronchial epithelial cells	Dexamethasone Triamcinolone Hydrocorticone	3, 4, 10
• Rat thymocytes	Methylprednisolone	5
• Mouse neuroblastoma cells	Corticosterone	6, 14
• Cichlid fish pituitary cells	Cortisol	7
• Mouse cortical collecting duct cells	Dexamethasone Aldosterone	11
• Rat vascular smooth muscle cells	Aldosterone Cortisol Dexamethasone	12, 13, 25
• Rat B103 neuroblastoma cells	Hydrocorticosone	15
• Guinea-pig cochlear spiral ganglion neurons	Dexamethasone	16
• Rat hippocampal neurons	Corticosterone Dexamethasone BSA-conjugated cortisol	19
• Mouse skeletal C2C12 cells	Dexamethasone	20
• Guinea-pig tracheal tissues	Budesonide	22
• Murine airway smooth muscle cells	Dexamethasone	23
• Guinea-pig mouse model of allergic asthma	Budesonide	24
• Human vascular endothelial cells	Dexamethasone	30

552

553 **Table 2:** Examples of the various cells types where GCs were reported to have non-genomic
554 effects due their rapid onset, insensitivity to GR blockade (RU486), and protein synthesis
555 inhibition (cycloheximide).

Signaling pathways	Cell types	GCs	References
<ul style="list-style-type: none"> PKA SERCA Ca²⁺-ATPases Adenylyl cyclase 	Human bronchial epithelial cells	Dexamethasone	10
<ul style="list-style-type: none"> PKC 	Mouse cortical collecting duct cells	Dexamethasone Aldosterone	11
<ul style="list-style-type: none"> IP3 accumulation PKC 	Rat vascular smooth muscle cells	Dexamethasone Aldosterone	12
<ul style="list-style-type: none"> PKA 	HT4 neuroblastoma cells	Corticosterone	6
<ul style="list-style-type: none"> PKC 	Rat B103 neuroblastoma cells	Corticosterone	15
<ul style="list-style-type: none"> CaMKII AMPK 	Mouse skeletal myotubes	Dexamethasone	20
<ul style="list-style-type: none"> PKC 	Tracheal smooth muscle tissues	Cortisol	21
<ul style="list-style-type: none"> Rho kinase 	Rat vascular smooth muscle cells	Dexamethasone	25
<ul style="list-style-type: none"> ROS/RNS (NO synthase) 	Human breast cancer cells	Cortisol	26
<ul style="list-style-type: none"> NO pathways 	Guinea-pig cochlear spiral ganglion neurons Human vascular endothelial cells Human umbilical endothelial cells	Dexamethasone	16, 30, 33
<ul style="list-style-type: none"> ERK1/2, P38MAPK, JNK 	PC12 cells Rat vascular smooth muscle cells	Dexamethasone	25, 37

• Src tyrosine kinase	Human breast cancer cells A549 cells	Cortisol Dexamethasone	26, 40
• PI3K/Akt	Human vascular endothelial cells	Dexamethasone	30, 33

556

557 **Table 3:** Examples of signaling pathways activated by GCs via nongenomic mechanisms that
558 were acute, sensitive (or insensitive) to GR blockade (RU486), and insensitive to protein
559 synthesis inhibition (cycloheximide).

560

561 **Figure legends.**

562

563 **Figure 1. Acute non-genomic effects of GCs on basal and agonist-induced Ca²⁺ responses.**

564 GCs have been described to differentially affect basal intracellular Ca²⁺ ([Ca²⁺]_i) homeostasis.
565 Depending on the cell type studied and GC applied, GCs can either reduce or augment basal
566 [Ca²⁺]_i. (A) GCs may decrease [Ca²⁺]_i by activating AC/PKA mediated mechanisms, likely
567 through events taking place at the cell membrane level and independent of GR stimulation,
568 ultimately leading to SERCA activation (thapsigargin-sensitive Ca²⁺-ATPase). (B) Conversely,
569 GCs can activate PLC/IP3 and PKC dependent signaling cascades resulting in enhanced basal
570 [Ca²⁺]_i; the involvement of GR in this process is currently unknown. (C) Agonist-induced
571 increases in [Ca²⁺]_i can be counteracted by GC-mediated activation of AC/PKA-induced
572 stimulation of SERCA pumps as described in ATP stimulated cells. In contrast, a functional
573 role for PKC was determined in the effects of GC on serotonin-induced Ca²⁺ responses,
574 suggesting that the acute inhibitory mechanisms of GCs are highly agonist specific. (D) Limited
575 studies are available on acute potentiating effects by GCs on agonist-induced Ca²⁺ responses;
576 in neuronal cells it was suggested that these effects are mediated via the rapid activation of
577 Ca²⁺-influx through ionotropic ATP-gated purinergic 2X receptors. Whether glucocorticoid-
578 mediated membrane receptors are involved in this pathway remains to be further investigated
579 (mGR?). These responses rely on the presence of external Ca²⁺. Abbreviations: AC, adenylyl
580 cyclase; AR, agonist receptor; IP3, inositol 1, 4, 5-triphosphate; GC, glucocorticoid; mGR,
581 membrane glucocorticoid receptor; PKA, protein kinase A; PKC, protein kinase C; SERCA,
582 sarco/endoplasmatic reticulum Ca²⁺ -ATPase.

583

584 **Glossary.**

585

586 **Muscle reactivity:** The ability of the muscle to respond to contractile agonists. It is impaired
587 during pathophysiological conditions such as asthma.

588 **Calcium mobilization:** Intracellular process triggered by external stimuli (e.g. contractile
589 agonists) where calcium is released to be engaged in different cellular functions such as increased
590 muscle reactivity and contraction. Calcium is usually acquired from extra-cellular sources
591 (calcium influx) or intracellular stores (e.g. endoplasmic reticulum).

592 **Genomic action:** Action that modulates the expression of genes. It involves transcriptional
593 processes where an activated transcriptional factor translocates to the nucleus and bind gene
594 promoters to modulate their expression. Such processes require certain time and are delayed.

595 **Glucocorticoid receptor (GR):** A nuclear receptor, which acts as a receptor and a transcriptional
596 factor. It is primary located in the cytosol. Glucocorticoids, through their lipophilicity, diffuse
597 across the cell membrane to bind GR in the cytosol. Such binding promotes the translocation of
598 GR to the nuclear where it binds gene promoters to modulate their expression. As described in this
599 article, several evidence demonstrate a membrane version of GR, not acting as a transcriptional
600 factor, but rather as a membrane receptor modulating the acute non-genomic effects of GC.

601 **Non-genomic Action:** Action that does not modulate the expression of genes. It does not involve
602 transcriptional processes or protein synthesis. Such action promotes rapid effects on events
603 proximal to the cell membrane to activate certain signal transduction pathways.

604 **Side effects of GC:** Due to their wide range of actions that include effects on the immune system,
605 metabolism, skeletal muscle, bone and eyes, to name just few, GC exert in addition to its intended
606 effect, some harmful effects especially when used in high dose and in long-term like in asthma
607 patients. Such effects usually require the genomic actions of GR.

608