Nitric Oxide 43 (2014) 3–7

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

What is next in nitric oxide research? From cardiovascular system to cancer biology

Ka Bian *, Ferid Murad **

Department of Biochemistry and Molecular Medicine, George Washington University, School of Medicine, 2300 Eye Street NW, Washington, DC 20037, USA

ARTICLE INFO

Article history:
Received 24 April 2014
Revised 7 August 2014
Available online 19 August 2014

Keywords:
Nitric oxide synthase (NOS)
Cyclic guanosine monophosphate (cGMP)
Soluble guanylate cyclase (sGC)
Particulate guanylyl cyclase (pGC)
Natriuretic peptide
Phosphodiesterase (PDE)
cGMP-dependent protein kinase (PKG)

ABSTRACT

The broad role of nitric oxide (NO) and cyclic GMP in biochemistry and biology as important messenger molecules is evident from the numerous publications in this research field. NO and cGMP have been known as components of the key signaling pathway in regulating numerous processes such as vascular dilation, blood pressure, neurotransmission, cardiovascular function, and renal function. In spite of almost 150,000 publications with nitric oxide and cyclic GMP, there are few publications regarding the effects of these messenger molecules on gene regulation, cell differentiation and cell proliferation.

Our research data with embryonic stem cells and several cancer cell lines suggest that nitric oxide, its receptor soluble guanylyl cyclase (sGC) and sGC's product cyclic GMP can regulate the processes of proliferation and differentiation. Furthermore, we have found that undifferentiated stem cells and some malignant tumors such as human glioma have decreased levels of sGC and translocation of the sGCβ1 subunit to the nucleus. We propose that sGC and cyclic GMP function as tumor suppressors. An understanding of the mechanisms of the translocation of the sGCβ1 subunit into the nucleus and the possible regulation of gene expression of NO and/or cyclic CMP could lead to novel and innovative approaches to cancer therapy and stem cell proliferation and differentiation.

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1. Introduction of NO–cyclic GMP research

Our studies with guanylyl cyclase started as early as 1970s and were focused to determine how some hormones and drugs regulated cyclic GMP synthesis [1–3]. We found that most tissue extracts possessed guanylyl cyclase activity in the high speed supernatant (sGC) and particulate fractions (pGC) of homogenates with different kinetic properties [4,5]. The soluble fraction (sGC) had typical kinetic double reciprocal linear plots with regard to substrate GTP while plots with pGC were curvilinear with a Hill coefficient greater than 1. We thought that we had isoforms of the sGC and pGC. We spent a number of years characterizing and purifying the 7 different isoforms of GC [6–8]. We found that the sGC was a heterodimer of alpha and beta subunits [9]. There can be two different alpha subunits, alpha 1 and 2, and two different beta subunits, beta 1 and 2.

Most tissues possess the alpha 1 and beta 1 heterodimer which we also found to be the receptor for nitric oxide [8,10–12]. NO binds to a heme prosthetic group that is liganded to histidine 105 of the beta subunit. The binding of NO to the heme prosthetic group activates the carboxy terminal catalytic domain about 200- to 400-fold (Vmax) and decreased the GTP Km. We also found that some of the particulate isoforms (pGCs) (natriuretic peptide receptors, or NPR 1,2,3) were receptors for the atriopeptins (ANP, BNP, and CNP) and *E. coli* heat stable enterotoxin (STa) [13–16]. We found that crude extracts of pancreas could activate one of the pGCs and presumably contained the natural ligand(s) (unpublished). However, others later found that guanylin and uroguanylin were the natural endogenous ligands for NPR3, pGC [17]. As stated above, we thought

* Corresponding author. Fax: 202-994-8974.
E-mail address: bcmkxb@gwu.edu (K. Bian).
** Corresponding author. Fax: 202-994-8974.
E-mail address: bcmfxm@gwu.edu (F. Murad).

http://dx.doi.org/10.1016/j.niox.2014.08.006
1089-8603/

from our earlier studies we had multiple isoforms of guanylyl cyclase in soluble and particulate fractions of tissue homogenates (sGC and pGC). We turned to complex biological models to see if the activities were regulated independently of each other. We studied fetal rat liver (a hematopoietic tissue), regenerating rat liver after partial hepatectomy, and Morris hepatomas and renal tumors [18–22]. There was little sGC in the tissue extracts uniformly in the proliferating tissues but the pGC activity was normal. Furthermore, NO and nitro vasodilators failed to increase cyclic GMP levels in these tissues or activate GC in the homogenates. We thought that there was some unknown relationship of cyclic GMP metabolism with cell proliferation and cancer. We conducted a number of descriptive studies during the 1970s and early 1980s including studies looking at cyclic GMP levels in urine and blood of rats and patients with tumors. We often found cyclic GMP urine levels elevated in rats and humans with tumors ([18,22] some data not published). We also began to look for cyclic GMP effects on the cell cycle of synchronized cultured cells (not published). We did not have the GC isoforms purified; we did not have antibodies to the GC isoforms; and we had not yet cloned the GC isoforms. Therefore we could not do the required mechanistic studies that were needed. Fortunately, our studies with nitric oxide and cyclic GMP with smooth muscle (airway smooth muscle, gastrointestinal smooth muscle and later vascular smooth muscle) began to rapidly move forward [8,10–13,15,23–27]. We found that nitrovasodilators such as nitroglycerin and nitroprusside were prodrugs to generate nitric oxide that activated sGC to produce cyclic GMP that in turn activated protein kinase G (PKG) to phosphorylate many smooth muscle proteins. After radiolabeling ATP in smooth muscle segments with P32, we were able to identify some of the P32 labeled proteins after separation on 2D gels and parallel immunoblots [23–27]. The resulting lowered cytosolic calcium decreased the activity of myosin light chain kinase (a calcium calmodulin dependent enzyme) resulting in the dephosphorylation of myosin light chain and smooth muscle relaxation [23–27]. We also found that Furchgott's endothelial derived relaxing factor (EDRF) acted as an "endogenous nitrovasodilator" to work through the same pathway and mechanism [23–30]. The only difference was that the "endothelial-dependent vasodilators" such as acetylcholine, histamine, bradykinin, etc. required the integrity of the endothelium because that is where their receptors were located. They were not on the smooth muscle.

Research with cyclic GMP and nitric oxide has expanded exponentially during the past 30–35 years. Our original work with NO and cyclic GMP in the 1970s and 1980s [4–6,8,10–13,16–18,20,21,25,26,29–38] has led to about 150,000 publications from numerous laboratories, about 80 biotechnology companies and many projects in multinational pharmaceutical companies. The popularity of the field is undoubtedly due to the many drugs that have been and will be developed for numerous disorders with the very broad role of NO and cyclic GMP as important messengers in biology. However, there remains much more to learn about this popular field. Our experience with NO and cyclic GMP research has been focused in recent years on cancer biochemistry and some novel approaches to cancer therapy with agents and methods to increase their formation and/or action and/or decrease their inactivation in tumor cells or stem cells.

2. Cyclic GMP and cancer biology: a shifted paradigm of key signaling pathway

The nitric oxide and 3′,5′-cyclic monophosphate (NO/cGMP) pathway is one of the best characterized signaling cascades and plays a central role in several physiological processes such as induction of vasodilation. Soluble guanylyl cyclase (sGC) is the major receptor for NO. The α1β1 heterodimer is the predominant isoform of sGC which is obligatory for catalytic activity. Nitric oxide binding

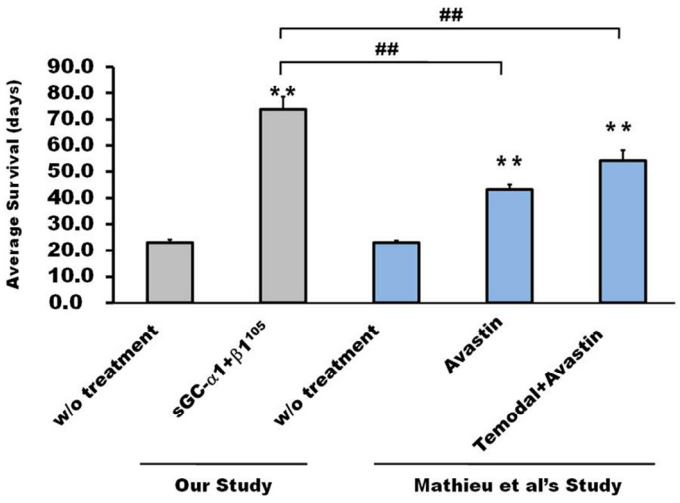

Fig. 1. Orthotopic xenograft of glioma cells with sGCαl/β1 stable clone in athymic mice increased the survival time. The survival time for the mice xenografted with $\alpha_1\beta^{Cys\text{-}105}$ sGC cells (n = 18) is significant longer than control (n = 6), avastin treated (n = 11), and avastin + temodal treated (n = 11). (mean ± S.E.M.). $^{\#\#}$ $p < 0.01$ (our study vs Mathieu's study), $^{**}p < 0.01$ (vs control of each study).

at histidine 105 of β1 subunit leads to sGC activity and cGMP production. The role of NO and cGMP signaling in tumor biology has been studied during the past three decades. Simply applying NO or cGMP regulating reagents to various cancer cell lines or animal models has generated controversial results and whether the pathway is beneficial or detrimental in cancer is still open to questions [39]. In this context, recently, we examined the NO/sGC/cGMP signaling molecules in human glioma tissues and cell lines compared to normal controls. We found that sGC α1 and β1 subunit expression (both gene and protein) is significantly lower in glioma preparations and cell lines [40]. Our analysis of GEO databases (National Cancer Institute) revealed a statistically significant reduction of sGC transcript levels in human glioma specimens. These findings generated our first hypothesis that "restoring sGC expression genetically will reverse the aggressive course of glioma".

To test the hypothesis, we first created $sGC\beta1^{Cys\text{-}105}$ mutant by substitution of His-105 with Cys. The heterodimer $\alpha1\beta1^{Cys\text{-}105}$ sGC is constitutively active with higher levels of cGMP produced. We then established 3 stable clones overexpressing $sGC\alpha1\beta1^{Cys\text{-}105}$ in U87 glioma cells which allows observation of the effect of sGC/cGMP with less inference by endogenous NO. The genetic restoration of sGC activity significantly inhibited glioma cell proliferation and colony formation [40]. Orthotopic implantation of glioma cells with $sGC\alpha1\beta1^{cys\text{-}105}$ in athymic mice increased the survival time by 4-fold over the control. We have compared our results with Mathieu et al.'s [41] study which used the U87 xenografts in the same athymic mice. The 50% survival time for the mice xenografted with $\alpha1\beta1^{cys\text{-}105}$ sGC glioma cells (n = 18) is markedly longer than control (n = 6), avastin (used in the clinic as an anti-angiogenic agent)-treated (n = 11), and avastin + temodal (alkylating agent for glioblastoma clinically)-treated (n = 11) groups (Fig. 1). Our above preliminary study established that suppressed expression of sGC, a key enzyme in NO/cGMP pathway, associated with an aggressive clinical course of glioma; and restoring sGC expression genetically leads to inhibition of glioma growth both *in vitro* and *in vivo*.

Our first hypothesis has been experimentally supported. We then attempted to assess the second hypothesis that "pharmacologically manipulating endogenous cGMP generation in glioma cells can overcome sGC defect and block the aggressive course of glioma (Fig. 2)". As well established, besides the activation of sGC, elevat-