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ROLE OF CYCLIC GMP IN GENE REGULATION

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1. ABSTRACT

Cyclic GMP is produced in response to nitric oxide and natriuretic peptides; cGMP is a key regulator of cell proliferation, differentiation, and apoptosis, and plays an important role in many (patho)physiological processes such as synaptic plasticity, angiogenesis, inflammation, and cardiac hypertrophy. The regulation of gene expression by cGMP has been recognized relatively recently, but cGMP-mediated increases or decreases in the mRNA expression of >60 different genes have been described, and gene expression profiling is just beginning to contribute to the growing list of cGMP-regulated genes. Deletion or over-expression experiments in mice involving components of the cGMP signaling pathway have contributed to our understanding of long-term effects of altered cGMP signaling, including the regulation of gene expression. We will discuss transcriptional and post-transcriptional mechanisms of gene regulation by cGMP, and review specific transcription factors and RNA binding proteins targeted by cGMP. Some of the effects of cGMP on gene expression are indirect, through cGMP modulation of other signaling pathways, e. g. mitogen-activated protein kinase pathways. However, some effects of cGMP can be directly attributed to cGMP regulation of specific transcription factors such as CREB, TFII-I or c-Fos, and are mediated by cGMP-dependent protein kinases. We will discuss specific genes regulated by cGMP in the context of their contribution to particular (patho)physiologic processes regulated by cGMP.

2. INTRODUCTION

Cyclic GMP is generated via two different pathways: (i) by cytoplasmic, soluble guanylate cyclases (sGCs) which are activated by nitric oxide (NO) and carbon monoxide (CO); or (ii) by receptor guanylate cyclases (rGCs) which are activated by natriuretic peptides (atrial natriuretic peptide, ANP; B- and C-type natriuretic peptides, BNP and CNP, respectively) and guanylins [Figure 1](1-3). NO is synthesized from L-arginine by three different NO synthases: the constitutively-expressed neuronal and endothelial isoforms (eNOS and nNOS) are activated by calcium to produce nanomolar amounts of NO, whereas the inducible isoform (iNOS) is transcriptionally activated in response to cytokines and other stimuli to transiently produce larger amounts of NO (1). CO is produced from heme by heme oxygenases (1). ANP and BNP are mainly released from the heart, while CNP is produced by endothelial cells and many other cell types and can act as a local hormone (1, 3). Guanylins are produced in the gut (1).

Break-down of cGMP is catalyzed by phosphodiesterases (PDEs), some of which are regulated by cGMP itself (3). Other cGMP effector proteins include PDEs which hydrolyze cAMP, cGMP-dependent protein kinases (PKG), cyclic nucleotide-regulated ion channels, and possibly cyclic nucleotide-regulated exchange factors that activate the small GTPases Ras and Rap (3, 4). While PKG is the major intracellular cGMP target in many cell types, high concentrations of cGMP can cross-activate cAMP-dependent protein kinases (PKAs) (1, 5, 6). Two different genes encode the soluble PKG I and membrane-bound PKG II, with two splice variants of PKG I differing in their first 100 amino acids (PKG I-alpha and I-beta) (3). PKG I is widely expressed with highest levels found in smooth muscle cells, platelets, and cerebellum; it is also found in cardiac myocytes, glomerular mesangial cells, in most endothelial cells, in different types of neuronal cells, epithelial cells, and white blood cells (5, 7-14). PKG II shows more limited expression, with highest levels in neuronal cells, bone and kidney (1, 5).

Deletion and transgenic overexpression experiments in mice have established important physiological roles of the cGMP signaling pathway in the cardiovascular system, in the nervous system, and in bone; some of the long-term effects of altered cGMP signaling involve the regulation of gene expression by cGMP. Mice deficient in nNOS and eNOS, and mice deficient in ANP or its rGC-A receptor demonstrate cardiac hypertrophy and in some cases, systemic hypertension (2, 15). In contrast, cardiomyocyte-specific expression of constitutively-active rGC-A prevents cardiac hypertrophy in response to aortic constriction (16). Mice harboring homozygous null mutations for PKG I die at a young age from severe intestinal dysfunction due to loss of NO/cGMP-dependent smooth muscle relaxation, but some longer-term survivors develop inflammation and other abnormalities (17, 18). The functional significance of NO/cGMP signaling in the nervous system is underlined by the finding that mice deficient in PKG I or doubly deficient in eNOS/nNOS demonstrate defects in neuronal differentiation and specific forms of learning, while PKG II-deficient mice demonstrate a defect in the circadian clock (19-21). Mice deficient in either CNP or PKG II develop dwarfism as a result of impaired endochondral ossification, whereas transgenic mice that overexpress CNP or BNP demonstrate skeletal overgrowth (22-26).

NO can regulate gene expression by multiple, including cGMP-*in*dependent, mechanisms; this has been reviewed recently (27, 28). Here, we will concentrate on regulation of gene expression by cGMP, and we will use the following criteria to define cGMP-mediated transcriptional and post-transcriptional regulation of gene expression: the effects should be observed with activators of sGCs or rGCs at physiologically relevant concentrations; NO's or CO's effect should be blocked by inhibition of sGC, while the effect of natriuretic peptides should be blocked by an appropriate rGC antagonist; and effects should be mimicked by membrane-permeable cGMP analogues. As a minimum criterium, we will consider changes in mRNA levels induced by an activator of sGC or rGC which are mimicked by a membrane-permeable cGMP analogue. Cyclic GMP-mediated regulation of gene expression can be attributed to the action of PKG, if it is prevented by specific PKG inhibitors in PKG-expressing cells and enhanced by over-expression of PKG; the effect should be absent in PKG-deficient cells and restored by transfection of PKG. Unfortunately, some PKG inhibitors exhibit variable effects in intact cells and tissues (5).

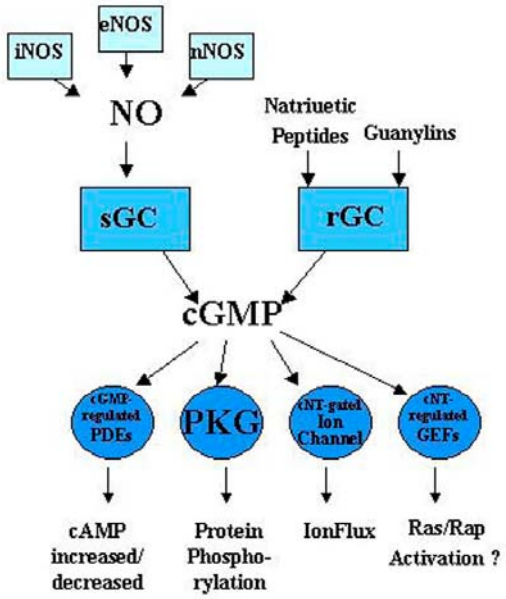

Figure 1. Cyclic GMP Signaling Pathway. Cyclic GMP is synthesized by soluble gunaylate cyclases in response to NO/CO, or by receptor guanylate cyclases which are activated by peptide hormones. Depending on the cell type, cGMP has several intracellular targets besides cGMP-dependent protein kinases (PKG I), as described in the text.

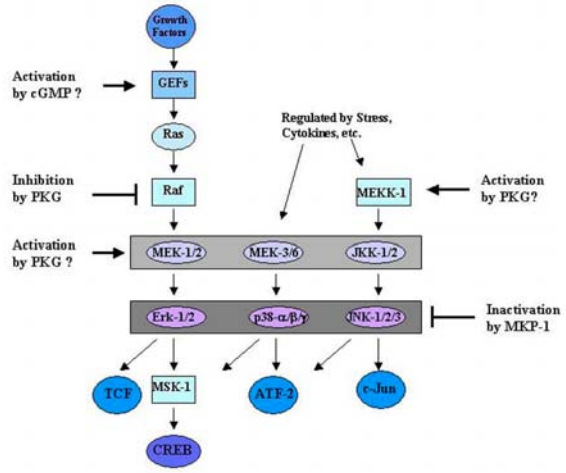

Figure 2. Regulation of MAP-kinase pathways by cGMP. The extracellular-signal regulated kinases Erk-1/2, the p38 isoforms alpha, beta, and gamma, and the c-Jun amino-terminal kinases JNK-1/2/3 represent three major MAP-kinase pathways involved in the regulation of gene expression by cGMP. Upstream regulatory kinases include MAP-kinase kinases MEK-1/2 and MEK-3/6 for Erks and p38, respectively, and JNK-kinases JKK-1/2 for JNKs. All three pathways are activated by multiple extracellular stimuli, including growth factors, cytokines and stress (such as oxidative stress and heat shock), and all three types of MAP-kinases are inactivated by MAP-kinase phosphatase-1 (MKP-1, see text). Some transcription factors targeted by MAP-kinases are shown; a more complete list has been reviewed (31). Cyclic GMP can either activate or inhibit each pathway, dependent on cell type and growth conditions (Table 2); see text for discussion of possible mechanisms of cGMP action.

We will first discuss transcriptional and post-transcriptional mechanisms of gene regulation by cGMP, including a review of specific transcription factors regulated by cGMP. After that, we will discuss specific genes regulated by cGMP in the context of their contribution to particular cGMP-regulated (patho)physiological processes. Regulation of gene expression by cGMP and PKG I in the cardiovascular system has been the subject of two previous reviews (29, 30).

3. MECHANISMS OF TRANSCRIPTIONAL REGULATION BY cGMP

Cyclic GMP can regulate transcription factors *directly* by inducing phosphorylation or by increasing expression of short-lived proteins. Examples of transcription factors directly controlled by cGMP-dependent phosphorylation include the cAMP response-element (CRE)-binding protein CREB, activating transcription factor-1 (ATF-1), the multi-functional transcription factor TFII-I, and possibly nuclear factor-kappaB (NF-kappaB; Table 1, and see below). Transcription factors whose expression is regulated by cGMP include the AP-1 family proteins c-Fos and JunB, the early growth response gene Egr-1, the growth arrest-specific homeobox gene GAX, and peroxysome-proliferator-activated receptor-gamma (PPAR-gamma) (Table 1).

In addition, cGMP can regulate transcription factors *indirectly*, through modulation of up-stream signal transduction pathways. Examples include cGMP inhibition of the calcineurin-dependent nuclear factor of activated T-cells (NF/AT), and cGMP inhibition of the RhoA-regulated serum response factor (SRF) [Table I]. Through activation or inhibition of mitogen-activated protein kinase (MAP-kinase) pathways, cGMP can regulate the activity of multiple transcription factors, including ternary complex factor (TCF), CREB, ATF-2 and c-Jun (Table 2 and Figure 2) (31). The effects of cGMP frequently show differences depending on the cell type and growth conditions examined; some apparently contradictory results may be explained by the fact that transcription factors can be subject to regulation by multiple signal transduction pathways with cross-talk and cell type-specific differences.

3.1. Direct regulation of transcription factors by cGMP-dependent phosphorylation

3.1.1. CREB and ATF-1

The transcription factor CREB belongs to a family of basic and leucine zipper (bzip) domain proteins that bind the DNA consensus sequence 5'-TGACGTCA-3' via the basic domain and homo/hetero-dimerize via the leucine zipper domain (32). CREB is activated by many different stimuli through phosphorylation of Ser^{133} in its kinase-inducible domain and recruitment of the transcriptional co-activator CREB binding protein (CBP). CREB is critical for proliferation, differentiation and survival of multiple cell types, and absence of CREB causes dwarfism and cardiac myopathy (32). Increased intracellular cGMP concentrations lead to increased CREB Ser^{133} phosphorylation in vascular smooth muscle cells (VSMCs),

Table 1. Transcription Factors Regulated by cGMP

Transcription Factor	Cell Type	Mechanism of Regulation	Effect of cGMP	References
Transcription Factors Directly Regulated by cGMP-dependent Phosphorylation				
CREB	Neuronal cells VSMCs BHK [1]	↑ phosphorylation of CREB Ser[133] (directly or indirectly PKG-mediated)	↑ transcriptional activity	34-38 33 39
ATF-1	VSMCs BHK [1]	↑ ATF-1 phosphorylation (PKG-dependent)	↑ transcriptional activity?	33 39
TFII-I	COS-7 [1], BHK [1] C2C12 Myoblasts	direct TFII-I phosphorylation by PKG / association with PKG	↑ transcriptional activity	48
NF-κB	T-Lymphocytes 293 cells [1] Neuronal cells Cardiomyocytes Endothelial cells Hepatocytes	↑ phosphorylation of p50 and p65 by PKG IκB phosphoryl./destabilization “ IκB stabilization “	↑ DNA binding/ transcriptional activity ↑ DNA binding/ transcriptional activity ↓ cytokine-induced DNA binding	54 59, 60 58 61-63 64
CGMP Regulation of Genes Encoding Transcription Factors				
AP-1 (c-Fos, Jun B)	Neuronal, Glial cells, REF52, Thyroid cells, BHK[1], Bronchial cells Neuronal, Glial cells[1], Osteoblasts VSMCs Cardiac Fibroblasts Endothelial cells	↑ *c-fos* transcription ↑ *junB* transcription ↑ *c-fos* transcription “	↑ mRNA, ↑ AP-1 DNA binding, ↑ AP-1 transcriptional activity (in serum-starved cells) ↑ Ca^{++}-stimulated *c-fos* mRNA ↑ FGF2-stimulated *cfos* mRNA ↑ ATII-stimulated *cfos* mRNA ↑ TNF-α-stim. AP-1 DNA binding	34, 36, 69-76 67, 68 40, 73, 80 44, 85-87 85 78 78 81
Egr-1	Neuronal cells	↑ *egr-1* transcription	↑mRNA, ↑ DNA binding, ↑transcriptional activity	70, 91
GAX	VSMCs	?	↑mRNA	95
PPAR-gamma, PGC-1	Adipocytes, VSMCs “	?	↑mRNA ↑mRNA	 96, 98, 99
Transcription Factors Indirectly Regulated by cGMP (selected)				
NF/AT MEF-2	Cardiomyocytes “	↓ calcium influx/ ↓ calcineurin activity	↓ NF/AT nuclear translocation ↓ transcriptional activity	100 100
SRF	VSMCs	↓Rho-dependent activation [2]	↓ SRF-dependent transcription [2]	7
C/EBP-beta	Osteoblasts, Glial cells[1]	?	↑ DNA binding/ transcriptional activity	85

[1] Transfection of PKG required for the effect in PKG-deficient cells, [2] SRF/TCF complexes on the *fos* and *egr-1* promoters are insensitive to Rho signaling

neuronal cells, and PKG I-transfected Baby Hamster Kidney (BHK) cells, but not in PKG I-deficient BHK cells (33-39). Cyclic GMP-induced CREB phosphorylation occurs at physiologically relevant PKG I levels and can occur independently of changes in intracellular Ca^{++}, activation of MAP-kinases (Erk-1/2 and p38), or cross-activation of PKA, thus ruling out the effect of other known CREB kinases (34, 39). PKG I can directly phosphorylate CREB on Ser[133] *in vitro*; the kinetics are slower than those of PKA, but comparable to those of Ca^{++}/calmodulin-dependent protein kinase IV (39). Correspondingly, PKG I is significantly less effective than PKA, but similar to Ca^{++}/calmodulin-dependent protein kinase, in stimulating cAMP-response-element (CRE)-dependent transcription in intact cells (39-42). In BHK cells treated with cGMP, the kinetics of CREB phosphorylation correlate with the kinetics of PKG I nuclear translocation (39). In these cells and in some neuronal cells, cGMP-mediated transactivation of the *c-fos* promoter is dependent on CREB phosphorylation and nuclear translocation of PKG I; PKG I constructs which are fully catalytically active but are excluded from the nucleus due to membrane-targeting or mutations in a nuclear localization signal are unable to activate *c-fos* (43, 44). Nuclear translocation of endogenous PKG I has been demonstrated in neuronal cells, neutrophils, macrophages and some embryonal smooth muscle cells (12, 13, 39, 43, 45). However, other investigators found no evidence of PKG I nuclear translocation in primary VSMCs, HEK293 and CV-1 cells, or observed nuclear PKG I only in a minority of the cell population (41, 46, 47). Based on recent findings, we speculate that PKG I may be retained in extra-nuclear compartments by binding to cell type-specific anchoring proteins (48). In cells where PKG I does not translocate to the nucleus, e.g. in primary VSMCs, cGMP/PKG I-induced CREB phosphorylation may be indirect, via activation of MAP-kinases, although this has not yet been formally documented (see below) (33, 49).

Table 2. Effect of cGMP on MAP-Kinase Activities

Cell Type	Erk-1/2	P38	JNK	References
Endothelial cells	↑(in serum-starved cells)	n.d.	↑ (in serum-starved cells)	108, 109, 113, 114
Neonatal cardiomyocytes	↑	no effect	no effect	115
Adult cardiomyocytes	no effect	↑	n.d. [2]	130
Adventitial fibroblasts	↑ (in serum-starved cells)	n.d.	n.d.	110
CHO cells [1]	↑ (in serum-starved cells)	n.d.	n.d.	116, 117
T-Lymphocytes	↑ (in resting cells)	↑	n.d.	14
Neutrophils	n.d.	↑	n.d.	134
HL-60, U937, THP-1 cells	n.d.	↑	n.d.	133
293 cells, COS7 cells [1]	n.d.	↑	n.d.	133
A549 lung carcinoma	↑	n.d.	n.d.	111
MCF7 breast carcinoma	↑	n.d.	n.d.	118, 119
SW280 colon carcinoma	n.d.	n.d.	↑	135
Neuronal cells	↑ (in serum-starved cells)	n.d.	n.d.	10, 71, 120, 121
VSMCs, early passage	↑ (in serum-starved cells)	↑	↑ (in serum-starved cells)	49, 112
VSMCs, late passage	↑ (in serum-starved cells transfected with PKG)	n.d.	↑ (in serum-starved cells transfected with PKG)	49
VSMCs, early passage	↓ (in PDGF-, EGF- or ATII-stimulated cells)	n.d.	n.d.	122-124
Aortic strips, Mesangial cells				125
VSMC lines (embryonal)	no effect	no effect	n.d.	39
Hepatic stellate cells	↓ (in PDGF-stimulated cells)	no effect	↓	126
BHK [1]	↓ (in EGF-stimulated cells)	n.d.	n.d.	128
NIH3T3 [1]	↓ (in serum-stimulated cells)	n.d.	↑	127, 135

[1] effect enhanced by transfection of PKG, [2] n.d., not determined

The closely related CREB family member ATF-1 is also phosphorylated in response to PKG I activation by cGMP, and ATF-1 appears to mediate transcriptional activation of the RhoA promoter by PKG I (33, 39).

3.1.2. TFII-I

TFII-I is a transcriptional regulator for many genes, including *c-fos* and so-called endoplasmatic reticulum stress-response genes; TFII-I is ubiquitously expressed and interacts with multiple transcription factors including SRF, TCF, ATF-6, USF-1, c-Myc, and NF-6B, and with histone deacetylases (50-52). In cooperation with these other proteins, TFII-I binds to several different DNA sequence elements, and it regulates transcription from initiator element-containing promoters through interaction with the basal transcription initiation complex (50). We found that TFII-I physically interacts with PKG I-beta, with these two proteins co-immunoprecipitating in C2C12 myoblasts (48). PKG I phosphorylates TFII-I *in vitro* and *in vivo*; PKG activation enhances the transactivation potential of wild type, but not phosphorylation-deficient mutant TFII-I on an SRF/TCF-dependent reporter gene, and PKG cooperates with TFII-I to transactivate the *fos* promoter (48). However, we found no effect of cGMP/PKG on Inr-dependent transcription.

3.1.3. Nuclear factor-kappa B (NF-kappa B)

NF-kappaB/Rel proteins are dimeric transcription factors that bind the DNA consensus sequence 5'-GGG(A/T)$_5$CC-3' through their Rel homology domain. Among other genes, NF-kappa B activates genes involved in inflammation, such as cytokines, cytokine receptors and adhesion molecules (53). NF-kappa B subunits are inactive when bound to cytoplasmic inhibitor(I)-kappa B proteins; multiple stimuli (including various cytokines) can induce I-kappa B kinase-mediated phosphorylation, ubiquitination, and proteosomal degradation of I-kappa B, which leads to activation and nuclear translocation of NF-kappa B (53). NO can both increase or decrease NF-kappa B activity depending on the NO concentration, redox milieu, cell type and co-stimulus, but the effects of high NO concentrations on NF-kappa B are generally cGMP-*in*dependent (27, 28, 56, 57). However, in cardiomyocytes, NO-donors *increase* NF-kappa B (p50/p65) DNA binding activity and induce expression of an NF-kappa B-responsive gene in a cGMP-dependent fashion; the effect is abrogated by inhibition of sGC or PKG I and is mimicked by a cGMP analogue (58). In these cells, cGMP induces phosphorylation and degradation of I-kappa B alpha, and direct phosphorylation of I-kappa Ḅ alpha by PKG I occurs *in vitro* (58). In primary neurons, NO donors also *increase* phosphorylation of I-kappa Ḅ alpha and nuclear translocation of NF-kappa B subunits (59, 60).

In contrast to the effect of NO/cGMP on "basal" NF-kappa B activity, cytokine-induced NF-kappa B activation in human endothelial cells is *inhibited* by NO donors and I-kappa Ḅ alpha is stabilized, and similar effects are observed with ANP, CNP, or cGMP analogues (61-63). Natriuretic peptides and cGMP analogues also *inhibit* NF-kappa B activation during hepatic ischemia/reperfusion injury through stabilization of I-kappa B; this correlates with cGMP induction of heat shock protein 70, and increased heat shock protein 70 association with I-kappa B (64).

A non-canonical pathway to NF-kappa B

activation has been proposed for cGMP/PKG I, involving direct phosphorylation of the NF-kappa B subunits p65, p50 and p52 leading to enhanced DNA binding and transcriptional activity (54). NF-kappa B activation through PKA-mediated phosphorylation of p65 involves recruitment of the co-activator CBP, but PKG appears to phosphorylate p65 on a different site (54, 55).

3.2. cGMP regulation of genes encoding transcription factors

3.2.1. Activating protein-1 (AP-1); regulation of *c-fos* and *junB*

The AP-1 transcription factor complex is composed of dimeric bzip proteins belonging to the Fos, Jun, Maf or ATF sub-families and recognizes the DNA consensus sequence 5'-TGA(G/C)TCA-3' (65). AP-1 plays an important role in growth factor-induced cell cycle progression, differentiation, apoptosis, and control of synaptic plasticity (65, 66). Resting cells typically have very low or undetectable levels of c-*fos* and *junB* mRNAs, but transcription of AP-1-related genes is rapidly induced in response to many stimuli (65). NO-releasing agents, natriuretic peptides, and cGMP analogues increase *c-fos* and *junB* mRNA expression in cultured cells and primary tissues, including fibroblasts, neuronal and epithelial cells (34, 36, 67-73). In intact animals, inhibitors of NOS, sGC, and PKG reduce *c-fos* expression in different areas of the nervous system after a variety of noxious stimuli, baro-receptor stimulation, or certain forms of learning (34, 72, 74-76). Cardiac expression profiling from eNOS-deficient mice demonstrate decreased *c-fos* mRNA expression compared to wild type mice (77). In early passage VSMCs and cardiac fibroblasts, cGMP-elevating agents modestly enhance *c-fos* induction by fibroblast growth factor (FGF)-2 or angiotensin II (AT II), respectively (78, 79).

As a consequence of NO/cGMP-induced *c-fos* and *junB* mRNA expression, AP-1 DNA binding activity is increased and transcription of AP-1-dependent reporters and endogenous genes are enhanced in NO/cGMP-treated cells (67, 69, 70, 80). In bovine pulmonary endothelial cells, NO-releasing agents and cGMP analogues increase AP-1 DNA binding activity only if cells are co-stimulated with TNF-alpha (81). However, high concentrations of NO can *de*crease DNA binding of recombinant Fos and Jun *in vitro* through direct S-nitrosylation and/or S-glutathionylation, but the *in vivo* significance of this effect is unknown (82, 83).

The mechanism of *c-fos* induction by NO/cGMP has been studied in some detail. We demonstrated that NO stimulation of the *fos* promoter is strictly cGMP-dependent and requires guanylate cyclase and PKG activity, with PKG I and II regulating transcription through different mechanisms (39, 84). PKG I targets three conserved *cis*-acting elements in the *fos* promoter: the CRE and the *fos* AP-1 (FAP) site which both bind CREB-related proteins; and the *fos* serum response element (SRE) which is composed of overlapping/ adjacent recognition sites for SRF, TCF, TFII-I and CCAAT enhancer-binding protein-beta (C/EBP-beta) (39, 40, 48). Studies with dominant negative transcription factors demonstrate that cGMP/PKG I transactivation of c-*fos* requires CREB-related proteins, but is independent of AP-1 and C/EBP-related transcription factors (39). PKG I-beta enhances the effect of TFII-I on the *fos* promoter (48). In some cell types, including fibroblastic and neuronal cells, PKG I-mediated transactivation of the *fos* promoter requires PKG I nuclear translocation and CREB Ser^{133} phosphorylation, but is independent of Erk-1/2 and p38 MAP-kinase pathway activity (39). In other cell types, activation of MAP-kinase pathways by cGMP/PKG I (see below) may contribute to *c-fos* induction through activation of TCF in the absence of PKG I nuclear translocation (65). In contrast, plasma membrane-bound PKG II can activate the *fos* promoter in cells of neuronal origin without inducing CREB Ser^{133} phosphorylation; this may involve indirect regulation of C/EBP-beta activity by cGMP/PKG II (44, 85).

Some cells of neuronal and osteoblastic origin show little or no *c-fos* mRNA induction in response to NO/cGMP, but co-stimulation with NO/cGMP and calcium-elevating agents leads to synergistic induction of high levels of *c-fos* mRNA (71, 85-88). Synergistic activation of the *fos* promoter by NO/cGMP and calcium is mediated more efficiently by PKG II than by PKG I, and requires co-operation between CREB and C/EBP-beta (44, 85). We found that C/EBP-beta is recruited to the *fos* promoter in response to cGMP/calcium; C/EBP-beta cooperates with CREB through direct protein/protein interaction, and transcriptional activity of the C/EBP-beta/CREB complex is regulated by cGMP and calcium, with cGMP inducing *de*-phosphorylation of C/EBP-beta and calcium increasing CREB Ser^{133} phosphorylation (85).

3.2.2. Early growth response gene-1 (*egr-1*)

Transcription of the *egr-1* gene encoding the zinc finger transcription factor Egr-1 (also called Zif268) is regulated by many growth factors and stress stimuli with similar induction kinetics as *c-fos* (89, 90). Like c-Fos, Egr-1 plays an important role in cell growth, differentiation, apoptosis, and control of synaptic plasticity (89, 90). In pheochromocytoma and neuroblastoma cells, NO-releasing agents and natriuretic peptides increase *egr-1* promoter activity, mRNA and protein expression, and increase transcription from an Egr-1-responsive reporter (70, 91). Moreover, cGMP analogues increase serum-stimulated Egr-1 DNA binding activity; this effect is enhanced by over-expression of PKG I and occurs in the absence of significant changes in MAP-kinase activity (9). In contrast, NO and cGMP analogues appear to have no effect on AT II-induced *egr-1* mRNA expression in cardiac fibroblasts (79), and high concentrations of NO may suppress *egr-1* induction by mitogens and cytokines in VSMCs, mesangial cells and macrophages (92-94).

3.2.3. The growth arrest-specific homeobox gene (*gax*)

GAX (also known as Mox-2) is a homeodomain-containing transcription factor whose expression in adult organisms is predominantly in cardiovascular tissues (90). GAX is expressed in quiescent VSMCs and rapidly down-regulated when cells are stimulated to proliferate by mitogens or following vascular injury; over-expression of GAX increases expression of the cell cycle inhibitor

$p21^{Waf1/Cip1}$ and induces G_0/G_1 growth arrest of stimulated cells (90, 95). CNP and cGMP analogues increase GAX mRNA expression in serum-starved primary VSMCs and largely prevent the decrease in GAX mRNA in ATII-stimulated cells (95). Although the physiological significance of these observations and the mechanism of cGMP action on GAX require further study, the regulation of GAX by cGMP may be important for VSMC differentiation.

3.2.4. The peroxysome proliferator-activated receptor-gamma (PPAR-gamma) and its co-activator (PGC-1)

NO, in a cGMP-dependent fashion, triggers mitochondrial biogenesis in many different cell types (96). This effect is mediated by NO/cGMP induction of PPAR-gamma coactivator-1 alpha (PGC-1) mRNA expression. PGC-1 interacts with a variety of other transcription factors, including PPAR-gamma and nuclear hormone receptors, to coordinate gene expression linked to energy homeostasis (96a). PGC-1 is a master regulatory factor of mitochondrial biogenesis in brown adipose tissue and in cardiac and skeletal muscle, and increases expression of other transcription factors involved in expression of nuclear and mitochondrial genes that encode mitochondrial proteins (96, 96a). Mitochondrial biogenesis induced by cold exposure is reduced in eNOS-deficient mice, which demonstrate a reduced metabolic rate and weight gain (96).

The nuclear receptor/transcription factor PPAR-gamma was initially described as an important regulator of adipogenic differentiation and glucose homeostasis; PPAR-gamma-activating agents also have anti-proliferative, anti-inflammatory and immunomodulatory effects (97). Compared to wild type mice, brown adipose tissue from eNOS-deficient mice contains reduced levels of PGC-1 and PPAR-gamma mRNA, and reducing cGMP levels in brown adipocytes with a sGC inhibitor diminishes expression of both mRNAs (96, 98). Treating primary VSMCs with cGMP analogues increases the expression of PPAR-gamma; this effect is not seen in PKG I-deficient VSMCs (99). The mechanism(s) whereby NO/cGMP increase expression of PGC-1 and PPAR-gamma remain to be determined.

3.3. Indirect regulation of transcription factors by cGMP

3.3.1. Inhibition of calcineurin-dependent transcription factors

Calcineurin is a Ca^{++}-dependent phosphatase which *de*phosphorylates and thereby activates the nuclear factor of activated T cells (NF/AT), myocyte enhancer factor-2 (MEF-2), and Elk-1 (a member of the TCF family) (94). These calcineurin-dependent transcription factors are involved in T lymphocyte activation, muscle hypertrophy and cell cycle control (94). Calcineurin-mediated *de*phosphorylation of NF/AT leads to nuclear translocation and transactivation of target genes, which include in cardiac myocytes hypertrophy-associated genes such as BNP (94, 100). PKG I activation inhibits calcineurin-dependent NF/AT nuclear translocation, phenylephrine-induced BNP promoter activity, and cardiomyocyte enlargement by interfering with Ca^{++} entry via the L-type Ca^{++} channel and possibly via other pathways (100). MEF-2-dependent transcription is inhibited similarly (100). Thus, PKG I-mediated inhibition of calcineurin signaling may contribute to cGMP/PKG I inhibition of cardiac myocyte hypertrophy (see Section 5.7.). Inhibition of calcineurin-dependent, NF/AT-induced interleukin-2 transcription could potentially also explain the finding that cGMP/PKG I inhibit interleukin-2 release and proliferation of anti-CD3-stimulated T lymphocytes (14).

3.3.2. Inhibition of rhoA-dependent serum response factor (SRF) activity

Serum response factor (SRF) is a widely expressed MADS domain-containing transcription factor that binds a cis-regulatory element termed CArG box [5'-$CC(A/T)_6GG$-3'] contained in the promoters of muscle-specific genes as well as mitogen-inducible immediate-early genes. SRF activates muscle-specific promoters in response to RhoA-induced changes in actin dynamics: when the small GTPase RhoA is activated downstream of G-protein-coupled receptors, it increases actin polymerization through a number of effector proteins; these changes in actin polymerization are sensed by the myocardin-related transcription factor MAL which functions as an SRF co-activator downstream of RhoA (101, 102). In contrast, the promoters of immediate-early genes such as *c-fos* and *egr-1* are *in*sensitive to RhoA signaling because on these promoters, SRF co-operates with transcription factors of the TCF family (103).

NO/cGMP signaling can inhibit RhoA functions such as Ca^{++} sensitization of smooth muscle cell contractility and RhoA-dependent actin polymerization, in part through PKG I-mediated phosphorylation of RhoA (7, 104, 105). We showed that PKG I inhibits SRE/SRF-dependent transcription by interfering with RhoA signaling in cardiomyocytes, VSMCs, and rat embryonal fibroblasts; PKG I acts both upstream of RhoA, inhibiting serum- and G alpha$_{12/13}$- induced Rho activation, and downstream of RhoA, inhibiting steps distal to the Rho targets ROK, PKN, and PRK-2 (7). PKG I also inhibits serum induction of vinculin mRNA, an endogenous RhoA-responsive SRF target gene (7). The vasodilator-activated phosphoprotein (VASP) functions downstream of RhoA to control actin dynamics and SRF activity, and VASP is a PKG I substrate with phosphorylation inhibiting its effects on actin polymerization (106, 107). Studies with phosphorylation-deficient VASP mutants suggest that VASP phosphorylation explains at least part of PKG's inhibitory effect on SRF-dependent transcription (107). Regulation of RhoA activity by PKG I is complicated by the fact that chronic PKG I activation leads to increased RhoA expression (see Section 5.8.2.).

3.3.3. Modulation of MAP-kinase-regulated transcription factors

Three different MAP-kinase pathways activate the extracellular signal-regulated kinases (Erk-1/2), p38 MAP-kinases, or c-Jun N-terminal kinases (JNKs), and regulate gene expression through direct or indirect phosphorylation of multiple transcription factors including TCF, CREB, ATF-2 and c-Jun (Figure 2) (31). Increased

intracellular cGMP concentrations can either lead to *in*creased or *de*creased activity of all three MAP-kinase pathways, depending on cell type and growth conditions (Table 2). Changes in MAP-kinase activity may explain the effect of cGMP on many genes involved in cell proliferation, differentiation, or apoptosis. In some cases, cGMP stimulation of gene expression has been clearly shown to depend on Erk-1/2 activation by cGMP: the increased expression of metalloproteinase-13 and heme oxygenase-1 in endothelial cells (108, 109); increased increases $p21^{Waf1/Cip1}$ expression in adventitial fibroblasts (110); and increased cyclooxygenase II expression in A549 lung cancer cells (111). In VSMCs, increased $p21^{Waf1/Cip1}$ expression is mediated by cGMP activation of p38 MAP-kinase (112).

3.3.3.1. cGMP regulation of erk-1/2 activity

In endothelial cells, NO donors stimulate basal Erk-1/2 activity in a cGMP-dependent fashion; this effect is mimicked by cGMP analogues and in some cases augmented by over-expression of constitutively-active PKG I (108, 109, 113, 114). Moreover, the mitogenic effect of VEGF on endothelial cells appears to be mediated by NO and cGMP, as VEGF-induced Erk-1/2 activation and proliferation is blocked by NOS and sGC inhibitors (113, 114). Stimulation of Erk-1/2 activity by cGMP has also been reported in neonatal cardiac myocytes, adventitial fibroblasts, Chinese hamster ovary cells (where the effect is augmented by transfection of PKG I), T-lymphocytes, and certain cancer cells (14, 110, 111, 115-119). cGMP can stimulate Erk-1/2 activity in some cells of neuronal origin, but not in others (9, 10, 71, 85, 120, 121).

In serum-starved, primary VSMCs, cGMP-elevating agents *stimulate* basal Erk-1/2 activity in a PKG I-dependent fashion (49); however, PDGF-, EGF-, and AT II-stimulated Erk-1/2 activities in early passage VSMCs and in aortic strips are *inhibited* by NO, ANP/CNP, with several studies demonstrating that this effect is not due to cross-activation of PKA (122-124). Inhibition of mitogen-stimulated Erk-1/2 activity has also been observed in mesangial cells, hepatic stellate cells and different fibroblast-like cells (125-128).

The mechanisms whereby cGMP-elevating agents modulate Erk-1/2 are incompletely understood (30). Erk-1/2 activity is stimulated when growth factor receptors activate Ras or Rap-1, Raf-kinases, and MEK-1/2 (Figure 2) (31). Whether cGMP *activation* of Erk-1/2 in resting cells includes activation of Ras or Rap-1 through cyclic nucleotide-binding guanine nucleotide exchange factors and/or activation of Raf kinase isoforms is controversial (4, 113, 115, 128, 129); however, cGMP activation of the pathway occurs at the level of MEK-1/2 and is dependent on PKG I activity (49, 115). On the other hand, Erk-1/2 *inhibition* by cGMP/PKG I in mitogen-stimulated cells may be explained by PKG I phosphorylation of Raf-1 and by cGMP/PKG I induction of the MAP-kinase inhibitor MPK-1 (Figure 2 and Section 5.4.1.) (128). Of note, under some conditions NO can activate Erk-1/2 in a cGMP-independent fashion (130, 131), and high intracellular cGMP levels can inhibit Erk-1/2 through cross-activation of PKA (6, 132).

3.3.3.2. cGMP regulation of p38 and JNK activity

NO activation of p38 in adult cardiac myocytes and 293 embryonal kidney cells is prevented by inhibitors of sGC or PKG and mimicked by cGMP analogues or by transfection of constitutively active PKG I (130, 133). Activation of p38 by cGMP is also observed in VSMCs (112), and in different hematopoietic cells (14, 133, 134), but there is no effect of cGMP on p38 activity in neonatal cardiomyocytes and hepatic stellate cells (115, 126). The cell type-specific activation of p38 by cGMP appears to involve activation of the upstream kinases MEK-3/6 by PKG I (133, 134) (Figure 2).

JNK activity is stimulated by cGMP in a PKG I-dependent fashion in VSMCs, fibroblasts and colon cancer cells (49, 135); the cGMP effects on JNK are prevented by a dominant negative MEKK-1 and may involve direct phosphorylation of MEKK-1 by PKG I (135), but this is a cell type-specific phenomenon (49, 109, 115).

4. POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION BY cGMP

4.1. Splicing

Regulation of gene expression at post-transcriptional levels includes regulation of pre-mRNA splicing, mRNA stability and translation. PKG I phosphorylates splicing factor 1 (SF1) *in vitro* and *in vivo*, and thereby inhibits pre-spliceosome assembly (45). However, it is presently unknown whether this phosphorylation results in alternative splice site selection or otherwise regulates RNA processing of specific genes.

4.2. Regulation of mRNA stability by cGMP

Several components of the NO/cGMP pathway appear to be controlled at a post-transcriptional level by cGMP, including iNOS, and sGC-alpha 1 and -beta 1 subunits, with elevated intracellular cGMP concentrations leading to mRNA *de*stabilization (see also Sections 5.2.3. and 5.3.1.) (136-138). In addition, treatment of cardiac fibroblasts with NO donors or cGMP analogues decreases TGF-beta 3 mRNA levels via message *de*stabilization (139). Recent work has established that stability of sGC-alpha 1 mRNA in VSMCs is regulated by the ubiquitous mRNA binding protein HuR; HuR binds to AU-rich elements in the 3'UTR and increases mRNA half-life, but cGMP-elevating agents decrease expression and RNA binding of HuR thereby *de*stabilizing sGC-alpha 1 mRNA (137). The rapid decrease in sGC- alpha 1 and TGF-beta 3 mRNA in NO/cGMP-treated cells is prevented by actinomycin D, suggesting that the mechanism of mRNA *de*stabilization requires active transcription of an unknown factor(s); this could be an RNA-*de*stabilizing protein or a factor involved in down-regulation of HuR (137, 139). NO/cGMP treatment of mesangial cells *de*creases the stability of matrix metalloproteinase-9 (MMP-9) by inhibiting the expression of HuR; NO/cGMP reduce HuR mRNA and protein levels to a degree that is sufficient to explain MMP-9 mRNA *de*stabilization which is reversed by the addition of

recombinant HuR protein (140).

HuR regulates mRNA stability of many different genes that are also regulated by cGMP, including iNOS, COX-2, VEGF, TNF-alpha, cyclins A and D1, and others (141-143). Therefore, down-regulation of HuR by cGMP could potentially explain the cGMP-mediated down-regulation of these mRNAs observed under some conditions (see below). Since many of these genes are also transcriptionally regulated by cGMP, cGMP could either increase or decrease mRNA expression, depending on whether cells express certain transcription factors and/or HuR and whether positive transcriptional or negative post-transcriptional regulation supervenes. cGMP can also *increase* mRNA stability, as illustrated by the example of the potassium chloride cotransporter-3 (144).

4.3. Translational regulation by cGMP

Translational regulation by cGMP has been most thoroughly studied for the asialoglycoprotein receptor (ASGR), a hepatocellular surface lectin (145-147), but the synthesis of insulin receptor subunits alpha and beta appears to be regulated by cGMP via a similar mechanism (146). Under conditions of increased intracellular cGMP, ASGR mRNA is shifted into a translationally active polysomal pool, whereas low intracellular cGMP results in the association of a negative trans-acting factor (called COPI) with the 5'UTR of the ASGR mRNA, which prevents ribosomal scanning at the site of translational initiation (145, 147). Cyclic GMP induced phosphorylation of COPI in intact cells is prevented by inhibition of PKG I and correlates with increased ASGR synthesis; PKG I-dependent phosphorylation of COPI may regulate association of the COPI/RNA complex (146).

5. REGULATION OF SPECIFIC GENES BY cGMP

5.1. Gene expression profiling

Presently, cGMP-mediated increases or decreases in mRNA expression fulfilling the minimal criteria described in the Introduction have been reported for > 50 different genes, but in most cases the mechanisms of cGMP regulation have not been explored. (Table 3). Gene expression profiling is beginning to contribute to the rapidly growing list of cGMP-regulated genes. Preliminary data from cDNA microarray analysis comparing PKG-expressing and PKG-deficient VSMCs suggest > 100 transcripts may be up-regulated more than three-fold by cGMP/PKG (148). Microarray analysis of gene expression in rat aorta or cardiomyocytes exposed to NO donors reveal hundred of genes changed by NO, but many of them may be regulated in a cGMP-independent fashion (149, 150). The hearts of eNOS-, nNOS-, and ANP-deficient mice have been compared to wild type hearts, and some of the divergent transcriptional programs may be related to changes in cGMP signaling (77, 151). Similarly, differential display-polymerase chain reaction has identified about 200 NO-regulated genes in hepatocytes (152, 153). Some of the genes differentially expressed in human endothelial cells over-expressing heme oxygenase-1 (HOX-1) may be related to increased intracellular cGMP levels in transduced versus control cells (154).

While gene array and differential display analyses provide large amounts of information regarding changes in gene expression in response to specific signals or genetic signaling defects, only detailed analysis of single genes will be able to distinguish direct versus indirect effects and determine the physiological significance of these changes.

5.2. cGMP signaling components

Several components of the cGMP/PKG signaling cascade are down-regulated by cGMP, leading to negative feedback or de-sensitization of the pathway. Elevated intracellular cGMP concentration lead to decreased expression of PKG I, rGC-A and sGC; negative cGMP regulation of iNOS, ANP and BNP is discussed in Sections 5.3.1. and 5.7.1. Whether expression of the cGMP-binding, cGMP-specific PDE5A gene is positively regulated by cGMP remains controversial (155, 156).

5.2.1. PKG I

In early passage VSMCs and in intact blood vessels, continuous exposure to NO-releasing agents, or cGMP analogues suppresses PKG I mRNA and protein levels by *decreasing* transcription without affecting mRNA stability (157, 158). Similarly, reduced levels of PKG I are found in cardiomyocytes exposed to cGMP analogues for 12 h, and in the aortas of transgenic mice over-expressing eNOS in the endothelium (8, 159). Down-regulation of PKG I in VSMCs exposed to inflammatory cytokines can be explained by increased iNOS expression and cGMP generation (160).

PKG inhibition, but not PKA inhibition, prevents down-regulation of PKG by NO in isolated pulmonary veins, and prolonged exposure to cAMP analogues does not affect PKG expression in these vessels (158). However, in primary aortic VSMCs, other investigators found that cAMP analogues decrease PKG I mRNA levels and PKG promoter activity more effectively than cGMP analogues, and the effects of both cyclic nucleotides are reversed by PKA inhibition, suggesting that high cGMP concentrations may inhibit the PKG I promoter through cross-activation of PKA (160, 161). Site-directed mutagenesis and electrophoretic mobility shift experiments suggest that two SP-1 sites are required for basal promoter activity, and SP-1 and SP-3 DNA binding activities appear to be reduced in cyclic nucleotide-treated cells (161).

5.2.2. Receptor guanylate cyclase A (rGC-A)

Ligand-dependent down-regulation of rGC-A is associated with a cGMP-mediated *reduction* in rGC-A mRNA level and promoter activity (162, 163). Correspondingly, rGC-A expression is up-regulated in ANP-deficient mice (164). Down-regulation of rGC-A mRNA and promoter activity by cGMP occurs in embryonal VSMCs and renal collecting duct cells, and is not mimicked by cAMP (162, 163). Transcriptional repression by cGMP is mediated by a 26 base-pair negative response element localized 1.3 kb upstream of the transcription start site; this element is conserved among

Table 3. Regulation of Specific Genes by cGMP

Gene[1]	Cell Type[2]	Culture Conditions[3]	Regulation by cGMP	PKG Mediated[4]	References
Gene Involved in cGMP Signaling					
PKG	VSMCs	Early passage	↓ mRNA, ↓ transcription	n.d.	157-161
rGC-A	VSMCs, RTCs	"	↓ mRNA, ↓ transcription	n.d.	162-165
sGC (alpha1,beta1)	VSMCs, RMICs	"	↓ mRNA stability (↓ HuR)	n.d.	136, 137, 167, 168
Genes Involved in Inflammation					
iNOS	CMs, VSMCs MCs MCs Macrophages	Cytokine-stim. Cytokine-stim. " LPS-stimulated	↑ mRNA ↑ mRNA (<12 h) ↓ mRNA stability (>24 h) ↓ mRNA stability	Yes n.d. n.d. n.d.	170, 171 138 138 172
COX-2	A549 Lung carcinoma RTCs MCs MCs Macrophages	 Salt-depleted Cytokine-stim. " LPS-stimulated	↑ mRNA ↑ mRNA ↑ transcription (<12 h) ↓ mRNA stability (>24 h) ↓ mRNA stability	? n.d. n.d. n.d. n.d.	111 175 173, 174 173, 174 172
HOX-1	Hepatocytes ECs	Normoxic Serum-starved	↑ mRNA, ↑ transcription "	? No	178 109, 177
TNF-α	Macrophages	LPS-stimulated	↓ mRNA stability	n.d.	180, 184
	Macrophages,Glia CMs, VSMCs	Unstimulated "	↑ mRNA "	n.d. Yes	181-183 58, 171
IL-12R-beta-2	T-Lymphocytes	Anti-CD3-stim.	↑ mRNA	n.d.	228
Genes Involved in Regulation of Cell Proliferation					
MKP-1	VSMCs, ECs, MCs, BHK	Serum-starved "	↑ mRNA "	Yes Yes	197, 200, 201 128, 199
Cyclins A, D1, E $p21^{Waf1/Cip1}$ $p16^{INK4a}$	VSMCs, MCs Adv. fibroblasts, VSMCs VSMCs	Serum-stimulated " "	↓ mRNA ↑ mRNA ↑ mRNA	n.d. n.d. n.d.	186, 187, 203 110, 112, 204 206
ET-1	ECs, Card. fibroblasts	Serum-starved	↓ mRNA	n.d.	188, 208, 210-212
CT-GF	MCs	Unstimulated	↓ mRNA	n.d.	209
Genes Involved in Apoptosis					
Mcl-1 Bcl-2	CMs Neuronal cells, B-lymphocytes	 Serum-starved	↓ mRNA ↑ mRNA "	n.d. Yes n.d.	214 10, 35 229
Tpx-1 BNIP3	Neuronal cells Hepatocytes	Serum-starved	↑ mRNA ↓ mRNA	Yes n.d	10, 227 153
Genes Involved in Angiogenesis and Extracellular Matrix					
VEGF	VSMCs, Hepatoma,Glioma cells	Normoxic "	↑mRNA "	n.d.	233 234
	VSMCs, ECs	Hypoxic	↓ mRNA, ↓ transcription?	n.d.	236, 237
TGF-beta-3 Fibronectin Collagen	Card. fibroblasts Card. fibroblasts, MCs Card. Fibroblasts, MCs	High glucose " High glucose	↓ mRNA stability ↓ mRNA ↓ mRNA	n.d. n.d. n.d.	139 241, 242 241, 242
Thrombospondin-1 Osteopontin	MCs VSMCs VSMCs	High glucose Dedifferentiated/ PKG transfected "	↓ mRNA, ↓ transcription ↓ protein, slight ↓ mRNA ↓ protein, slight ↓ mRNA	Yes Yes Yes	240, 241 239 239
MMP-9	MCs	Cytokine-stim.	↓ mRNA stability (↓ HuR)	n.d.	140
MMP-13	Ecs	Resting	↑ mRNA, ↑ transcription	Yes	108
Genes Associated with Cardiac Hypertrophy					
ANP BNP Skeletal α-actin β-MHC MLP	CMs " " " "	PE-stimulated " " " ET-I stim.	↓ mRNA ↓ mRNA, ↓ transcription ↓ mRNA ↓ mRNA ↓ mRNA	Yes Yes n.d. n.d. Yes	8, 16 16, 100 246 246 150
Genes Associated with VSMC Differentiation and Function					

SM-MHC-2 SM-α actin SM-calponin FGFR-1/2	VSMCs “ “ “	Dedifferentiated/ transfected with PKG “	↑ protein, ↑ transcription? “ “ ↓ mRNA	Yes “ “ “	206, 250-253 250, 251 250, 251 251
RhoA	VSMCs		↑ mRNA, ↑ transcription	Yes	33
Genes Associated with Neuronal Cell Differentiation and Function					
GnRH Somatostatin mPer-1 mPer-2	Hypothalamic neurons “ Suprachiasmatic nucleus “	 Light induced “	↓ mRNA ↓ transcription ↑ mRNA ↓ mRNA ↑ mRNA	Yes n.d. PKG II PKG II	87, 166 270 21 21
Genes Associated with Osteoblast Differentiation and Function					
Osteocalcin Collagen I Alkaline Phosphatase	Osteoblasts “ “		↑ mRNA ↑ mRNA ↑ mRNA	n.d. n.d. n.d.	280-284 284 280, 281, 283-285
Miscellaneous Genes					
Erythropoietin	Hepatoma cells	Hyp/normoxic	↑ mRNA, ↑ transcription	Yes	288
γ-Globin	Erythroblasts		↑ transcription	?	289
Neuroglobin	Neuronal cells		↑ mRNA	?	290
PAI-1	VSMCs, ECs	ATII-stim.	↓ mRNA	n.d.	99, 293, 294
KCl Cotransporter-3	VSMCs		↑ mRNA stability	n.d.	144
p11	Bronchial epithelium		↑ mRNA	Yes	80

[1] CT-GF, Connective tissue growth factor; ET-1, Endothelin-1; FGFR, Fibroblast growth factor receptor; MCP, Muscle LIM protein; MHC, Myosin heavy chain; MKP-1, MAP-kinase phosphatase-1; MMP, Matrix metalloprotease; PAI-1, plasminogen activator inhibitor-1; please see text for other abbreviations, [2] Adv. Fibroblasts, Adventitial fibroblasts; CMs, Cardiomyocytes; ECs, Endothelial cells; MCs, Mesangial cells; RTCs, Renal tubule cells, RMICs Renal medullay interstitial cells, [3] ATII, angiotensin II; LPS, lipopolysaccharide; PE, Phenylephrine; if not otherwise noted, cells were grown in standard serum-containing medium and not specifically stimulated, [4] ? indicates that KT5823 was used as a PKG inhibitor

species and has some resemblance to a negative cGMP-responsive element in the gonadotropin-releasing hormone promoter (165, 166). The *trans*-activating factors mediating cGMP's transcriptional repressor effect have not been identified, but binding of nuclear proteins to the negative response element is reduced in cells treated with ANP or cGMP analogues (165).

5.2.3. Soluble guanylate cyclase (sGC)

Low concentrations of NO donors, ANP, or cGMP analogues *decrease* mRNA and protein levels of both sGC-alpha 1 and - beta 1 subunits in early passage VSMCs as well as other cell types; sGC mRNA levels decrease within 2-4 h and by >90% within 24 h (136, 167). Correspondingly, sGC expression is up-regulated in eNOS-deficient mice (168). cGMP accelerates the decay of sGC-alpha-1 and beta-1mRNA; stability of sGC-alpha-1 mRNA is regulated by HuR, which binds to AU-rich elements in the 3'UTR (see Section 4.2.) (136, 137). Since cAMP-elevating agents and cAMP analogues also destabilize sGC mRNA and can reduce HuR protein, it will be important to determine the role of PKA versus PKG I in this process (137).

5.3. Inflammation

Inflammatory cytokines, including tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1) and interferon-gamma (IF-gamma) transcriptionally activate inducible NOS (iNOS) and cyclooxygenase-2 (COX-2) to produce NO and prostaglandin H2 (the precursor of eicosanoids), both considered important mediators of inflammation (56). Heme oxygenase-1 (HOX-1, also named heat shock protein 32) is similarly induced by inflammatory cytokines, to produce CO (which can activate sGC), biliverdin and free divalent iron, which may serve as an anti-oxidant defense mechanism during inflammation (169). cGMP can have both positive and negative effects on the expression of iNOS, COX-2 and TNF-alpha, in some cases leading to bi-phasic regulation, e.g. early transcriptional up-regulation followed by later down-regulation due to mRNA destabilization; the latter effect could be related to cGMP-mediated down-regulation of HuR (described in Section 4.2.).

5.3.1. Inducible NO synthase (iNOS)

In neonatal rat cardiomyocytes, VSMCs, and mesangial cells, *cytokine-induced* iNOS mRNA levels are *enhanced* by NO donors, ANP/BNP or cGMP analogues, and reduced by NOS inhibitors (138, 170, 171). In the absence of cytokines, the cGMP-elevating agents have very little or *no* effect on *basal* iNOS expression. The mechanism whereby cGMP enhances cytokine induction of iNOS remains unclear; in cardiomyocytes this effect is prevented by a specific PKG I inhibitor, and it could potentially involve cGMP/PKG stimulation of NF-kappa B activity (described in Section 3.1.3.) (138, 170). In mesangial cells, cytokine induction of iNOS mRNA peaks at 12-24 h and persists > 48 h, but in the presence of cGMP analogues, peak induction occurs *earlier* and mRNA levels *decrease* after 24 h, due to a shortening of the iNOS mRNA half-life in the presence of cGMP analogues (138). *De*stabilization of iNOS mRNA by cGMP has been

confirmed in other cell types but was not observed in cardiomyocytes (142, 170, 172).

5.3.2. Inducible cyclooxygenase-2 (COX-2)

The effects of NO on cytokine-induced COX-2 and iNOS expression are similar with both stimulatory and inhibitory effects described, depending on cell type and time course (56). In mesangial cells, NO is necessary for the transcriptional induction of COX-2 mRNA by cytokines, and NO donors, ANP and cGMP analogues synergistically *enhance* the effect of IL-1beta on COX-2 mRNA at early time points (173, 174). However, at later time points, NO donors *decrease* cytokine-induced COX-2 mRNA levels, reminiscent of the biphasic effect of NO on iNOS expression in these cells (138, 174). *De*stabilization of COX-2 mRNA by ANP and cGMP analogues has been described in LPS-stimulated macrophages (172).

In whole animal models, most of the evidence suggests a positive role of NO on COX-2 expression, and the importance of NO generation for prostaglandin synthesis has been confirmed in iNOS-deficient mice (56). Induction of COX-2 mRNA by salt depletion and/or angiotensin-converting enzyme inhibition in renal cortex is blocked by NOS inhibitors, and this effect is relieved by cGMP analogues; correspondingly, NO donors and cGMP analogues *increase* basal COX-2 mRNA levels in renal tubule cells (175).

5.3.3. Inducible heme oxygenase-1 (HOX-1)

Several studies in VSMCs and endothelial cells found that NO *induces* HOX-1 mRNA and protein expression in a cGMP-*in*dependent fashion (169, 176). However, others reported that ANP and cGMP analogues *induce*-HOX 1 mRNA in endothelial cells, and that the effect is dependent on activation of AP-1 and blocked by MAP-kinase pathway inhibitors (109, 177). In primary rat hepatocytes, NO-donors and cGMP analogues increase HOX-1 mRNA and promoter activity to a similar extent, and the effect is dependent on an intact CRE (178); however, perfusion of intact rat livers with cGMP analogues does not appear to affect HOX-1 mRNA levels (179).

5.3.4. Tumor necrosis factor-alpha (TNF-alpha)

TNF- alpha is an important cytokine involved in the pathogenesis of many inflammatory diseases, including sepsis (180). In early passage rat aortic VSMCs, cGMP analogues *induce* basal TNF-alpha mRNA and enhance IL-1-induced TNF-alpha mRNA (171). Similarly, NO increases TNF-alpha mRNA in a cGMP- and PKG I-dependent fashion in cardiomyocytes, and this effect correlates with increased NF-kappa B activity and is mimicked by cGMP, but not cAMP analogues (58). In resting macrophages and glial cells, basal TNF- alpha mRNA and protein synthesis are also *up*-regulated by cGMP-elevating agents (181-183). However, in LPS-stimulated macrophages, induction of TNF-alpha mRNA and protein is *attenuated* by NO, ANP, and cGMP analogues due to RNA *de*stabilization; this could be related to HuR down-regulation (141, 180, 184).

5.4. Cell proliferation

cGMP can have pro- or anti-proliferative effects, depending on the cell type and growth conditions studied. *Anti*-proliferative effects of cGMP in VSMCs, mesangial cells, fibroblasts, neuronal cells, and epithelial cells have been correlated with cGMP inhibition of growth factor-induced Erk-1/2 activity (see Section 3.3.3., Table 2), increased expression of MAP-kinase phosphatase-1 (MKP-1), modulation of cell cycle-regulatory genes and in some cases, reduction of growth factor synthesis (110, 120, 122, 124, 185-189). The effects of natriuretic peptides on proliferation are cGMP dependent, but NO can also have cGMP-independent effects (190, 191). Most data indicate that cGMP *inhibits* the proliferation of growth factor-stimulated VSMCs through PKG I, with over-expression of PKG I enhancing the effect (122, 185, 192, 193); under some conditions, cGMP may inhibit growth by cross-activating PKA (6, 132, 191). However, there are two studies reporting cGMP *stimulation* of FGF- or PDGF-induced proliferation in primaryVSMCs; this effect was not observed in VSMCs from PKG-deficient cells (78, 99)

The *pro*-proliferative effects of cGMP in endothelial cells correlate with cGMP stimulation of Erk-1/2 activity (Section 3.3.3. and Table 2), and may be in part related to increased synthesis of vascular endothelial growth factor (VEGF, discussed in Section 5.6.1.) (113, 114, 194, 195). The effects of cGMP on chondroblast and osteoblast proliferation are discussed in Section 5.10.

5.4.1. MAP-kinase phosphatase-1 (MKP-1)

MKP-1 is a ubiquitously-expressed protein phosphatase with dual specificity toward phospho-Tyr and phospho-Thr, which *in*activates Erk-1/2, JNK, and p38 MAP-kinases *in vitro* and *in vivo* (Figure 2) (196, 197). MKP-1 mRNA is expressed at very low levels in quiescent cells, but its transcription is rapidly induced by various mitogenic stimuli through the Ras/Raf/Erk pathway; its expression serves as a feed-back mechanism to terminate MAP-kinase activation (198). MKP-1 mRNA is also *induced* by NO-releasing agents, ANPs, and cGMP analogues in endothelial cells, VSMCs and breast cancer cells (197, 199-202). MKP-1 mRNA induction by cGMP can occur in the absence of Erk-1/2 stimulation (128). Cyclic GMP, via PKG I, induces MKP-1 mRNA sufficiently to inhibit TNF-alpha-induced p38 activity and growth factor-induced Erk-1/2 activity, and the MKP-1 induction contributes to the anti-proliferative effect of cGMP-elevating agents (128, 198, 199). Insulin induction of MKP-1 in VSMCs is mediated via the NO/cGMP/PKG I pathway and is necessary for insulin-mediated inhibition of PDGF-directed VSMC migration (200, 201).

5.4.2. Cell cycle regulatory proteins

cGMP-mediated inhibition of cell proliferation is associated with G1 cell cycle arrest or delay in G1/S transition, and *de*creased proliferative cell nuclear antigen (PCNA) expression (120, 122, 186, 189, 203-205). cGMP *de*creases expression of cell cycle-promoting genes, including cyclin A, D1, and E (186, 187, 203). In addition, cGMP can *in*crease expression of cell cycle inhibitors, such as $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ (110, 112, 204, 206). CO inhibition of VSMC proliferation requires sGC activation and is mediated by cGMP; the anti-proliferative effect of

CO/cGMP requires p38 MAP-kinase activation and increased $p21^{Waf1/Cip1}$ expression, because CO/cGMP does not inhibit proliferation in cells isolated from mice deficient in the p38 upstream kinase MAP-kinase kinase-3 (these cells also failed to increase p21) and in $p21^{Waf1/Cip1}$-deficient cells (112). CO/cGMP inhibition of VSMC proliferation is also associated with a reduction in the levels of E2F-1, a transcription factor essential for cell cycle progression (207). Under some conditions, cGMP-induced changes in expression of cell cycle-associated genes appear to be dependent on inhibition of Erk-1/2 (110, 124, 187).

5.4.3. Growth factors (endothelin-1, connective tissue growth factor)

Anti-proliferative effects of cGMP in smooth muscle cells and cardiac fibroblasts have been correlated with inhibition of growth factor synthesis, e.g., cGMP *inhibits* the expression of ET-1 and connective tissue growth factor (CT-GF) (188, 208, 209). VEGF is discussed in Section 5.6.1.

Endothelin-1 (ET-1) is a potent vasoconstrictor and mitogenic peptide produced by proteolytic cleavage from an inactive precursor and secreted by endothelial cells (208, 210). Basal prepro-ET-1 mRNA levels and peptide synthesis are *negatively* regulated by cGMP, because incubation of endothelial cells with NOS inhibitors, NO scavenger molecules, or sGC inhibitors increase prepro-ET-1 synthesis and ET-1 peptide secretion, and this effect is reversed by cGMP analogues (208, 210). Thrombin- or AT II-mediated increases in prepro-ET-1 mRNA levels are also inhibited by natriuretic peptides and cGMP analogues, but the mechanism of this suppressive effect is unknown (188, 211, 212).

5.5. Apoptosis

Apoptosis, the process of programmed cell death, is critical for normal development and many physiological and pathophysiological processes (213). cGMP regulates several apoptosis-associated genes and, depending on the cell type, cGMP can have either pro- or anti-apoptotic effects.

5.5.1. Pro-apoptotic effects of cGMP

NO (in a sGC-dependent manner), and cGMP-elevating agents can *induce* apoptosis in VSMCs, cardiac myocytes, endothelial and epithelial cells (185, 213-220). In cardiomyocytes, cGMP sharply *de*creases mRNA expression of the anti-apoptotic Bcl-2 homologue Mcl-1 (214). In many cases, the pro-apoptotic effect of cGMP appears to be mediated by PKG I (185, 213, 216, 221).

In human colon cancer cells, rGC-activating guanylin peptides and certain cGMP-specific phosphodiesterase inhibitors *induce* apoptosis at concentrations that cause a prolonged increase in the intracellular cGMP concentration and activation of PKG I (217, 218, 221). Cyclic GMP may increase apoptosis via PKG I-dependent activation of JNK (65, 135, 221). In addition, PKG I activation correlates with increased beta-catenin phosphorylation and degradation (222). Since beta-catenin promotes anti-apoptotic gene expression, down-regulation of beta-catenin-dependent genes may be partly responsible for the pro-apoptotic effects of cGMP. Over-expression of an N-terminal beta-catenin fragment reduces cGMP-induced beta-catenin degradation, and prevents down-regulation cyclin D1 mRNA and apoptosis (222). While these observations may point to a novel mechanisms of gene regulation by cGMP via beta-catenin phosphorylation, further studies will be required to confirm the mechanisms of beta-catenin regulation by cGMP.

5.5.2. Anti-apoptotic effects of cGMP

Cyclic GMP has *anti*-apoptotic effects in neuronal cells, hepatocytes, and lymphocytes, and a number of studies point to Bcl-2 and Bcl-2-associated proteins as mediators of cGMP's anti-apoptotic effects. In cerebellar neurons, prolonged inhibition of NOS or sGC induces apoptosis which is prevented by cGMP analogues; cGMP's protective effect is associated with *in*creased CREB phosphorylation and *in*creased mRNA and protein expression of the apoptosis inhibitor Bcl-2 (35). Apoptosis of trophic factor-deprived neuronal cells can also be prevented by cGMP, and this is associated with cGMP blocking JNK activation, inhibiting caspase-3 activation, and stimulating the anti-apoptotic phosphatidylinositol 3-kinase/ protein kinase B (Akt) pathway (223-226). PKG I activation by cGMP appears to protect neuronal cells from lipid peroxidation during growth factor deprivation by *in*creasing mRNA and protein expression of the oxidative stress-related proteins thioredoxin and thioredoxin peroxidase (Tpx-1), which subsequently leads to *in*creased Bcl-2 expression (10, 227). In hepatocytes, cGMP inhibits apoptosis by *down*-regulation of mRNA and protein levels of the pro-apoptotic Bcl-2-binding protein BNIP3, among other mechanisms (153). In B-lymphocytes, NO/cGMP prevent antigen-induced apoptosis through mechanisms involving *in*creased Bcl-2 mRNA and protein expression (229).

5.6. Angiogenesis and extracellular matrix

Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis, the formation of new blood vessels in response to tissue ischemia, tumor growth, or wound healing, which appears to be positively regulated by cGMP/PKG, at least under conditions of normal oxygen tension. VEGF expression is modulated by cGMP (see below), and the angiogenic response to VEGF requires the NO/cGMP pathway, because it is defective in eNOS-deficient mice and is blocked by NOS, sGC and PKG inhibitors (113, 114, 230). The VEGF-induced increase in endothelial cell proliferation and migration appears to be mediated by cGMP/PKG-induced activation of Erk-1/2 and PKB (Akt) (113, 114, 194, 231). An *in vivo* model of embolic stroke suggest a positive role for cGMP in angiogenesis which is dependent on VEGF (195). Ischemia-induced angiogenesis and endothelial regeneration after vascular damage *in vivo* are also positively regulated by cGMP/PKG, with ischemia-induced angiogenesis enhanced in PKG I-overexpressing mice and diminished in PKG I-deficient mice (194, 206, 232).

5.6.1. Vascular endothelial growth factor (VEGF)

In *normoxic* VSMCs, hepatoma and glioma cells, NO donors *increase* basal VEGF mRNA levels in a cGMP-dependent fashion (233, 234). VEGF expression is induced

by hypoxia through transcriptional mechanisms involving the hypoxia-inducible transcription factor HIF-1alpha and through post-transcriptional mechanisms involving mRNA stabilization by HuR (141, 235). Treatment of *hypoxic* VSMCs or endothelial cells with NO, CO, or a membrane-permeable cGMP analogue *decreases* VEGF mRNA, HIF-dependent transcription, and DNA binding activity (236, 237). The effect of NO/cGMP on VEGF mRNA stability and HuR binding has not yet been determined.

5.6.2. Thrombospondin-1 and other extracellular matrix proteins

Thrombospondin-1 is a cellular matrix protein and a potent inhibitor of angiogenesis in its native, full-length form; it is secreted by many different cell types including endothelial cells, mesangial cells and VSMCs (238, 239). Thrombospondin-1 is also a major physiological regulator of tumor growth factor-beta (TGF-beta) activity, which in turn regulates extracellular matrix, including fibronectin and collagen synthesis (240). In mesangial cells, high glucose stimulates thrombospondin-1 expression at the transcriptional level and activates TGF-beta through down-regulation of the NO/cGMP/PKG pathway; conversely, NO/cGMP or expression of constitutivley active PKG I prevents the glucose effects and *suppresses* thrombospondin-1 promoter activity (240, 241). PKG-deficient, de-differentiated VSMCs express high levels of thrombospondin-1 and osteopontin, which are down-regulated when constitutively active PKG I is re-introduced by transfection (239). NO/cGMP also *down*-regulate fibronectin and collagen mRNA levels in cardiac fibroblasts and mesangial cells (241, 242). Correspondingly, ANP-deficient cardiomyocytes demonstrate increased expression of thrombospondin, osteopontin and collagen mRNA (151).

5.6.3. Matrix metalloproteinases (MMPs) and their tissue inhibitor (TIMP)

Angiogenesis requires degradation of matix proteins through regulated expression of matrix metalloproteinases (MMPs) and their tissue inhibitor (TIMP). NO and cGMP *de*crease the expression of MMP-9 through mRNA destabilization via down-regulation of HuR (see Section 4.2.) (140). In contrast, NO and cGMP *in*crease the expression of MMP-13 and MMP-1, -2, and -3; cGMP stimulation of the MMP-13 promoter is mediated by PKG I stimulation of the Erk-1/2 MAP-kinase pathway (108, 243). A preliminary report suggests up-regulation of TIMP-2 by NO/cGMP/PKG (244).

5.7. Cardiac hypertrophy

Cardiac hypertrophy involves myocyte enlargement, changes in myofibrillar assembly, and a "hypertrophic pattern" of gene expression, i.e., increased expression of ANP, BNP and skeletal alpha-actin (245). Stimuli for myocyte hypertrophy include ET-1, AT II, and alpha-adrenergic agonists such as phenylephrine; signaling pathways that promote hypertrophy include MAP-kinases, Ca^{++}/calcineurin, and RhoA (245). Mice deficient in either ANP or rGC-A develop increased ventricular mass out of proportion to the mild changes in blood pressure (2), and mice deficient in NOS-1 develop cardiac hypertrophy with normal systemic blood pressure (15). These and many other studies suggest that NO/cGMP *negatively* regulate cardiac hypertrophy.

5.7.1. ANP, BNP, skeletal alpha-actin and beta-myosin heavy chain

Over-expression of PKG I enhances the anti-hypertrophic effects of NO in phenylephrine-stimulated cardiomyocytes *in vitro* and prevents phenylephrine-induced increase in prepro-ANP mRNA (8). Similarly, natriuretic peptides and cGMP analogues *suppress* the phenylephrine-induced hypertrophic pattern of gene expression including skeletal alpha-actin and beta-myosin heavy chain gene expression (16, 246). Increased expression of extracellular matrix proteins in the hypertrophic hearts of ANP-deficient mice could be explained by the lack of cGMP/PKG suppression of thrombospondin and TGF-beta activity, as discussed in Section 5.6.2. (151, 241). Since RhoA signaling is necessary for phenylephrine-induced hypertophic gene expression, some of PKG I's anti-hypertrophic effects may be through inhibition of RhoA signaling to SRF (see Section 3.3.2.) (7, 245). NO/cGMP activation of PKG I also inhibits Ca^{++}-dependent activation of the hypertrophic calcineurin-NFAT pathway, preventing NFAT induction of BNP mRNA expression and promoter activity (see Section 3.3.1.) (100).

5.7.2. Muscle LIM protein (MLP)

Expression profiling of genes stimulated by the hypertrophic peptide ET-1 in the presence or absence of NO demonstrate *down*-regulation of muscle LIM protein (MLP) by NO (150). This effect is at least partially mediated by cGMP/PKG I and may explain some of PKG I's anti-hypertrophic effects, because MLP expression is necessary and sufficient for ET-1-induced hypertrophy (150).

5.8. VSMC differentiation and function

VSMCs change their state of differentiation from a highly differentiated, "contractile" phenotype to a *de*-differentiated "synthetic" phenotype in response to vascular injury and during *in vitro* culture; de-differentiated VSMCs acquire the capacity to proliferate, migrate, and produce extracellular matrix proteins such as osteopontin and thrombospondin, and contribute to neointima formation after vascular injury (29). The mechanism(s) responsible for VSMC phenotypic modulation are incompletely understood, but *in vitro*, *de*-differentiation to the synthetic phenotype is associated with loss of PKG I expression and transcriptional down-regulation of contractile proteins such as smooth muscle (SM) myosin heavy chain-2 (MHC-2), SM-alpha-actin, and SM-calponin (29). After vascular injury *in vivo*, transiently reduced PKG I expression has been observed by two groups, but not by a third; PKG I expression appears to decrease in synthetic, proliferating VSMCs in the neointima coincidentally with the transcriptional down-regulation of contractile marker expression and increased synthesis of osteopontin (193, 247-249).

5.8.1. SM-myosin heavy chain-2, SM-alpha-Actin, SM-calponin, and FGF receptor

When synthetic, PKG-deficient VSMCs are transfected with constitutively active or wild type PKG I

(with cGMP stimulation), a more contractile phenotype is restored with fusiform morphology, *in*creased expression of SM-MHC-2, SM-alpha-actin, and calponin protein, and *de*creased expression of osteopontin, thrombospondin, and FGF receptors-1/2 (29, 239, 250, 251). SM-MHC-2 mRNA and protein expression is also increased in CNP peptide-overexpressing VSMCs *in vitro* and *in vivo* after angioplasty (206), and down-regulation of the SM-MHC promoter by platelet-derived growth factor is prevented by NO (252). The mechanism of cGMP/PKG I-mediated induction of contractile protein expression has not yet been determined, but a preliminary report suggests that PKG I activation may activate the SM-MHC promoter (253). FGF receptor protein and mRNA levels are both dramatically and proportionally decreased in the PKG I-transfected cells compared to control-transfected cells (251). Preliminary data from cDNA micro-array analyses comparing PKG I-transfected and control-transfected, late passage VSMCs suggest that > 100 transcripts may be up-or down-regulated more than three-fold by cGMP/PKG I (148). Thus, cGMP/PKG-regulated gene expression may play an important role during VSMC phenotypic modulation, but an existing controversy regarding the role of cGMP/PKG in artherosclerosis will need to be resolved in future studies (29, 99, 193, 206).

5.8.2. RhoA

RhoA plays a major role in physiological processes associated with changes in the actin cytoskeleton, such as VSMC contractility and motility, and during smooth muscle cell differentiation (104, 254-256). Treating primary VSMCs with NO donors or cGMP analogues *in*creases RhoA mRNA and protein levels within 3-6 h with a peak effect at 24 h (33). This increase is mediated by PKG I and involves increased *rhoA* transcription as well increased RhoA protein stability due to PKG I phosphorylation. The transcriptional effect of cGMP/PKG I is associated with increased ATF-1 phosphorylation, and appears to be mediated by ATF-1 binding to a CRE in the RhoA promoter (33). Experiments in intact animals suggest that basal release of NO is necessary to maintain RhoA expression and function in VSMCs (33). However, cGMP/PKG I can also inhibit the activation (GTP-loading) and down-stream functions of RhoA in mitogen-stimulated cells, as described in Section 3.3.2. (7).

5.9. Neuronal cell differentiation and function

5.9.1. Neuronal differentiation

The differentiation of neural plate cells during development of the nervous system is regulated through the coordinated action of inductive proteins, including Sonic hedgehog (Shh), which direct activation and repression of transcription factors in a manner appropriate to the cells' position along the dorsoventral axis of the neuronal tube (257). cGMP *enhances* Shh signaling, thereby enhancing differentiation of neural plate cells into ventral neural cell types, while cAMP has the opposite effect (257). Additional roles of cGMP/PKG I in neuronal differentiation have been described that likely involve cGMP-mediated regulation of gene expression (120, 258, 259).

5.9.2. Synaptic plasticity

Synaptic plasticity refers to the capacity of synaptic connections to become persistently strengthened or weakened in response to external stimuli, and is thought to be involved in learning and memory (260). Long-term changes in synaptic strength are thought to require changes in neuronal gene expression, including induction of CREB phosphorylation and c-Fos expression (32, 66, 261). Multiple studies have implicated NO/cGMP/PKG signaling in specific forms of synaptic plasticity, i.e., hippocampal and cerebellar long-term potentiation (LTP) and depression (LTD), respectively (260, 262-264). Deletion of nNOS and eNOS in mice interferes with LTP and spatial learning (265). Hippocampus-specific ablation of PKG I causes a defect in late-phase LTP, and ablation of PKG I in cerebellar Purkinje cells results in impairment of LTD (19, 20). Induction of CREB phosphorylation and/or *c-fos* expression by the NO/cGMP/PKG I pathway has been documented in different neuronal tissues including hippocampus and cerebellum, and CREB and c-Fos are likely mediators of long-term changes in synaptic plasticity (36, 69, 70, 75, 76, 88, 266-268). In two different models of learning in rats, intraventricular injection of a NOS inhibitor blocks the formation of memory and the increase in c-Fos expression in the hippocampus and cerebral cortex (76). NOS inhibitors also attenuate stress activation of *c-fos* expression in the rat hypothalamus (74). In the mollusc Aplysia, NO/cGMP/PKG I and a CREB-dependent pathway are necessary and sufficient to trigger transcription-dependent, nociceptive sensitization, i.e. long-term hyperexcitability after brief noxious stimulation (269).

5.9.3. Hypothalamic hormone synthesis

Growth hormone-releasing factor *stimulates* somatostatin mRNA levels and somatostatin release in the hypothalamus via increased NO and cGMP synthesis; somatostatin mRNA levels are also increased in response to cGMP analogues (270). Somatostatin suppresses pituitary growth hormone secretion, but may also function as a neuromodulator in other areas of the nervous system (270).

Transcription of the gonadotropin-releasing hormone (GnRH) gene in hypothalamic neurons is *repressed* by the glutamate/NO/cGMP/PKG I pathway; cGMP-mediated transcriptional repression localizes to a 300 bp neuronal-specific enhancer element in the promoter which binds the transcription factors Oct-1 and C/EBP- (87, 166).

5.9.4. Circadian rhythm

The circadian clock of mammals is located in the hypothalamic suprachiasmatic nuclei (SCN), and the Period genes 1 and 2 (mPer1 and mPer2) are involved in adjusting the clock in response to nocturnal light (21). cGMP levels and PKG activity in the SCN change with a circadian rhythm, and a role of cGMP/PKG signaling in the processing of light signals and transcriptional regulation of genes in the mammalian circadian system has been suggested by several studies (38, 271, 272). This role was confirmed in PKG II-deficient mice which lack the ability to re-set their circadian clock in response to nocturnal light; light induction of the mPer2 gene in the SCN is significantly reduced, whereas mPer1 induction is

elevated in these animals (21).

5.10. Bone development and homeostasis

5.10.1. Chondroblast growth and differentiation

Mice deficient in CNP or PKG II develop dwarfism as a result of impaired chondroblast proliferation and differentiation in the growth plates of long bones (22, 23). Targeted expression of CNP in growth plate chondrocytes can rescue the CNP-deficient phenotype only in the presence of PKG II (25). In contrast, transgenic mice that overexpress CNP or BNP demonstrate skeletal overgrowth (24-26). Studies on cGMP/PKG II-regulated genes in chondroblasts are lacking. However, cGMP and calcium synergistically stimulate *c-fos* expression in osteoblasts; this effect is mediated more efficiently by PKG II than PKG I, and involves cGMP/calcium-regulated cooperation between the transcription factors CREB and C/EBP-beta (85). Since c-Fos, CREB and C/EBP-beta are key regulators of chondrocyte growth and differentiation, these transcription factors may be important targets of cGMP/PKG II during bone development (273-276).

5.10.2. Osteoblast differentiation

Mice with targeted disruption of eNOS demonstrate defects in osteoblast maturation and activity; they show defective postnatal bone formation and anabolic responses to estrogens, and at least part of this phenotype may be due to defective cGMP generation (277-279). cGMP-elevating agents and cGMP analogues promote osteoblast differentiation in culture, increasing mRNA expression of differentiation-associated genes and promoting mineralization, while cAMP appears to have the opposite effect (280-283). In primary osteoblasts and in osteoblastis cell lines, NO donors, CNP, and cGMP analogues increase osteocalcin, collagen I and alkaline phosphatase mRNA and protein expression (280-285). Osteocalcin synthesis is synergistically increased by cGMP and vitamin D (286).

5.11. Miscellaneous genes regulated by cGMP

5.11.1 Erythropoietin

Erythropoietin regulates growth and differentiation of erythroid cells, and its expression is *induced* by hypoxia and cGMP (235). In hepatoma cells, hypoxia increases NO production, intracellular cGMP levels, erythropoietin promoter activity and secretion of erythropoietin; the effect of hypoxia on erythropoietin expression is attenuated by inhibitors of NOS, sGC, and PKG I and correlates with decreased GATA-2 binding to the erythropoietin promoter (287, 288). cGMP analogues may also *in*crease erythropoietin levels in *normoxic* hepatoma cells (288). NOS inhibition decreases the elevated erythropoietin level of anemic and ex-hypoxic/polycythemic mice; these findings may explain decreased erythropoietin synthesis in uremic patients who have elevated plasma levels of the endogenous NOS inhibitor N^G-monomethyl-L-arginine (287, 288).

5.11.2. Globin genes

Treatment of erythroid cells with hemin stimulates transcription and translation of hemoglobin and other erythroid-specific genes, and hemin increases the intracellular cGMP concentration, possibly via stimulation of heme oxygenase (289, 290). Hemin and cGMP analogues *stimulate* transcription of the gamma-globin gene in primary erythroblasts and erythroleukemia cell lines; hemin's effect is prevented when sGC is inhibited, but not when hemin-induced Erk-1/2 activation is inhibited (289). cAMP has the opposite effect on gamma-globin expression (291). Hemin also increases neuroglobin mRNA and protein expression in immortalized hippocampal neuronal cells, and myoglobin expression in L6 muscle cells, possibly via the same mechanism (290, 292).

5.11.3. Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is produced by VSMCs and endothelial cells, and elevated levels are found in atherosclerotic and balloon-injured vessels (293). NO donors, natriuretic peptides, and cGMP analogues *reduce* AT II-induced PAI-1 mRNA levels without affecting mRNA stability (293, 294). cGMP treatment reduces basal PAI-1 mRNA levels in primary VSMCs from wild type mice, but not from PKG I-deficient mice (99).

6. FUTURE PERSPECTIVES

Regulation of gene expression by cAMP has been studied intensely for several decades, but transcriptional regulation by cGMP has been recognized only relatively recently. Gene expression profiling is already contributing to the rapidly increasing list of cGMP-regulated genes, and is likely to contribute more in the future. We are just beginning to understand the mechanisms of transcriptional and post-transcriptional regulation by cGMP. Many of the effects of cGMP on gene expression are indirect, involving cross-talk with other signaling pathways (such as MAP-kinase pathways), but some effects of cGMP can be directly attributed to cGMP regulation of specific transcription factors (such as CREB and c-Fos). While there are many studies describing stimulation or repression of gene expression in response to increasing intracellular cGMP concentrations, future studies should concentrate on elucidating the mechanisms behind these phenomena. A more complete understanding of the effects of cGMP on specific transcription factors and RNA binding proteins may allow us to deduce long-term effects of cGMP signaling on gene expression programs, such as during bone development or in cardiac hypertrophy.

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