The Use of Nanosized Cortisol-Polymer Complex for Analysis of Mechanisms of Regulation of Functional Activity of Skin Fibroblast

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Cortisol in concentrations within the therapeutic range inhibits calcium response of fibroblast to angiotensin II. In physiological concentrations, cortisol potentiates the effects of angiotensin II via modulation of membrane mineralocorticoid receptors. The inhibitory effects of glucocorticoids on cell proliferation and collagen synthesis are manifestations of its genomic effects mediated by intracellular receptors. The use of glucocorticoid preparation based on nanosized polymer structure made it possible to distinguish between the genomic and nongenomic mechanisms of regulation of activity of target-cells.

Key Words: *glucocorticoids; aldosterone; calcium ions; skin fibroblasts; nanopharmacology*

Glucocorticoids (GC) are widely used in clinical practice as immunodepressant and antiinflammatory preparations. However, these products have substantial disadvantages determined by low specificity of action and manifesting as side effects of hormone therapy (Cushing's syndrome, immunity impairment, healing deceleration, psychosis-like reactions, *etc*.).

Predetermined modification of drugs using nanotechnologies includes two main approaches: synthesis of nanosized drugs on the basis of their polymer (conjugation of drugs with polymer molecules) [15] and incorporation of drugs into nanostructures presented by micelles, dendrimers, spheres (fullerenes) [5]. Conjugation of drugs with polyethyleneglycol (PEG), or pegilation, increases drugs solubility due to its intrinsic hydrophilicity, increases the size and weight of the particles, thereby reducing their renal excretion. PEG limits drugs availability for proteolitic enzymes and antibodies. Examples of such products approved by FDA (Food and Drug Administration): PEG-asparaginase (Oncaspar) designed to treat lymphocytic leukemia and other lymphoid tumors; PEG-adenosine deaminase (Adagen) designed to treat severe combined immunodeficiency disorders.

The development of drug modification by using dendrimers is now in progress. The following drug modifications are most intensively developed: NSAID (non-steroidal anti-inflammatory drugs), antimicrobial and antiviral products, anti-tumor compounds (cytostatics, radionuclides, photosensitive compounds). This can be explained by high epidemiological significance of corresponding pathologies, rather than physicochemical properties of these drugs.

Investigation of molecular mechanisms of early membranotropic effects of GC on the target cells is a new trend associated with the creation of synthetic nanosized GC structures (nanosteroids). A series of publications about fast extragenomic membranotropic effects of steroids appeared in recent years [6,13]. They include inotropic and vasoconstrictive effects of aldosterone, fast neuronal effects of progesterone, and early effects of vitamin D3 on calcium and cAMP levels in bones. The involvement of membrane estrogen and gestagen receptors into regulation of membrane-bound enzymes adenylate cyclase, protein kinase C, 5'-nucleotidase under normal conditions and during tumor growth was also demonstrated [8,9]. We previ-

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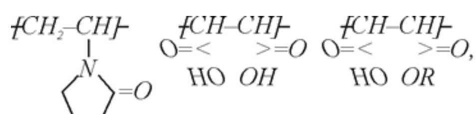
ously showed that complexation with polymer materials, particularly, with polyvinyl pyrrolidone increases affinity of steroid hormones to membranes and modifies their genotropic activity [3]. Polyvinyl pyrrolidone-glucocorticoid complex (PVP-GC) does not enter the cell, but can potentiate the effects (permissive action) of adrenomimetics (isadrine) and unbound GC on the model of rat thymocytes: it increases cAMP level and cytostatic activity of GC. Creation of complexes, which do not enter the cell, but potentiate, for example, the effects of adrenomimetics and antiallergic compounds, will allow to develop new antiallergic and anti-inflammatory compounds with selective action.

Here we studied genomic and non-genomic mechanisms of regulation of activity of skin fibroblasts using polymer nanosized cortisol preparation.

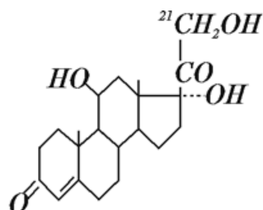
MATERIALS AND METHODS

Fibroblasts were chosen as the object of research, because they are the target cells for pharmacological action of GC and are involved in inflammatory and allergic reactions. Regulation of functional activity of fibroblasts at the membrane level opens new prospects for therapeutic modulation of inflammation and remodeling processes in the skin under normal and pathological conditions.

Polymer GC derivatives were synthetized in Institute of High Molecular Weight Compounds, Russian Academy of Science. By their chemical structure, polymer derivatives of cortisol (hydrocortisone) correspond to water-soluble triple copolymer of vinylpyrrolidone, maleic acid, and monosubstituted steroid maleate.



where R — hydrocortisone.



Mean molecular weight of copolymers is 24,000, hydrocortisone content is 4.4 molar % (13.2 weight %). Ester bond between the macromolecule and steroid is formed by polymer anhydride group and C(21) hydroxyl group of GC.

Fibroblasts were isolated from rat skin as is described previously [1]. The cells were cultured in 96-well plates at 5% CO_2 and 37°C in DMEM supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml; "Gibco"). Fibroblasts were stimulated by incubation with 100 nM angiotensin II (AII) for 24 h. The test GC preparations in a final concentration range from 10 nM to 1 μM, PVP-GC, cortisol, dexamethasone, antagonist of mineralocorticoid receptor spironolactone, and antagonist of GC receptors mifepristone in a final concentration of 10 μM were added to the medium 1 h before AII.

Intracellular levels of free calcium ions were measured using Fura-2/AM fluorescent dye according to previously described method [2]. Proliferative activity of fibroblasts was assessed by [^{3}H]-thymidine incorporation [7], collagen synthesis was evaluated by [^{3}H]-proline incorporation [14]. Statistical analysis was performed using Student's test. The data are represented as mean±confidence interval (p=0.05).

RESULTS

The studied genomic mechanisms of regulation of fibroblast activity included: effects of GC on basal and AII-stimulated cell proliferation; effects of GC on basal and induced collagen synthesis assessed by incorporation of tritium-labeled proline.

AII significantly increased incorporation of labeled thymidine by 57% and proline by 31%. Proliferative effect of AII (100 nM) and its effects on collagen synthesis in fibroblasts are mediated by AT-1 membrane receptors [11], AII effects can be abolished by concurrent AT1-receptor antagonist irbesartan (1 μM).

GC in a concentration range from 1 nM to 10 μM inhibited [^{3}H]-thymidine incorporation into fibroblast DNA. In concentrations >10 nM GC significantly decreased AII-stimulated DNA synthesis; starting form the concentration of 1 μM, GC reduced basal level of labeled thymidine incorporation (Fig. 1, *a*). The effects of GC on collagen synthesis are determined mainly by inhibition of AII-induced synthesis, basal level of labeled proline incorporation decreased by no more than 10-15%. Comparison of GC activity showed that dexamethasone was 4-5-fold more potent in inhibiting both cell proliferation and collagen synthesis compared to cortisol. The effects of GC were abolished in the presence of 10-fold excess of intracellular GC receptor antagonist mifepristone. PVP-GC had no significant effects on basal and AII-stimulated synthetic activity of fibroblasts (Fig. 1, *b*).

The extragenomic mechanism of regulation of fibroblast activity studied by us included effects of GC on basal and AII-stimulated intracellular level of free calcium ions ($[Ca^{2+}]_{cyt}$). PVP-modified cortisol was used as a tool in the study of non-genomic membrane-mediated effects of GC.

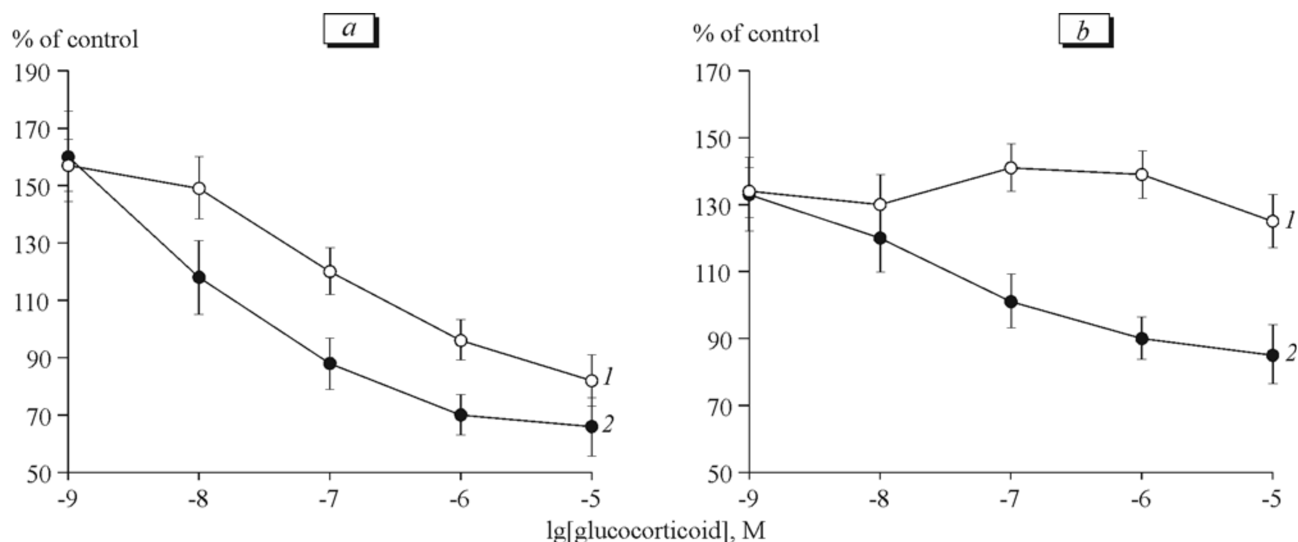


Fig. 1. Effects of GC on basal and AII-induced DNA (*a*) and collagen (*b*) synthesis in fibroblasts. *2*) dexamethasone. For *a*: *1*) cortisol; for *b*: *1*) PVP-cortisol. AbscissaOrdinate: [^{3}H]-thymidine incorporation (*a*) or [^{3}H]-proline incorporation (*b*); Ordinateabscissa: $\log_{10}$ of final steroid concentration in the incubation medium.

$[Ca^{2+}]_{cyt}$ in resting cells did not exceed 73 nM (61±12 nM). Addition of AII 100 nM to the cell suspension led to a maximal increase in intracellular calcium concentration to 209±32 nM (*n*=6) after 15-20-sec incubation. Preliminary addition of PVP-GC to fibroblasts significantly changed Ca-response of the cells to AII.

PVP-GC in nanomolar concentrations (2-10 nM) potentiated the calcium-stimulating effect of AII: the dose-response curve was shifted to the left (Fig. 2). One may assume that the effects of GC are mediated by membrane receptors of mineralocorticoids, because the potentiating effect of PVP-GC was abolished by mineralocorticoid receptor antagonist spironolactone, while mineralocorticoid aldosterone in concentration 0.5 nM potentiates the effects of AII (Fig. 2). It should be noted, that synthetic GC dexamethasone was far inferior to cortisol by its activity.

PVP-GC in micromolar concentrations 0.5-2.0 μM (pharmacological concentrations) inhibited the induced increase in $[Ca^{2+}]_{cyt}$ and had no effect on basal calcium level in fibroblasts. Latent period was not required for calcium-blocking effect of GC.

These findings suggest that the extragenomic GC effect on $[Ca^{2+}]_{cyt}$ had a clear-cut two-phase pattern. The detected differences in the action of hormone cortisol and synthetic GC dexamethasone at the level of membrane receptors correspond to previously established facts of different sensitivity of membrane steroid receptors to natural hormones and their synthetic analogues [6].

Thus, the use of nanosized cortisol preparation revealed two types of extragenomic GC effects on fibroblasts: potentiating effect of GC on AII effect mediated through mineralocorticoid receptors and antagonistic membranotropic effect of GC manifesting when pharmacological concentrations of steroids were used.

It was previously accepted that anti-inflammatory, anti-allergic, antitoxic, and immunodepressive effects of GC are realized through one receptor and therefore inseparable from each over. However, molecular mechanisms of the effects of GC are still intensively investigated and subtypes of intracellular GC receptors were identified (isoforms α and β) [12]. Since receptor dimerization is necessary for genotropic activity of GC, it is obvious that three variants of dimers can appear, and their ratio in different cells can influence the effect of glucocorticoids in different tissues.

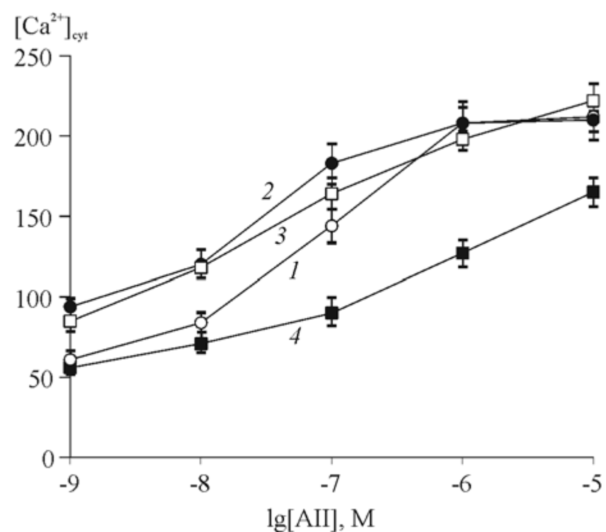


Fig. 2. Effects of steroids on AII-induced calcium level in fibroblasts. *1*) control (20 μl of DMEM medium); *2*) aldosteron; *3*) PVP-GC, 10 nM; *4*) PVP-GC, 1 μM. Ordinate: intracellular concentration of free calcium ions, nM; abscissa: $\log_{10}$ of final AII concentration in the incubation medium.

The therapeutic response to GC and the development of resistance to GC depend on dimerization pattern, down-regulation of receptor β-isoform and repression of transcriptional factor NF-κB [10]. Fluorinated steroids (dexamethasone) more rapidly form dimers and induce genome-mediated apoptosis of hypothalamic cells, while prednisolone derivatives stimulate the formation of both dimers of GC receptors and their heterodimers with mineralocorticoid receptors, thus providing smoother and more physiological effects on regulation of the hypothalamic–pituitary–adrenal axis or proliferation of epidermis cells [4].

It can be assumed that the use of PVP-modified steroids (nanosteroids) will help to selectively regulate functional activity of fibroblasts at the level of membrane receptor system, which opens new vistas for further evolution of topical GC used in dermatology using nanosized design of original molecular complexes.

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