Tissues cIMPly do not lie…

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If one tells the truth, one is sure, sooner or later to be found out

Oscar Wilde

Abstract

We recently suggested that inosine 3',5'-cyclic monophosphate (cIMP) synthesized by soluble guanylyl cyclase (sGC) is the mediator of hypoxic augmentation of coronary vasoconstriction. Here we explain why we actually believe that cIMP may be considered as a new second messenger.

Cyclic guanosine 3', 5'-monophosphate (cGMP) has long been viewed as the only signaling molecule synthesized by soluble guanylyl cyclase (sGC) (Friebe and Koesling, 2009; Waldman and Murad, 1987). However, in addition to cGMP, purified sGC can synthesize several other cyclic nucleotides including inosine 3',5'-cyclic monophosphate (cIMP) (Beste et al., 2012). The latter study, by Seifert and his colleagues, came as an illumination to us as we were trying over the years to explain why in isolated arteries hypoxia augments contractions in a counter-intuitive manner that requires the presence of the endothelium, the production of nitric oxide (NO) and the subsequent activation of sGC, but not the presence of cGMP (De Mey and Vanhoutte, 1983; Rubanyi and Vanhoutte, 1986; Graeser and Vanhoutte, 1991, Pearson et al., 1996; Chan et al., 2011). Hence, in a recent study, we tested and, we believe, proved right, the hypothesis that cIMP may act as a mediator in hypoxic augmentation of coronary vasoconstriction (Chen et al., 2014). In his editorial commentary, Roland Seifert (this issue) expressed his skepticism about such a second messenger role for cIMP based on two major arguments: cIMP levels are far below detection limit, and there the absence of known target protein

for cIMP (Seifert, this issue). We believe that this skepticism largely arises from overlooking our findings in isolated porcine coronary arteries that cIMP levels are markedly elevated by hypoxia and that cIMP at rather low concentrations activates Rho kinase (ROCK), which subsequently promotes hypoxic vasoconstriction (Chen et al., 2014).

The evidence reiterated by Seifert to support his view that cIMP is far below detection level was obtained in cultured HEK293 cells over-expressing the isoform A of particulate guanylyl cyclases (pGC-A) (Beste et al., 2013). In a separate study on nucleotidyl cyclase activity in HEK293 cells over-expressing sGC and in RFL-6 rat fibroblasts endogenously expressing sGC, no measurement on cIMP is mentioned (Bähre et al., 2014). Our experiments were performed *ex vivo* on relatively intact porcine coronary arteries. In such “fresh” tissues, cIMP was detected using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). The level of cIMP was elevated by NO in arteries without endothelium, as well as by inosine 5’-triphosphate (ITP, the substrate for cIMP formation) or by hypoxia in arteries with endothelium. These effects were prevented by ODQ, a selective inhibitor of sGC. The level of cIMP increased also in arteries without endothelium when exposed to hypoxia in the presence of Bay 58-2667, a NO-independent and heme-independent activator of sGC (Follmann et al.,2013). Taken in conjunction these results prompted the unavoidable interpretation that the increased levels of cIMP are produced by sGC (Chen et al., 2014). The most novel finding of our study is that the sGC-dependent formation of cIMP in the arteries is stimulated by hypoxia. We believe that these findings are solid and will withstand the test of time, as has the NO- and the sGC-dependency of the hypoxic augmentation (Graeser et

al., 1991; Pearson et al., 1996; Chan et al., 2011; Chen et al., 2014).

However, we still do not know how and why hypoxia causes sGC to switch substrate. An increased availability of ITP may in part be involved, as supported by the increased ITP level under hypoxia (Chen et al., 2014). ITP is primarily derived from ATP deamination (Behmanesh et al., 2009; Sakumi, et al., 2010). An increased intracellular level of cIMP occurs in response to exogenous application of ATP (Ferguson et al., 1973). ATP is the most abundant nucleotide inside the cell (Gribble et al., 2000). It has been estimated that 10-25% of the ATP pool can be converted to ITP by deamination (Sakumi, et al., 2010). Under normal conditions, ITP is largely degraded by ITPase, and the intracellular level of ITP is rather low (Behmanesh et al., 2009; Sakumi, et al., 2010). However, if hypoxia were to promote ATP deamination and/or suppress the activity of ITPase, sufficient ITP could accumulate for sGC to synthesize cIMP (Bähre et al., 2014).

The role of cIMP as a mediator in hypoxic augmentation of vasoconstriction is also questioned by Seifert for the lack of downstream target. As he points out, the affinity of cIMP is rather low for activation of either cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA), classical targets for cyclic nucleotides (Seifert, this issue). We agree that hypoxic augmentation by cIMP is not likely to involve either PKG or PKA as this has been excluded by our previous experiments using various inhibitors of sGC, adenylyl cyclase, PKG, and PKA (Chan et al., 2011). The hypoxic augmentation of vasoconstriction in arteries with, or in those without endothelium but treated with cIMP, is blunted by the Rho-kinase inhibitor Y27632 (Chan et al., 2011; Chen et al., 2014). The phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Thr853, an indicator of the activation of ROCK (Gao et al., 2007; Somlyo and Somlyo,

2003) is stimulated in arteries with endothelium by hypoxia and arteries without endothelium exposed to cIMP. Moreover, in homogenates of coronary arteries, cIMP activates ROCK starting at a concentration of 10^{-7} M (Chen et al., 2014), which is well within the physiological range for the activation of kinases by cyclic nucleotides (Dhanakoti et al., 2000; Wolter et al., 2011). Activation of ROCK leads to reduced activity of myosin light chain phosphatase (MLCP), consequently increasing the Ca^{2+} sensitivity of the myofilaments, and thus augmenting vasoconstriction (Gao et al., 2007; Somlyo & Somlyo, 2003).These findings prompts the suggestion that Rho-kinase is indeed a downstream target for cIMP. However, although the effect of cIMP is prevented by ROCK inhibitors (Chan et al., 2011; Chen et al., 2014), we also still have to determine how ROCK eventually is activated (directly or indirectly) by the cyclic nucleotide. Current knowledge implies that the enzyme can be activated directly only by GTPase-RhoA, arachidonic acid, sphingosine phosphorylcholine, caspase-3, and granzyme B (Duong-Quy et al., 2013).In our article we used “mediator” rather than “second messenger” to describe the role of cIMP in hypoxic augmentation of vasoconstriction (Chen et al., 2014). It was tempting to indeed propose cIMP as a new second messenger, but we agree with Seifert that this should not be done lightly. To be designated as such, according to Sutherland (Sutherland et al., 1968), a second messenger molecule should meet the following criteria: (1) its intracellular levels should change in response to a physiologically relevant stimulus; (2) when reaching intracellular compartments it should mimic the effect of an extracellular stimulus; (3) the effects of the extracellular stimulus should be blocked by antagonism of the action of the messenger; (4) the molecule should be synthesized and metabolized; and (5) specific intracellular

binding sites should be present (Aley et al., 2013; Sutherland et al., 1968). In our study the formation of cIMP by sGC was stimulated by NO under hypoxic conditions. These changes were associated with the activation of ROCK and hypoxic augmentation of vasoconstriction. The extracellular administration of cIMP also caused a similar phenomenon, presumably following diffusion across the cell membrane (Werner et al., 2011). Studies using human phosphodiesterases show that cIMP is hydrolyzed (Reinecke et al., 2011) and phosphodiesterase inhibition potentiated the hypoxic augmentation (Chen et al., 2014). Hence, it appears that cIMP meets, or nearly meets all the criteria recommended by Sutherland, except that there is currently no agent available to specifically block the synthesis of cIMP (without affecting that of cGMP).

Soluble guanylyl cyclase is a critical enzyme involved in various functional responses to NO (Friebe and Koesling, 2009; Waldman and Murad, 1987). Currently, cGMP is considered to be the only molecule responsible for all the actions resulting from the activation of sGC. However, the available evidence indicates that hypoxia has no significant effect on cGMP formation (Chan et al., 2011; Chen et al., 2014).Taken in conjunction (Figure 1), our *ex vivo* findings over the years strongly suggest that, at least in isolated porcine coronary arteries, this enzyme can generate another signaling molecule, cIMP, which promotes vasoconstriction under hypoxic conditions, in sharp contrast to the vasodilator role of cGMP (Graeser et al., 1991, Pearson et al., 1996; Chan et al., 1011; Chen et al., 2014). Tissues do not lie, but one has to understand their language.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

References

Aley PK, Singh N, Brailoiu GC, Brailoiu E, Churchill GC (2013) Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger in muscarinic receptor-induced contraction of guinea pig trachea. J Biol Chem 288:10986-10993.

Bähre H, Danker KY, Stasch JP, Kaever V, Seifert R (2014) Nucleotidyl cyclase activity of soluble guanylyl cyclase in intact cells. Biochem Biophys Res Commun 443: 1195-1199.

Behmanesh M, Sakumi K, Abolhassani N, Toyokuni S, Oka S, Ohnishi YN, Tsuchimoto D, NakabeppuY (2009) ITPase-deficient mice show growth retardation and die before weaning. Cell Death Differ 16: 1315-1322.

Beste KY, Spangler CM, Burhenne H, Koch KW, Shen Y, Tang WJ, Kaever V, Seifert R (2013) Nucleotidyl cyclase activity of particulate guanylyl cyclase A: Comparison with particulate guanylyl cyclases E and F, soluble guanylyl Cyclase and bacterial adenylyl cyclases CyaA and edema factor. PLoS ONE 8:e70223.

Chan CK, Mak J, Gao Y, Man RY, Vanhoutte PM. Endothelium derived NO, but not cyclic GMP, is required for hypoxic augmentation in isolated porcine coronary

arteries (2011) Am J Physiol Heart Circ Physiol 301: H2313-H2321.

De Mey, J. G. and P. M. Vanhoutte (1983). Anoxia and endothelium-dependent reactivity of the canine femoral artery. J Physiol 335: 65-74.

Dhanakoti S, Gao Y, Nguyen MQ, Raj JU (2000). Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP. J Appl Physiol. 88: 1637-1642.

Duong-Quy S, Bei Y, Liu Z, Dinh-Xuan AT (2013) Role of Rho-kinase and its inhibitors in pulmonary hypertension. Pharmacol Ther 137: 352-64.

Ferguson DR, Price RH (1973) The production of cIMP by toad bladder, and its effects on transport of water and salt. FEBS Lett 34: 207-212.

Follmann M, Griebenow N, Hahn MG, Hartung I, Mais FJ, Mittendorf J, Schäfer M, Schirok H, Stasch JP, Stoll F, Straub A (2013) The chemistry and biology of soluble guanylate cyclase stimulators and activators. Angew Chem Int Ed Engl 52: 9442-9462.

Friebe A, Koesling D (2009) The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. Nitric Oxide 21: 149-156.

Gao Y, Portugal AD, Negash S, Zhou W, Longo LD, Raj JU (2007) Role of Rho kinases in PKG-mediated relaxation of pulmonary arteries of fetal lambs exposed to chronic high altitude hypoxia. Am J Physiol Lung Cell Mol Physiol 292: L678-L684.

Graeser T,Vanhoutte PM (1991) Hypoxic contraction of canine coronary arteries: role of endothelium and cGMP. Am J Physiol Heart Circ Physiol 261: H1769-H1777.

Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG, Ashcroft FM (2000). A novel

method for measurement of submembrane ATP concentration. J Biol Chem 275: 30046-30049.

Pearson PJ, Lin PJ, Schaff HV, Vanhoutte PM (1996) Augmented endothelium-dependent constriction to hypoxia early and late following reperfusion of the canine coronary artery. Clin Exp Pharmacol Physiol 23:634-641.

Reinecke D, Burhenne H, Sandner P, Kaever V, Seifert R (2011) Human cyclic nucleotide phosphodiesterases possess a much broader substrate-specificity than previously appreciated. FEBS Lett. 585: 3259-3262.

Rubanyi GM, Vanhoutte PM (1985) Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J Physiol 364: 45-56.

Sakumi K, Abolhassani N, Behmanesh M, Iyama T, Tsuchimoto D, Nakabeppu Y (2010). ITPA protein, an enzyme that eliminates deaminated purine nucleoside triphosphates in cells. Mutat Res 703: 43-50.

Seifert R (2014) Is cIMP a second messenger with functions opposite to those of cGMP? Naunyn Schmiedebergs Arch Pharmacol, this issure.

Somlyo AP, Somlyo AV (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 83: 1325-1358.

Sutherland EW, Robison GA, Butcher RW(1968) Some aspects of the biological role of adenosine 3',5'-monophosphate (Cyclic AMP). Circulation 37: 279-306.

Waldman SA, Murad F (1987) Cyclic GMP synthesis and function. Pharmacol Rev 39: 163-196.

Werner K, Schwede F, Genieser HG, Geiger J, Butt E (2011) Quantification of cAMP and

cGMP analogs in intact cells: pitfalls in enzyme immunoassays for cyclic nucleotides. Naunyn Schmiedebergs Arch Pharmacol 384: 169-176.

Wolter S, Golombek M, Seifert R (2011) Differential activation of cAMP- and cGMP-dependent protein kinases by cyclic purine and pyrimidine nucleotides. Biochem Biophys Res Commun 415: 563-566.

Figure legend

Proposed mechanism for cIMP acting as a mediator in hypoxic augmentation of vasoconstriction. In such a response hypoxia causes a marked increase in the synthesis of cIMP by soluble guanylyl cyclase (sGC). Cyclic IMP in turn activates rho kinase (ROCK), resulting in reduced activity of myosin light chain phosphatase (MLCP), consequently increased Ca^{2+} sensitization of myofilaments and augmented vasoconstriction. Cyclic GMP may counteract the effect of ROCK by stimulating the activity of MLCP via cGMP-depedent protein kinase (PKG). A critical role of cIMP is appreciated by testing the effect of hypoxia on vasoconstriction by: 1) activating sGC by endothelium-derived nitric oxide (NO), an exogenous NO donor (DETA NONOate) and Bay 58-2667 (a NO-independent,heme-independent activator of sGC); or by suppressing activation of sGC by inhibition of endothelial NO synthase (eNOS, with nitro-L-arginine), removal of the endothelium and ODQ (selective sGC inhibitor); and 3), activating ROCK by exogenous cIMP or inhibiting ROCK with Y27632. Full arrows: activation. Dotted arrows: inhibition.