RECEPTORS AND CELL SIGNALING, INTRACELLULAR RECEPTORS — STEROID HORMONES AND NO

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OUTLINE

INTRODUCTION

Soluble extracellular molecules that cross the cell membrane, bind an intracellular receptor, and alter cellular function are essential regulators of mammalian endocrine biology. This article reviews two of the most well-studied and prototypical intracellular receptors: steroid hormone receptors, which are transcriptional regulators, and the nitric oxide receptor, which is soluble guanylate cyclase. These two systems are quite different and represent the diverse mechanisms by which animal cells respond to diffusible mediators that cross the plasmalemma. While steroids alter gene and cell regulation on a time scale of minutes to days, nitric oxide (NO) has diverse effects (on a time scale of seconds to hours) such as vasodilation, inhibition of platelet activation, neurotransmission, and cytotoxicity. In some instances, crosstalk exists between NO and steroid receptor pathways. The biochemistry and molecular biology of steroids and NO can impact numerous biotechnological applications such as gene therapy, tissue engineering, and bioreactor dynamics, particularly in relation to the external control of cellular metabolism and gene expression.

STEROIDS AND THEIR BIOSYNTHESIS

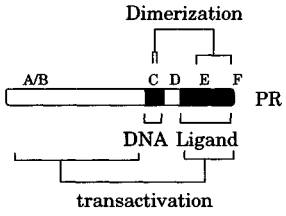
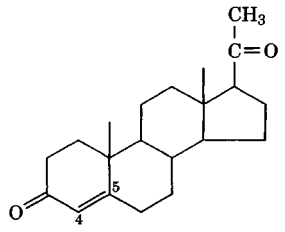
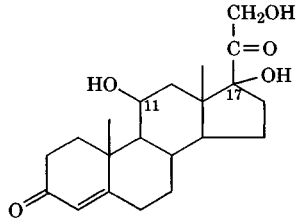
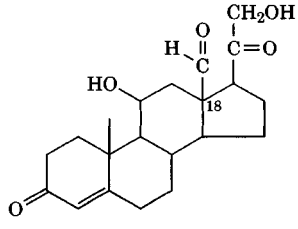
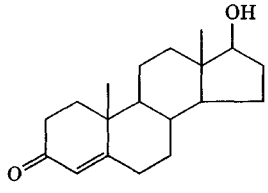
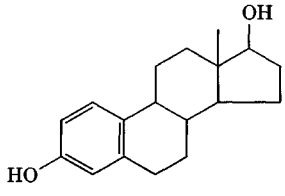
Steroid hormones are small hydrophobic metabolites of cholesterol (C_{27}), and are broadly divided into five classes (Table 1): *progestagens, glucocorticoids, mineralocorticoids, androgens,* and *estrogens*. Cleavage of the C_6 unit from cholesterol involves the hydroxylation of C-20 and C-22 followed by cleavage by desmolase to produce pregnenolone (C_{21}). Conversion of pregnenolone to progesterone requires oxidation of the 3-hydroxyl group to a 3-keto group and isomerization of the C-5–C-6 double bond to the C-4–C-5 position. Progesterone can be metabolized to the major glucocorticoid, cortisol (by multiple hydroxylations), or to the major mineralocorticoid, aldosterone (by multiple hydroxylations and oxidation of the C-18 methyl group to an aldehyde). Alternatively, progesterone can be modified to androgens and estrogens via specific hydroxylation, chain cleavage, and reduction reactions. Thyroid hormones, retinoids, and vitamin D are not derived from cholesterol and are unrelated in structure to steroid hormones, but also diffuse across the cell membrane to bind their intracellular receptor in target tissues.

STEROID RECEPTORS AND GENE REGULATION

Steroid regulation of gene expression involves steroid molecules crossing the plasmalemma via passive transport (Fig. 1). Once in the cytoplasm, the steroid binds its receptor with high affinity ($K_d \sim 1$ nM), which causes the release of chaperones [including heat shock proteins hsp70 and hsp90, p23, and various peptidylprolyl isomerase (PPIase)-active immunophilins/cyclophilins (CyP) such as FKBP52 (hsp56), CyP18, and CyP40] from the receptor, and consequent exposure of nuclear localization sequences and phosphorylation sites in the steroid receptor (1,2). The chaperones maintain the receptor in a conformation that is competent for steroid binding but lacks DNA regulatory activity. The receptor with bound steroid (termed the steroid receptor complex or liganded steroid receptor) undergoes nuclear import, after which the liganded steroid receptor dimerizes. The dimer binds its specific hormone response element (HRE or SRE for steroid response element) to activate or repress within 30 min about 50 to 100 *primary response* genes via its interactions with numerous other transcriptional factors and consequent changes in chromatin structure. Other *secondary response genes* can then be regulated over the course of hours or days through mechanisms requiring expression of the primary response genes. The ability of a liganded steroid receptor to find the handful of promoters containing HRE among the ~100,000 genes in the mammalian genome represents an extraordinary diffusional search process. The dimer may undergo one-dimensional diffusion along DNA segments or DNA–intersegment transfer facilitated by the bivalent interactions made possible with a dimer complex (3).

The steroid receptor has three functional domains: the N-terminal domain (site of transcriptional activation and binding of transcriptional factors; A/B domain in Table 1), a DNA binding domain (which also confers dimerization; C/D domain in Table 1), and a hormone binding domain at the C-terminus of the protein which also participates in transcriptional activation (E domain in Table 1) (4).

Table 1. Steroids and their receptors

Steroid	Receptor	Response element	Steroid structure
Progestagens (C_{21}) Pregnenolone 17-hydroxypregnenolone Progesterone 17-hydroxyprogesterone		Same as *GRE*	 **Progesterone**
Glucocorticoids (C_{21}) Corticosterone Deoxycorticosterone Cortisol 11-Deoxycortisol Cortisone 18-hydroxycorticosterone 1-α-hydroxycorticosterone Aldosterone		GRE 5′-**AGAACA**nnn**TGTTCT**-3′	 **Cortisol**
Mineralocorticoids (C_{21}) Aldosterone		Same as *GRE*	 **Aldosterone**
Androgens (C_{19}) 11-β-hydroxyandrostene dione 5-α-Androstanediol Androsterone Epiandrosterone Andronosterone Testosterone Epitestosterone 5-α-dihydrotestosterone 5-β-dihydrotestosterone 11-keto-testosterone		Same as GRE	 **Testosterone**
Estrogens (C_{18}) Estrogen Estrone Estradiol Estriol		ERE 5′-**Pu**GGTCA**nnn**TGACC**Py**-3′	 **Estradiol**

The DNA binding domain and hormone binding domain are highly conserved across the steroid receptor family, while the N-terminus, which controls transcriptional regulatory activity, is not. Crystal structures are widely available for many mammalian steroid receptors (see *http://nrr.georgetown.edu/NRR/NRR.html*).

The liganded steroid receptor dimer bound to its HRE has an extremely complicated and not fully characterized set of interactions with other proteins. Once in the nucleus, steroid hormone receptors can interact in three-dimensional assemblies with general transcription factors (TBP, $TAF_{II}30$, $dTAF_{II}110$, TFIIB), coactivators (ARA_{70}, RIP140, ERAP, TIF-2, SRC-1, RSP5, TIF1, SUG1, GRIP170), sequence-specific transcriptional factors (fos/jun, GATA-1, RelA, PML, OTF-1), and chromatin regulators (hBrm, BRG1, Spt6, and HMG1) (5–11). Coactivators can bridge the dimer with other DNA binding proteins of the transcriptional initation complex that assemble on the TATA box of the promoter (Fig. 1). In contrast to activation, gene repression is commonly

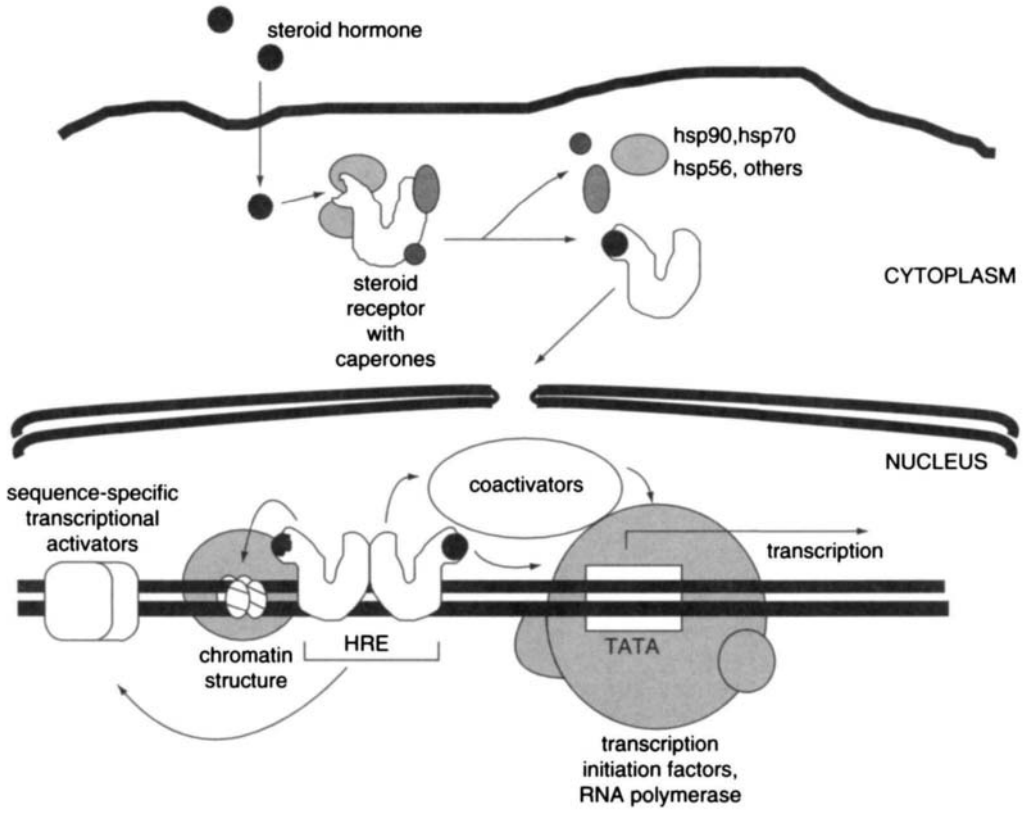


Figure 1. Steroid receptor function.

viewed as an action of the steroid hormone dimer that: (*1*) sterically blocks DNA binding sites used by other factors, such as fos/jun (AP1 complex); (*2*) inactivates normally competent transcription factors; or (*3*) serves as a binding sink for factors needed for transcription (4). In addition to these issues of assembly on the promoter, steroid receptors may function by altering the structure of the chromatin via its effects on hBrm and BRG1 (5–7). hBrm is the human homolog of *Drosophila Brama* (*brm*) and yeast SWI/SNF complex and is a 180-kDa nuclear protein that mediates ATP-dependent nucleosome disruption. BRG1 (205 kDa) is a second human homolog of yeast SWI/SNF that binds the retinoblastoma gene product Rb, on which assembly processes occur. Interestingly, hBrm is not expressed in all cells.

The human progesterone receptor (PR) can bind steroid receptor coactivator 1 (SRC-1A) for up-regulation of transcription. Interestingly, SRC-1A has histone acetyltransferase activity. Histone acetylation opens up the chromatin structure for transactivating factors. Human PR can also bind p300/CREB-binding protein (CBP)-associated factor, which has histone acetyltransferase activity. The localization of histone acetyltransferase activity by PR suggests an important role in chromatin reorganization since histone deacylase activity can repress the PR activation of gene expression (12). Similar interactions are found for the human estrogen receptor (13).

The HRE are typically palindromes containing two 6-base consensus half-site sequences separated by a spacer region (Table 1), although direct repeats of the consensus sequence are possible. A promoter may contain several HREs. Receptors for glucocorticoids, mineralocorticoids, progesterone, and androgens utilize the same 6-base DNA sequence 5′-AGAACA-3′ (4). The estrogen receptor utilizes the sequence 5′-AGGTCA-3′, which is also the sequence used by nonsteroid nuclear receptors. The DNA binding domain of the steroid receptor contains two zinc binding motifs—the *P Box* binds the HRE half-site and the *D Box* facilitates dimerization. Liganded steroid receptors predominantly form homodimers on the HRE in vivo. The nonsteroidal thyroid hormone response elements contain copies of a core consensus motif: 5′-AGGTCA-3′ commonly in a head-to-tail repeat separated by four base or inverted tail-to-tail repeats. Numerous variations exist naturally or have been constructed artificially. The response elements for one or more hormone receptors may exist on a single promoter to cause synergistic activation of the gene. In promoter construct experiments, a single glucocorticoid response element (GRE) (or estrogen response element, ERE) upstream of a TATA box confers a steroid responsive promoter, with two GRE (or two ERE) elements resulting in synergistic gene activation. Such experiments indicate that the steroid hormone receptor dimer can interact directly with TATA binding factors to cause gene activation, independent of other transcriptional factors. Mutations in steroid receptors and their associated proteins are implicated in a variety of developmental, inflammatory, and carcinogenic processes. For example, the androgen receptor has a critical role in male sexual differentiation, and its mutations may play a role in prostate cancer (10).

NITRIC OXIDE AND NITRIC OXIDE SYNTHASE

The pioneering discovery by R. Furchgott in 1980 (14) that acetylcholine-induced vasodilation was endothelial-dependent set the stage for the identification of endothelial-derived relaxing factor (EDRF) as nitric oxide. By 1998, over 22,000 publications have defined in great detail the biochemistry, molecular biology, and physiology of the nitric oxide pathway. In mammalian cells, L-arginine is cleaved by nitric oxide synthase (NOS) to NO and citrulline (Fig. 2). Three isoforms of NOS have been cloned and their gene products fully characterized: neuronal NOS (nNOS, Type I isoform, 155 kDa); inducible NOS (iNOS, Type II isoform, 130 kDa); and endothelial NOS (eNOS, Type III isoform, 140 kDa). The genes are highly homologous across species, and the three isoforms are about 50% homologous with each other (15). For full NO synthesis activity, the eNOS enzyme requires three co-substrates, L-arginine, O_2, and NADPH, along with several cofactors, FMN, FAD, tetrahydrobiopterin (BH_4), calmodulin and a heme group, as well as, superoxide dismutase and glutathione. Distinct from iNOS, the isoforms eNOS and nNOS are activated when intracellular calcium is elevated above resting levels. The eNOS gene does not contain a TATA box, which is not uncommon for constitutive genes. The eNOS promoter contains two AP-1 sites, five SP1 (GC-rich) sites, and SSRE and NF1 sites. The SP-1 sites in the eNOS promoter have been shown to be functional by gel shift assays (16). The iNOS gene is dramatically induced in macrophages, smooth muscle cells, and other cell types by cytokines (IFN-γ, TNF-α, and IL-1) as well as bacterial endotoxin. The transcriptional factor NF-κB is critical for the induction of iNOS.

In several bioprocess or biomedical environments, the cellular response to mechanical forces can regulate cellular function. Nitric oxide can play an important role in the response of mammalian cells to fluid mechanical forces. The eNOS mRNA and protein levels can be elevated several fold in endothelial cells when the cells are exposed to laminar shear stress (15 or 25 dyn/cm^2) (17,18). The eNOS enzyme can be activated by fluid shear stress via a tyrosine kinase–dependent mechanism that is largely independent of elevation of intracellular calcium (19) or binding of hsp90 (20). During chemical activation of endothelium, eNOS can be phosphorylated, palmitoylated, and myristoylated at specific sites on the enzyme. In light of eNOS activation by shear stress, several changes in the post-translational state of eNOS are expected. (see Whiteley et al.—Protein Processing, Processing in the Endoplasmic Reticulum and Golgi Network).

NITRIC OXIDE: cGMP-DEPENDENT SIGNALING

Nitric oxide rapidly diffuses through cellular membranes to bind its intracellular receptor, soluble guanylate cyclase, leading to the production of cGMP. Guanylate cyclase is a heme-containing protein. Nitric oxide is a highly reactive species that exerts autocrine and paracrine action over very short distances of a few hundred micrometers due to its short half-life in biological fluids. However, recent evidence has raised the possibility that NO can bind to oxyhemoglobin in an S-nitrosothiol complex (21).

Endothelial-produced NO, elicited by shear stress, can act as an autocrine factor to elevate cGMP levels (22). NO released by endothelium can diffuse through the internal elastic lamina to reach smooth muscle cells in the media of the vessel. In smooth muscle cells, the elevated levels of cGMP caused by NO binding and activating soluble guanylate cyclase results in a potent smooth muscle cell relaxation and consequent vasodilation. The signaling pathway downstream of guanylate cyclase activation includes the activation of cGMP-dependent protein kinases and subsequent phosphorylation of several targets such as calcium-sensitive potassium channels (K_{Ca}). Additionally, there exist cGMP-gated ion channels and cGMP-regulated cyclic nucleotide phosphodiesterases.

NITRIC OXIDE: cGMP-INDEPENDENT SIGNALING

During the bioreactor cultivation of animal cells, sublytic fluid shear forces may have significant effects on cellular metabolism. Hydrodynamic shear stress elevates the release of NO and prostacyclin (PGI_2) from endothelial cells in culture (23,24). Recent reports have suggested that elevated levels of NO can lead to the activation of constitutive cyclooxygenase (COX1, also named constitutive PGH synthase 1) (25–27). Davidge et al. (25) tested whether NO-activated PGI_2 production required cGMP using several different approaches. Their work indicated that NO stimulation of PGI_2 production is most likely independent of cGMP levels. This is consistent with earlier reports that elevated levels of cGMP do not enhance PGI_2 production in bovine endothelial cells (28). Treatment of bovine aortic endothelial cells in culture

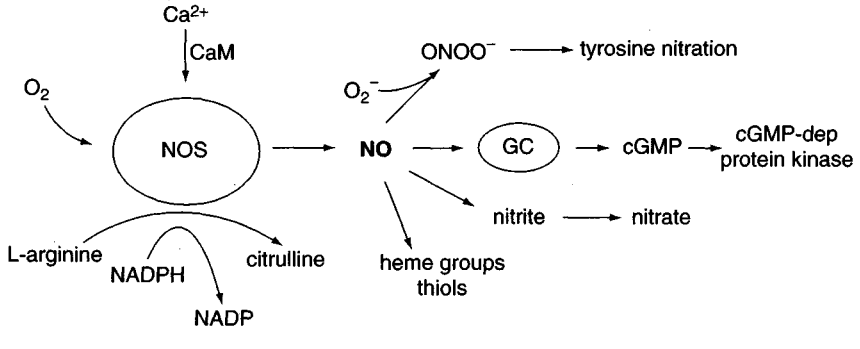


Figure 2. The NO pathway. Activation of nitric oxide synthase (NOS) results in the production of nitric oxide (NO), which can bind and activate soluble guanylate cyclase (GC), resulting in the production of cyclic GMP (cGMP). The activation of cGMP-dependent protein kinase leads to phosphorylation events that alter cellular function. Through nonenzymatic routes, NO rapidly reacts with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), which can react with tyrosine residues to form nitrotyrosine.

(BAEC) with glyceryl trinitrate, sodium nitroprusside, or 3′-morpholinosydnonimine (SIN-1, a donor of NO and O_2^-, and consequently peroxynitrite) dramatically enhanced by four- to six fold the release of PGI_2 from arachidonic acid–stimulated BAEC via cGMP-independent pathways (27). In calcium ionophore A23187-treated bovine microvessel endothelial cells, both NO and PGI_2 production were increased several fold in a cGMP-independent manner, but inhibition of NO production with N^G-nitro-L-arginine methyl ester (LNAME) attenuated the PGI_2 release by half (25). Recently, peroxynitrite generated from the reaction of NO with O_2^- was shown to activate purified ram seminal vesicle COX1 and recombinant human COX2 by serving as a substrate for the peroxidase activity of the enzymes (29). While BAEC posseses a soluble guanylate cyclase that can be activated by nitric oxide to elevate intracellular cGMP levels (22), there is some uncertainty if human umbilical endothelial cells or BAEC possess cGMP-dependent protein kinases (30). However, a more recent report has demonstrated cGMP-dependent protein kinase type I in BAEC (31). In light of the above observations, Wang and Diamond (32) tested the hypothesis that shear induced nitric oxide was responsible for the elevated release of PGI_2 when endothelial cells were exposed to arterial levels of shear stress. They found that blocking shear-induced NO production caused ~50% decrease in shear-induced production of PGI_2.

While the COX enzymes are potential targets for NO activation because they contain an iron-heme center at their active site, Tsai et al. (33) found no activation of cyclooxygenase activity at NO concentrations ranging from micromolar to millimolar added to purified ovine seminal vesicle COX1 enzyme. This is in contrast to a report of heme-independent S-nitrosation of cysteines in the active site of COX1 (34). Furthermore, in the mouse macrophage cell line RAW264.7, lipophilic superoxide dismutase–mimetic agents decreased the prostaglandin production without affecting the level of NOS or COX or by inhibiting the release of arachidonic acid (29). Clearly, nitric oxide has diverse effects as an intracellular signaling molecule, many of which are independent of soluble guanylate cyclase.

NITRIC OXIDE, PEROXYNITRITE, AND NITROTYROSINE

Excessive production of nitric oxide has also been associated with cellular injury (35). Nitric oxide–related tissue injury may be at least partially due to peroxynitrite ($ONOO^-$), a relatively long-lived, strong cytotoxic oxidant that is generated by the diffusion-limited reaction between nitric oxide (NO) and superoxide anion (O_2^-) with a second-order rate constant of $6 \times 10^9\ M^{-1}\ sec^{-1}$ (36). High levels of NO production can result in the formation of significant amounts of $ONOO^-$, because nitric oxide is capable of outcompeting superoxide dismutase for the metabolism of cellular superoxide.

Oxidative stress and reactive oxygen species (ROS) may contribute to the response of mammalian cells to various environmental stimuli, either chemical or mechanical. The antioxidant N-acetyl cysteine (NAC) has been shown to impair the induction of endothelial intracellular adhesion molecule (ICAM-1) by shear stress (37) via elevated production of ROS. Similarly, NAC impairs the induction of ICAM-1, monocyte chemotactic protein-1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1) in endothelium exposed to cyclic stretch (38–40). It is unclear why ROS generated by stretch (but not shear stress) can induce the PAI-1 gene. Interestingly, the MCP-1 gene, which is induced by shear stress via AP-1 elements in the promoter (41) by mechanisms that likely involve ROS (38), is also induced by NO (42). The activation by shear stress of AP-1 activity, and shear stress mediated induction of ICAM-1, MCP-1, and PAI-1 through ROS pathways, may involve NO and peroxynitrite.

Protein nitration by peroxynitrite has been hypothesized to be a major mechanism of oxidative modification of proteins associated with atherosclerosis (43). Peroxynitrite nitrates the aromatic ring of free tyrosine to produce a stable product, 3′-nitrotyrosine, which accumulates in proteins and acts as a cumulative index of peroxynitrite production. This reaction occurs spontaneously but is also catalyzed by low-molecular-mass transition metals as well as superoxide dismutase. This aberrant addition of a nitrate group to the *ortho* position of the hydroxyl group of tyrosine represents one outcome of cellular oxidative stress, rendering nitrated proteins dysfunctional and disrupting the phosphorylation of tyrosine residues in proteins involved in cell signaling networks. More specifically, peroxynitrite was recently reported to have vasoregulatory significance by relaxing vascular tissue through nitrosylation of tissue glutathione (or other thiols), subsequently releasing NO over prolonged time periods (44,45).

CROSSTALK BETWEEN NITRIC OXIDE AND STEROID SIGNALING

The nuclear factor NF-κB exists as an inactive cytoplasmic heterotrimer of p50 and p65 complex bound by the inhibitor IκB. Upon phosphorylation of IκB by tyrosine kinases or IκB kinase [potentially activated by protein kinase C isoforms that are activated by shear stress (46)], the heterodimer p50,p65 is released as an active DNA binding complex, which rapidly translocates to the nucleus. NF-κB activity is inhibited by dexamethasone, sodium salicylate, and aspirin via distinct mechanisms (47). The expression of the iNOS gene is dramatically elevated by cytokines and endotoxin through a NF-κB pathway. The induction of iNOS is markedly blocked by the steroid dexamethasone (48). Thus NF-κB and IκB regulation represents an important point of crosstalk between NO/NOS and steroid pathways. Furthermore, it has been shown that nitric oxide donors such as S-nitroso-glutathione decrease cytokine induction of endothelial vascular cell adhesion molecule (VCAM-1) and endothelial-leucocyte adhesion molecule (ELAM) (49). Promoter construct experiments and gel shift assays indicated that NO partially inhibited the activation of NF-κB. This is consistent with the role of NO as an anti-inflammatory and antiatherosclerotic agent (50) similar in function to steroids such as estrogen. While NF-κB activity is induced at early times of shear stress exposure (51), probably through PKC and mitogen activated

protein (MAP) kinase signaling, NF-κB may eventually be downregulated through the continued elevated production of NO by shear-stressed endothelium. There have been several reports of endothelial genes (for example, PAI-1) being less inducible by cytokines after shear stress exposure (52). It is attractive to speculate that the lack of cytokine inducibility in sheared cells is NO dependent. This scenario, however, is in contrast to NO causing MAP kinase and p21ras activation, and NF-κB induction in Jurkat cells, as well as activation of G proteins (53–55). Also, NO increases the amount of GTP-bound p21ras in human T cells, likely through S-nitrosylation of a critical cysteine in p21ras that enhances guanine nucleotide exchange (53–55). Finally, an important example of NO-steroid crosstalk is the antiatherosclerotic effects of estrogen. This activity of estrogen is ascribed to the enhanced production by endothelial cells of NO through estrogen-dependent inhibition of superoxide anion production (56).

SUMMARY: RELEVANCE TO BIOTECHNOLOGY

Numerous expression vectors utilize hormone response elements to confer external regulation of the gene of interest. These vectors are used in bioprocessing applications to time the induction of protein expression, independent of other growth-dependent phases of the bioreactor dynamics. In biotechnology applications where expression of a gene is linked to the cell cycle, it is difficult to optimize simultaneously the cell growth rate, protein expression per cell, and overall reactor productivity. For example, expression of the heavy- and light-chain mRNA by hybridomas may display cell-cycle dependency, with high expression during stationary phase. The use of an HRE-rich promoter for these genes would allow a precisely timed induction of the genes or utilization of a continous process for protein expression, thus avoiding batch configurations typical of hybridoma technology. Furthermore, recent evidence of growth factor activation of kinases that phosphorylate steroid receptors suggests important crosstalk between steroids and growth factors that may be exploited to improve bioreactor operation.

In gene therapy, retroviral and adenoviral vectors can contain hormone response elements to help control the tissue distribution, timing, and level of expression of the therapeutic gene (57). Steroid-regulated promoters can be constructed with as little as 300 bp of DNA and thus can meet the packing constraints imposed by viral-based gene transfer. Additionally, the level of expression can be controlled by the level of the administered hormone and/or the number of repeats of the response element in the promoter. A limitation of this approach is that wild-type genes would also be susceptible to the hormonal induction during the clinical implementation. Important goals include the design of: (*1*) synthetic steroid ligands that are neither agonists nor antagonists of endogenous receptors; (*2*) novel HREs; and (*3*) novel ligand and DNA binding specificities of engineered hormone receptors. An extremely specific genetic switch would then be possible by co-expressing the modified receptor with a gene construct containing a modified HRE. Addition of the steroid analog (that has no activity for or against the endogenous receptor) would activate the modified receptor that would then bind the novel HRE of the transgene of interest. While chimeras utilizing the human steroid binding domain and yeast transactivator GAL4 are useful tools of study, the expression of a yeast protein in humans would present likely immunological complications. Thus the human steroid receptor gene requires mutation to confer novel ligand and DNA binding properties. Both rational design and combinatorial/screening strategies may produce optimal steroid analog/receptor constructs that meet these goals.

The biology of nitric oxide may impact gene therapy, tissue engineering, and biocultivation dynamics of human cells. Nitric oxide synthase has been an important target for gene therapy of blood vessels to prevent restenosis after balloon angioplasty. Nitric oxide is widely viewed as an inhibitor of smooth muscle cell division (a major source of wall thickening) and an inhibitor of platelet and neutrophil activation and adhesion. The diffusible nature of nitric oxide in conjunction with its short half-life and local zone of action makes the eNOS gene an attractive candidate for gene therapy. Furthermore, a major challenge in tissue engineering an artificial blood vessel requires the creation of a nonthrombogenic lining. The antiplatelet activity of nitric oxide is a primary motivation for seeding tissue constructs with endothelium or engineered smooth muscle cells expressing nitric oxide synthase. Thus nitric oxide, when locally produced at the correct level, may prevent intimal hyperplasia, accelerated atherosclerosis, and thrombosis that would reduce the patency of implanted grafts. Finally, in the biocultivation of complex human cells such as endothelium, stem cells, or neurons, the intracellular signaling generated through nitric oxide pathways may be an important consideration. This is especially true in cellular systems where the NO pathways are highly sensitive to chemical perturbations in oxygen or glucose or to mechanical pertubations common to bioreactors.

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