Indian Journal of Biochemistry & Biophysics
Vol. 28, June 1991, pp. 159-163

Minireview

Glucocorticoid actions and biomodulators: An integrated biological control

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Received 16 October 1990; accepted 11 March 1991

Glucocorticoids produced by adrenal cortices under the control of the brain via pituitary, hypothalamus, and limbic system have a multitude of effects within the body. The major effects of glucocorticoids are to reduce inflammation, stabilize blood glucose, maintain muscle strength and promote fluid excretion. Glucocorticoids generally have anabolic effects on protein and RNA metabolism in liver and catabolic effects in other tissues like muscle, lymphatic and adipose tissues, skin and bone. In liver, they stimulate enzymes and increase protein and glycogen contents. In other tissues, including muscle, their catabolic actions inhibit synthesis and stimulate degradation of protein and RNA[1]. Prolonged high levels of glucocorticoids can lead to cell death of susceptible cells and account for muscle wasting and immunodeficiency[2]. Excessive amounts of glucocorticoids promote lypolysis in some areas (extremities) and lipogenesis in others (face and trunk). Glucocorticoids exert permissive effects on the action of many other hormones. Anti-inflammatory effect of glucocorticoids is known to be mediated by induction of lipocortin, an inhibitor of phospholipase A_2. Lipocortin prevents the production of fatty acid precursors for prostaglandins, prostacyclin, thromboxanes, and leucotrienes, some of which mediate the process of inflammation and pain[3]. Glucocorticoids are necessary for maintenance of normal blood pressure and cardiac output. They have been shown to stimulate the uptake of Na^+ into tubular epithilial cells of the large intestine and kidney and to maintain the fluid and electrolyte balance. Glucocorticoids are the key regulators of homeostasis and adaptation[4] and have an important role in the development, growth and aging processes[5-9].

Glucocorticoid Action Mechanism

Glucocorticoids exert their cellular and molecular actions through a cascade of regulatory events initiated by high affinity binding to their intracellular receptors. The hormone-receptor complexes traverse the nuclear membrane and bind to their acceptor sites usually located 100-300bp upstream from the RNA polymerase start site, ultimately causing the transcription of specific genes and thus modulate gene expression[10] (Fig.1). These acceptor sites are regions of DNA sequences known as glucocorticoid regulatory elements (GREs). An analysis of the GREs in the DNA of various genes such as rat tyrosine aminotransferase, tryptophan oxygenase, growth hormone and chicken lysozyme gene yields the consensus sequence of 15 nucleotides (5′-GGTACAnnnTGTTCT-3′). In genes that are negatively regulated by glucocorticoids, an imperfect copy of the GRE is found[11]. The glucocorticoid

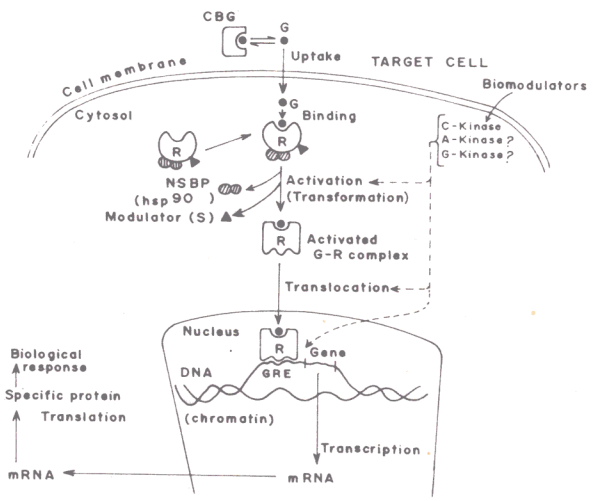

Fig. 1—Schematic representation of glucocorticoid action and its possible modulation. [Glucocorticoid (G) is bound to blood carrier protein (cortico-steroid-binding globulin; CBG). It is the free hormone which diffuses across the membrane of target cell and binds to specific receptor (R) protein in the cytoplasm. The hormone-receptor complex undergoes activation (transformation) with the removal of non-steroid binding protein (NSBP/hsp90) and other modulator(s). The activated glucocorticoid-receptor (G-R) complexes translocate to the nucleus where they bind to high-affinity binding sites termed as glucocorticoid regulatory elements (GREs), upstream from the regulated gene. This changes the synthesis of mRNA which then transported into the cytoplasm where it is translated into specific protein and elicit the biological response. The biomodulators may influence the glucocorticoid action at the level of activation and/or translocation. They may also influence some *cis/trans* acting factor(s) involved in gene expression in presence of glucocorticoid]

receptor is a ligand-dependent transcription factor, which regulates the expression of target genes in response to glucocorticoids. Although the receptors without the bound hormone are able to interact specifically with DNA *in vitro*, binding of hormone is needed for transcriptional activation *in vivo*[11].

The untransformed native glucocorticoid receptor (GR) is found in the cytosol as a 9-10s complex with a molecular weight of about 320 kDa[12-14]. This complex is a hetero-oligomer that contains a single molecule of the steroid binding protein[15] and two molecules of a non-steroid binding protein, identified as the 90 kDa heat-shock protein (hsp 90)[16-18]. The steroid binding protein is made of 795 amino acid residues. Sequence analysis and gene transfer experiments have defined the modular structure of this protein[19]. It consists of a carboxyl terminal hormone-binding domain, a central DNA-binding domain and an amino terminal immunologic/modulatory domain.

The exposure of crude cytosol to physiological temperature or to a high salt concentration in the cold, transform the hetero-oligomeric hormone-receptor complex to a monomer of 4s subunit with the molecular weight of 94 kDa, which is a nuclear binding form[20,21]. Activation/transformation of glucocorticoid-receptor complexes is one of the initial events that occur after the binding of the hormone to the specific cytosolic receptor in the target cells. Several factors have been implicated in the process of activation of glucocorticoid receptor from a non-DNA binding form to a form that binds to the nucleus or DNA. Although the exact mechanism of activation of receptor is still under active investigation, it is thought to be controlled by subunit dissociation[12,22], endogenous heat-stable cytoplasmic/nuclear factors[23-25], phosphorylation/dephosphorylation[26,27] and presence of small RNA molecules[28]. Hormone-dependent dissociation of hsp90 has been suggested to trigger the process of activation, possibly by unmasking the previously obscured DNA binding surface on the receptor protein[29]. Once the activated form of receptor is generated, it translocates to the nucleus and binds to acceptor sites on the nuclear chromatin. Interaction of activated hormone-receptor complexes with enhancer sequences upstream from the transcription start alters the local configuration of DNA and/or chromatin, which may modulate the transcription of the regulated gene[10].

Biomodulators of Glucocorticoid Action

It is now being considered that the hormone action on target cells is controlled not only by the concentration of hormones and their cognate receptors but also by modulators of these hormonal actions[30]. It was the pioneer work of Katunuma's group during early 70s which observed the existence of modulators that specifically enhanced the action of glucocorticoids in target organs. They found that the administration of a small dose of glucocorticoid induced liver tyrosine aminotransferase activity in germ-free rats less than that in conventional rats and isolated a fraction from enteric flora of rats that specifically enhanced the sensitivity of target organs or cells to glucocorticoid hormone without itself having any glucocorticoid-like action. This fraction was named as glucocorticoid sensitivity amplifier (GSA). The factor was isolated, purified from *Proteus mirabilis* and characterized as having pseudouridine, oleamide and phosphate[31,32]. Later, other compounds that markedly enhance the action of glucocorticoid, even with glucocorticoid concentrations having a maximum effect, were discovered[30] and termed as glucocorticoid potency amplifier (GPA). GPA themselves have no glucocorticoid-like activity. The GPAs reported so far are the potent activators of Ca^{2+}-/phospholipid-dependent protein kinase (protein kinase C), such as 1,2-racemic dioctanoyl glycerol (1,2-DG), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), guanisine 3′-diphosphate and epidermal growth factor (EGF)[30-32]. These natural compounds (GSAs and GPAs) that modulate glucocorticoid actions have been named as glucocorticoid action biomodulators[30].

Diacylglycerol (DAG), a potent activator of protein kinase C, specifically enhanced the induction of tyrosine aminotransferase (TAT) and ornithine decarboxylase (ODC) by even maximally effective doses of dexamethasone, but itself had no effect on these enzyme inductions in the absence of glucocorticoid. DAG did not affect the induction of these enzymes by glucagon and insulin[33]. It was indicated that DAG affects specifically the induction by glucocorticoids. In adrenalectomized rats, the tumor-promoting phorbol ester (TPA) markedly enhanced the inductions of TAT and ODC by glucocorticoids even at a maximally effective concentration of the hormone[34]. These findings speculated that protein kinase C may have some role in the action of glucocorticoid and that the modulators of the protein kinase C activity may regulate the action of glucocorticoid.

To ascertain further the role of protein kinase C in mediation of glucocorticoid action, Kido *et al.* reported that the induction of TAT by dexamethasone in rat hepatocytes was inhibited by 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H-7), an inhibitor of protein kinase C, but not by

N-[2-(methylamino) ethyl]-5-isoquinolinesulphonamide (H-8), an inhibitor of cyclic nucleotide protein kinase[35]. H-7 also inhibited the accumulation of glucocorticoid-receptor complexes in the nuclear fraction with a concomitant accumulation of these complexes in the cytoplasmic fraction[35]. It did not affect incorporation of glucocorticoid in hepatocytes. Based on these findings, the involvement of protein kinase C was attributed to the translocation of glucocorticoid-receptor complexes in the nuclei[35]. A similar phenomenon of reduced nuclear binding of labelled dexamethasone was also observed using rat liver slices. Sphingosine, a known selective inhibitor of protein kinase C[36,37], inhibits the induction of TAT and tryptophan oxygenase (TO) by dexamethasone in primary culture of rat hepatocytes[38]. It did not inhibit the induction of TAT by dibutyryl-cAMP. Finding indicated that sphingosine, an endogenous modulator of protein kinase C, may influence the expression of glucocorticoid action. Sphingosine, the backbone moiety of sphingomyelin, gangliosides and other complex sphingolipids is a potent and reversible natural inhibitor of protein kinase C activity *in vitro* and in cell systems and that inhibition of protein kinase C requires the hydrophobic character and the positively charged amines[36,37]. Protein kinase C, a Ca^{2+}-and phospholipid-dependent protein kinase, plays a central role in the transduction of extracellular signals into a cellular response[39]. Its activity is regulated by various putative lipid second messengers such as diacylglycerol, sphingosine and lysosphingolipids. A possible involvement of protein kinase C in mediation of glucocorticoid action expression has been advanced[30,38].

It has been demonstrated that endotoxin, the lipopolysaccharide component of the cell wall of Gram-negative bacteria, suppresses the glucocorticoid-mediated induction of phosphoenolpyruvate carboxykinase, tryptophan oxygenase, glucose 6-phosphatase and fructose 1,6-diphosphatase. The precise mechanism of the inhibitory effect of endotoxin remains unknown[40]. The same group later reported that hormone penetration into the cell is not affected in endotoxemia[41]. Further, it was shown that endotoxin has no effect on the glucocorticoid hormone penetration in the cells and also on the receptor binding capacity[42]. No difference was observed in the cytosol labelling in control and endotoxin-treated rats[43]. However, *in vitro* as well as *in vivo* experiments showed reduced nuclear binding of labelled dexamethasone in endotoxin-treated rats. Once the mechanism of action of endotoxin is known, it can be used in modulating glucocorticoid actions.

Administration of glucocorticoids is known to result in a rapid decrease in the number of circulating lymphoblasts and regression and disintegration of certain normal and malignant lymphoid tissues[44,45]. These effects are the basis for the use of glucocorticoids in treatment of certain cancers and some other lymphoid diseases. Anti-tumour effect of glucocorticoid on L5178J lymphoblasts *in vivo* is enhanced by diacylglycerol, an activator of protein kinase C. Diacylglycerol may be useful in enhancing the anti-tumour effect of a low dose of glucocorticoid[46].

Phosphorylation of Glucocorticoid Receptor

To assign the functional site of protein kinase C in glucocorticoid action, it is needed to dissect the phosphorylation/dephosphorylation state of glucocorticoid receptor. Phosphorylation of glucocorticoid receptor has been studied in a number of cells, tissues and animals under variety of conditions. In most of the studies, phosphorylation has been shown to play a role in the binding of hormone to the receptor[47]. Although phosphorylation is widely studied modification in regulating the activities of enzymes as well as non-enzymatic proteins, the conclusive evidence on the role of phosphorylation in regulating glucocorticoid receptor function is not clear. In the initial studies, Munck *et al.*[48] proposed that in cells deprived of ATP the receptor is present in a form that cannot bind hormone, the 'null receptor' form. It was also proposed that there is a cyclic event between the hormone binding/and non-binding form dependent on phosphorylation. Inactivation of receptors in intact cells was inhibited by phosphatase inhibitors such as molybdate, fluoride, vanadate and ATP[49]. These studies pointed out that inactivation of receptors may result from dephosphorylation. Later, it was speculated that if phosphorylation of the receptor is required for steroid binding, activation of glucocorticoid-receptor complexes may involve a dephosphorylation[50]. The importance of dephosphorylation in activation of glucocorticoid-receptor complexes was also studied with the use of phosphatase inhibitors such as molybdate and tungstate, which block the heat-induced receptor activation. The exact mechanism(s) by which molybdate and tungstate block the activation of bound hormone-receptor complexes is not well understood. The potential role of dephosphorylation in glucocorticoid receptor activation has been questioned[51,52]. The number of phosphate groups is

almost the same in both the non-DNA binding and the DNA binding forms of the receptor[53,54]. No quantitative change in the extent of receptor phosphorylation was observed in the process of activation. There are three phosphorylation sites in the glucocorticoid receptor: one within the steroid-binding domain and one/two in the immunologic domain and none in the DNA-binding domain[54]. Although uncertainty exists as to the precise location, and function, of the phosphorylated group(s), there is still no direct evidence that a change in phosphorylation state is involved in receptor function. A non-hormone-binding form of the glucocorticoid receptor has been identified in the nuclei of ATP- depleted WEHI-7 cells that appears to have lost one phosphate group[54,55].

Although glucocorticoid receptor is a phosphoprotein[56-58], the protein kinase(s) which phosphorylate glucocorticoid receptor and/or glucocorticoid receptor has intrinsic kinase activity is not yet known. Kinase activity intrinsic to rat liver glucocorticoid receptor has been reported[58-60]. Reported kinase activity associated with glucocorticoid receptor varies in its requirement for divalent cations. Purified rat liver glucocorticoid receptor exhibits a Mg^{2+}-dependent protein kinase activity[59]. Ca^{2+}-dependent protein kinase activity associated with rat liver glucocorticoid receptor has also been reported[60]. The purified preparation of rat liver glucocorticoid receptor also phosphorylates exogenous substrates in presence of Mg^{2+} (ref. 58). Using immunoaffinity purified preparation of glucocorticoid receptor, it has been shown that glucocorticoid receptor does not exhibit intrinsic kinase activity, while a Mg^{2+}-dependent protein kinase contaminant was observed[61-63]. Besides, neither the protein kinase(s) involved in the phosphorylation of glucocorticoid receptor has been identified nor the role of receptor phosphorylation in DNA binding, trans-activation, and recycling of the receptor established. Once the role of receptor phosphorylation/dephosphorylation and the kinase(s) and phosphatase(s) involved therein are fully understood, it will become relevant target for modification by various modulators.

Concluding Remarks

At present it is difficult to attribute a direct functional role of protein kinase C in glucocorticoid action. Alterations in glucocorticoid responsiveness by protein kinase C modulators show that Ca^{2+}-/phospholipid-dependent protein kinase is directly/indirectly involved in glucocorticoid action. The exact site(s) of phosphorylation by protein kinase C of glucocorticoid receptor and/or non-receptor components are not known. *Albeit,* the possibility of phosphorylation by protein kinase C of some *cis/trans* acting factor(s) involved in glucocorticoid regulation of gene expression cannot be ruled out[64-66]. A careful dissection of the involvement of protein kinase C in glucocorticoid receptor phosphorylation and its role in receptor function is of immediate interest. It seems plausible that the cascade mechanism of glucocorticoid action may not be a completely isolated phenomenon; instead the influence of various endogenous regulators of protein kinases in general and protein kinase C in particular may be considered as an integrated biological control. It is an exciting area of investigation to achieve the greater action of glucocorticoid even at a minimal dose to avoid the toxic effect of higher dose of the hormone.

Acknowledgement

I am grateful to Prof. Nobuhiko Katunuma and Dr Hiroshi Kido of the Institute for Enzyme Research, Tokushima University, Japan for inviting me as a visiting professor where I worked on the biomodulators of glucocorticoid action. I am also thankful to the Ministry of Education, Science and Culture of Japan for supporting visiting professorship and to the North-Eastern Hill University, Shillong for granting me a leave of absence during course of my study.

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