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Cardioprotective actions of cyclic GMP: lessons from genetic animal models

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Abstract

Most common diseases and/or their consequences, including hypertension and susceptibility to end-organ damage, have genetic components. Contrary to hereditary Mendelian diseases (where the existence of a rare mutation within a single gene usually dictates whether signs of the disease are present), the manifestations of common diseases correspond to what is defined in genetics as “quantitative complex traits”. Such traits show within populations continuous variation from low to high values, and are shaped by the interactions of a great number of genes (each typically having small effects on their own) with environmental factors. Consequently, identification of genetic variants contributing to complex traits must rely on methods that are different than those used for the identification of Mendelian genetic mutations (which can be performed by following within pedigrees the hereditary transmission of markers linked to the mutated gene). Among several available tools, genetic animal models have proved particularly useful to identify the effects of naturally occurring genetic variants and their effects within mammalian organisms. Indeed, inbred strains are organisms that carry identical copies of each gene. Accordingly, it is possible by performing crosses between them to reduce the complexity of the problem, since the progeny of crosses will carry only 2 possible variants of any gene and experimental and environmental conditions can be controlled.

Complex traits and genetic animal models

The pioneering work of Lewis K. Dahl constitutes an early and illustrative example of the utility of such models. Dr. Dahl had hypothesized from epidemiologic studies in human populations that elevated blood pressure may arise from the combination of high dietary salt with individual genetic susceptibility factors. To test that hypothesis experimentally, Dahl began a breeding program where he fed outbred rats with highly salted food. As some rats developed high blood pressure in response to high salt diet but others did not, he selected breeders on the basis of their response to the salt challenge and within 3 generations, he obtained the clearly separated salt-sensitive (S) and salt-resistant (R) rat lines. [1] Subsequently, John Rapp developed inbred strains from Dahl's outbred stocks. [2] This general two-step procedure (phenotypic-driven selection of breeders in outbred stocks

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Disclosures

None

followed by inbreeding of strains with desired characteristics) has constituted the basis for the creation of several models of inbred hypertensive strains that can be compared to normotensive counterparts, the most widely used ones arguably being the spontaneously hypertensive rats (SHR) and their Wistar-Kyoto (WKY) normotensive counterparts. [2] Among hypertensive models, some investigators have also developed strains that are more sensitive to end-organ damage, as for instance the SHR stroke-prone (SHR-SP) [2] and the SHR heart failure (SHHF) [3] strains.

In addition to having higher blood pressure, SHR also display higher levels of locomotor activity than WKY. To test whether this trait co-segregates with hypertension, E.H. Hendley performed a phenotypic-driven selection of the progeny of SHR/WKY crosses, and thus generated 2 novel inbred strains: the WKY-derived hyperactive (WKHA) and the WKY-derived hypertensive (WKHT) rats. [4] A posteriori, it now appears that the designation of the WKHA strain may be partly misleading: indeed, SHR and WKHA display higher locomotor activity than WKY, but the difference is due mostly to the fact that WKY are markedly hypoactive in comparison to many other rat strains. [5] Nonetheless. one additional useful trait of WKHA rats was that, although both strains have normal and equivalent levels of blood pressure, they show the characteristics of concentric left ventricular hypertrophy (LVH) in comparison to WKY rats. [6, 7] This phenotypic difference between the strains was exploited in the genetic studies described below.

Left ventricular mass and natriuretic peptides

Since it has been first reported in 1990 by the Framingham heart study [8], the importance of increased left ventricular mass (LVM) has repeatedly been recognized as an important and independent risk factor that correlates very closely with cardiovascular risk [9] and has strong prognostic implications. [10, 11] The fact that genetic factors contribute importantly to the variance of LVM is underlined by its high heritability (in fact higher than 50% in most studies). [12, 13] To identify natural genetic variants that contribute to the variance of LVM, we therefore studied the progeny of WKHA/WKY crosses to identify quantitative trait loci (QTL) that link to LVM. Even prior to completing the linkage study, we observed that there was a negative correlation between LVM and LV concentration of atrial natriuretic factor (ANF) peptide and its corresponding mRNA transcript (despite the fact that other known markers of LVH correlated positively with LVM). [14] At the time of our report, the gene coding for ANF [natriuretic peptide precursor A (*Nppa*)] was known as one whose expression was affected both quickly and reliably in response to hypertrophic stimuli [15], to the point that increased *Nppa* expression was considered as almost synonymous with LVH. It was therefore relatively surprising to observe that *Nppa* expression could dissociate from LVM. However, experiments in mice had shown that inactivation of either *Nppa* [16] or *Npr1* (the gene coding for the ANF receptor) [17, 18] results in increased LVM that is disproportionate compared to the accompanying increase in blood pressure. Altogether, it thus appeared that early activation of *Nppa* by hypertrophic stimuli might in fact represent a cardioprotective response, and conversely, that deficits in *Nppa* signaling might associate with an increased hypertrophic response. We further showed that WKHA rats harbored a single nucleotide polymorphism in the *Nppa* minimal promoter region that reduced its transcriptional activity [6]. Likewise, the *Nppa* locus was within the confidence interval of the

overlapping QTLs linked to either LV ANF expression or LVM (identified by the symbol *Cm24* in the Rat Genome Database) in the male progeny of WKHA/WKY crosses. [6] These linkage results were further validated by physical mapping in congenic rats, where introgression of the *Nppa/Cm24* locus from WKHA into the WKY background both decreased LV ANF concentration and increased the size of cardiac ventricular myocytes [19]. Of note, the *Nppa/Cm24* locus was linked to LV ANF concentration in both the male and female progeny of WKHA/WKY crosses, but it showed linkage to LVM only in male rats. [19, 20]

Beyond the effect of the *Nppa/Cm24* locus on LVM under basal conditions, we wanted to test whether this locus may associate with cardiac outcome under pathologic conditions. We therefore challenged the hearts of WKY and their congenic WKY.WKHA-*Cm24* counterparts by volume overload. Although this maneuver had either no or little effects in WKY rats, it induced adverse LV remodeling and dysfunction in the hearts of their congenic counterparts harboring *Cm24* from WKHA. [21] Moreover, this effect of the *Nppa/Cm24* locus on cardiac deterioration was found in both the male and female progeny, despite the fact that the locus did not link under basal conditions with LVM in the female progeny. This finding had three implications: 1) it illustrated the fact that the effect of a QTL on a phenotypic trait may become manifest only under particular environmental conditions (in this case, the combination of sex and volume overload); 2) it provided a proof-of-principle demonstration that a naturally occurring variant of the *Nppa* gene may constitute a genetic risk factor linked to cardiac outcome; and 3) it showed that, beyond its effect on LVM under basal conditions, the *Nppa* pathway may have important cardioprotective effects in pathologic conditions.

Cardioprotective effects of ANF and/or cyclic GMP

Inactivation of the ANF pathway could possibly affect LVM via either a local direct effects of ANF on the heart or the possible hemodynamic effects of decreased levels of circulating ANF. However, genetic alterations targeted specifically to the heart showed that inactivation of ANF increased LVM primarily via a cardiac-specific effect. [22, 23] Most of the biologic actions of ANF are believed to be mediated via cyclic GMP (cGMP) [24, 25], whose intracellular production is increased after binding of ANF to its plasma membrane guanylate cyclase (GC) receptor (known as either NPR-A or GC-A). Consequently, to test whether the generation of cGMP within cardiomyocytes could also prevent left ventricular hypertrophy (LVH) *in vivo*, we engineered transgenic (TG) mice that express a constitutively activated guanylate cyclase domain of the NPR-A receptor exclusively in cardiomyocytes, resulting in a selective increase in intracellular cGMP. [26] While expression of the transgene had no effect on cardiac performance under basal conditions, it attenuated the effects of either pharmacologic or mechanical hypertrophic stimuli on both LVM and cardiomyocyte size. [26] Cyclic cGMP thus appeared to constitute a negative intrinsic modulator of the hypertrophic response. Can this signaling pathway therefore be exploited therapeutically to improve cardiac functions in pathologic situations? The first indication in this regard came from the work of the group of David Kass: they showed that pharmacologic blockade of intracellular cGMP degradation by administration of the phosphodiesterase-5A (PDE5A) inhibitor sildenafil also suppresses pressure overload-induced LVH and restored LV chamber

function to normal. [27] The applicability of cGMP-dependent cardioprotection may apply to cardiomyopathies other than that caused by pressure overload, as others have shown in mice that PDE5A inhibition with sildenafil: 1) preserves the deterioration of LV function after myocardial infarction by limiting necrosis and apoptosis [28]; and 2) protects the heart against doxorubicin toxicity. [29] Recently, we have tested whether this approach could also improve the cardiomyopathy cause by dystrophin deficiency in mice carrying the *mdx* mutation. [30] When cGMP signaling was enhanced either pharmacologically (with sildenafil treatment) or genetically (by crossing *mdx* mice with our heart-specific guanylate cyclase transgenics), it was possible to improve the contractile performance, myocardial metabolic status and sarcolemmal integrity of dystrophic hearts. [30]

Other cGMP-generating pathways

In addition to stimulation of plasma membrane GC receptors and regulation of intracellular degradation, increases in intracellular cGMP may result from activation of other signaling pathways. Most notably, cytoplasmic soluble GC can be activated by gaseous substances such as carbon monoxide (CO) and mostly nitric oxide (NO), the latter being produced by a family of enzymes known as nitric oxide synthases (NOS). There are 3 different genes coding for 3 distinct isoforms of NOS, i.e. neuronal NOS (nNOS, also known as NOS1), inducible NOS (iNOS, or NOS2), and endothelial NOS (nNOS, or NOS3). All three NOS isoforms can be found in cardiomyocytes, although NOS1 and NOS3 are expressed constitutively whereas NOS2 is induced by stimulation by cytokines. [31, 32] Although not all cellular actions of NO are mediated by cGMP, genetic alteration of the expression of NOS genes in either knockout or transgenic mice has shown that NOS affects LVM and cardiomyocyte size in a manner parallel to the effects seen with alterations of the ANP signaling pathway. Thus, NOS3 −/− mice display increased LVM and cardiomyocyte volume (as compared to their wild-type counterparts) either during aging [33], during the post-myocardial infarction period [34] or after pressure overload [35], whereas overexpression of NOS3 in transgenic mice attenuates the hypertrophic effects of chronic isoproterenol infusion. [36] In aging NOS1 −/−, LVM and cardiomyocyte volume are increased when compared to wild-type counterparts in the absence of any change in blood pressure. [33]. Although the above genetic manipulations were not cardiac-specific, experiments using isolated cardiomyocytes from the same animals showed that the cardiac effects of NOS may result (at least in part) from local actions [37].

The importance of these other signaling pathways relates in part to the fact that they provide other avenues by which intracellular cGMP concentration can be modulated pharmacologically. For instance, it has been reported that a drug that is an orally active stimulator of soluble GC can reduce angiotensin II-induced cardiac remodeling. [38] Likewise, inhalation therapy with low doses of CO has been shown to improve doxorubicin-induced cardiomyopathy in part via stimulation of soluble GC. [39] Of note, in the particular cases of dystrophin deficiency and doxorubicin toxicity, it is believed that deficits in NO- and/or ANP-mediated signaling are partly causal to the disease [30, 39], and may thus represent examples where enhancing cGMP signaling may prove particularly effective. However, one important additional caveat in the interpretation of the results obtained with genetically modified animals is the fact that subcellular pools of cGMP are determined by the respective

locations of soluble and particular guanylate cyclases (GC) and phosphodiesterases. [40] For instance, cGMP derived from either NOS1 and NOS3 affect cardiac contractility in very different ways. [37]

Mechanisms mediating the cardioprotective actions of cGMP

Initial studies on the mechanisms of action of cGMP in the cardiovascular system have dealt mostly with its actions in vascular smooth muscle cells, endothelial cells and/or platelets [41-43]. However, given the more recent findings on the cardiac effects of cGMP, there is now increasing information concerning its mechanisms of action in the heart. [40, 44] Firstly, there seems to be agreement between studies using either pharmacologic [45] or genetic [46, 47] tools that the cardioprotective effects of cGMP are mediated by cGMP-dependent protein kinase [also known as protein kinase G (PKG)], with phosphorylation of ERK1/2 appearing to be one important one obligatory step [45, 48, 49]. Of note, this dependency on PKG may be in contrast to some actions of cGMP elsewhere in the cardiovascular system, since it is for instance dispensable for the protective actions of cGMP in restenosis-associated vascular remodeling. [50] Once PKG is activated in cardiac cells, its cardiprotective actions appear to result from effects on two main downstream systems. Firstly, it improves calcium handling in cardiomyocytes [51], presumably via combined effects on phosphorylation of several sarcoplasmic proteins (including phospholamban, the IP3 receptor and the ryanodine receptor) and calcium entry through LTCC calcium channels [40, 51, 52], and also possibly phosphorylation of a novel family of cation channels called “transient receptor potential proteins” (TRP) [53], the latter having been recently implicated in cardiac hypertrophy [54]. Intracellular calcium is well-known to play central roles in the genesis and development of LVH. [55]. The other important target of cGMP within cardiomyocytes appears to be the mitochondria, as cGMP signaling prevents opening of the mitochondrial permeability transition pore via several convergent effects, including: 1) activation of the mitochondrial ATP-sensitive K channel. [56, 57]; 2) direct phosphorylation of an unidentified protein on the mitochondrial outer membrane. [56]; and 3) upregulation of the antiapoptotic protein Bcl-2 [43, 48]. Finally, the protective effects of cGMP signaling in the heart are not restricted to only cardiomyocytes. For instance, it exerts anti-fibrogenic effects by inhibiting the TGF-β-induced transformation of fibroblasts into myofibroblasts via PKG-dependent phosphorylation of Smad3 in these cells [58].

Since cGMP acts upon multiple effectors, the spectrum of its cardioprotective actions appears to be fairly large, which is compatible with thae fact that it improves both cardiac remodeling and function in several types of cardiomyopathies. In addition, there may be redundancy between these various pathways. For instance, even though calcineurin is (downstream of increased cellular calcium) one of the primary targets mediating the antihypertophic effects of cGMP-PKG signaling [46, 59], the antihypertophic effects of this pathway are maintained in the hearts of knockout mice effectively lacking calcineurin activity. [60] Between studies, there are also sometimes discrepancies between some of the modes of actions of cGMP, one possible cause being that the cardioprotective effects of cGMP have been examined in different types of cardiomyopathies (such as for instance ischemic or pressure overload cardiomyopathies), and that there may some differences

between the various models used. Nonetheless, a better understanding of the mode of action of cGMP may lead to new therapeutic modalities for the treatment of cardiac dysfunction.

Genetic determinants of cardiovascular risk

Given the high heritability of LVM, there are ongoing efforts to identify gene variants that contribute to the variance of this trait. Accordingly, a recent linkage study has reported on chromosome regions linked to LVM [61], while a meta-analysis of genome-wide association studies (GWAS) has identified several genetic loci linked to either LV mass or geometry. [62] There was no indication in either study of association or linkage with genes associated with the generation of intracellular cGMP. Nonetheless, the latter trait can be controlled by a variety of genes. For instance, in addition to the ones discussed in the above paragraphs, precursors to natriuretic peptides are processed by the protease corin [63], are cleared from the circulation via the natriuretic peptide clearance receptor (encoded by *Npr3*), and are metabolized in the circulation by the membrane metallo-endopeptidase (encoded by *Mme*). Interestingly, a polymorphism within the *corin* gene and found almost exclusively in black individuals has been reported to associate with LVM in subjects with hypertension. [64] In addition, circulating levels of natriuretic peptides are themselves under genetic control. Accordingly, common genetic variants within the *NPPA-NPPB* locus have been found to associate with circulating natriuretic peptide concentrations. [65] In the future, if genes associated with the cGMP/PKG pathway turn out to associate with LVM, detection of corresponding variants may be helpful to stratify cardiovascular risk in patients, or to identify patients most susceptible to benefit from therapies that enhance cGMP signaling.

From animal models to human studies

Altogether, the combined investigations on animal strains carrying natural variants of *Nppa*, on their corresponding congenic strains and on either transgenic or knockout mice have: 1) made it possible to discover the cardioprotective effects of cGMP; 2) enabled studies aimed at understanding the mechanisms of action of cGMP; and 3) have provided models to test the therapeutic potential of the cGMP/PKG pathways. These studies have progressed to the point that the National Institutes of Health have recently initiated a multicenter clinical trial of sildenafil for the treatment of heart failure with preserved ejection fraction (Clinical Trials.gov identifier: NCT00763867). Moreover, GWAS performed in human populations are beginning to report on genetic loci that associate with LVM. Despite these successes, we are still limited in our ability to understand how variants discovered by GWAS influence complex traits, and one of the challenges lying forward will be to identify the biological context in which these statistically significant candidate variants act. [66] In this regard, genetic animal models will continue to constitute invaluable tools, either to discover naturally occurring genetic variants influencing complex traits or to complement GWAS in order to discover which genes within a given region are linked mechanistically to a given trait.

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