

Calcium blood level modulates endogenous nitric oxide action: effects of parathyroidectomy in patients with hyperparathyroidism

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Abstract

Platelet cyclic guanosine monophosphate (cGMP) is produced by soluble guanylate cyclase (sGC), the activity of which is modulated by the activity of nitric oxide (NO) constitutive synthase (cNOS) which, in turn, is activated by a calcium/calmodulin complex. In primary hyperparathyroidism (H-PTH) an increase in platelet free calcium levels is present. In this study we evaluate the platelet cGMP levels, as an expression of NO production, in the presence of 3-isobutyl-1-methylxanthine (IBMX) alone (IBMXcGMP) and after stimulation by ionomycin (IONO; IONOcGMP) and sodium nitroprusside (SNP; SNPcGMP), in eight subjects affected by H-PTH before and after removal of adenoma. Platelet cGMP levels were also measured in seven normal subjects. IBMXcGMP and IONOcGMP were elevated in H-PTH patients compared with normal subjects (1.9 ± 0.3 vs 0.8 ± 0.2 fmol/ 10^6 platelets and 2.7 ± 0.4 vs 1.4 ± 0.3 ; $P < 0.02$ and $P < 0.05$ respectively) but SNPcGMP was unaffected (3.9 ± 0.6 vs 2.5 ± 0.5). After parathyroidectomy, blood levels of intact parathyroid hormone (i-PTH), total calcium

(t-Ca), IBMXcGMP and IONOcGMP all decreased (177.5 ± 23.9 vs 45.0 ± 8.8 pg/ml, $P < 0.005$; 6.5 ± 0.5 vs 4.6 ± 0.1 mEq/l, $P < 0.005$; 1.9 ± 0.3 vs 0.8 ± 0.2 , $P < 0.005$; 2.7 ± 0.4 vs 1.8 ± 0.3 , $P < 0.05$ respectively), while SNPcGMP was not modified (3.9 ± 0.6 vs 4.3 ± 0.9). t-Ca and i-PTH were directly correlated with IBMXcGMP ($P < 0.02$, $r_s = 0.613$; $P < 0.02$, $r_s = 0.576$ respectively) and i-PTH was also correlated with t-Ca ($P < 0.001$, $r_s = 0.840$). In conclusion: (1) levels of IBMXcGMP and IONOcGMP are high in subjects with H-PTH; (2) after surgery both IBMXcGMP and IONOcGMP decrease to normal values. As IBMXcGMP expresses basal cGMP and IONOcGMP expresses the cGMP after cNOS stimulation, it can be speculated that the increase in NO production could be a mechanism to downregulate the vasoconstriction which may be caused by the high calcium levels in smooth muscle cells. After surgery, together with the normalization of calcium levels, NO production also returned to normal values.

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Introduction

Cyclic guanosine monophosphate (cGMP) in the platelet is produced by soluble guanylate cyclase (sGC), the activity of which is modulated by nitric oxide (NO) levels. Nitric oxide is synthesized by an enzyme, constitutive NO synthase (cNOS), which converts the amino acid L-arginine to NO and citrulline (Palmer *et al.* 1988, Palmer & Moncada 1989). cNOS is calcium/calmodulin-dependent (Mayer *et al.* 1989) and it releases NO for short periods in response to receptor or physical stimulation (Mulsch *et al.* 1989, Moncada *et al.* 1991). NO acts on vascular smooth muscle (Rapoport & Murad 1983) and platelets (Mellion *et al.* 1981) through the stimulation of sGC that converts guanosine triphosphate (GTP) to cGMP. The cyclic

nucleotide causes smooth muscle relaxation, chiefly via a decrease in intracytosolic calcium (Fig. 1) which, in turn, leads to a decrease in the contractility of the calcium-dependent apparatus (Waldman & Murad 1987, Lincoln *et al.* 1990). NO synthesis occurs in response to a variety of substances such as the calcium ionophore, ionomycin (IONO) (Furchgott 1984) that forms calcium-permeant channels in the plasma membrane (Knowles *et al.* 1989), and some nitrovasodilators, such as sodium nitroprusside (SNP) which spontaneously releases NO which activates sGC (Feelisch & Noack 1991). In primary hyperparathyroidism (H-PTH) a variable grade of hypercalcemia and an increase in platelet free calcium levels are present. The aim of this study was to assess the activity and regulation of enzymes involved in NO and cGMP production in

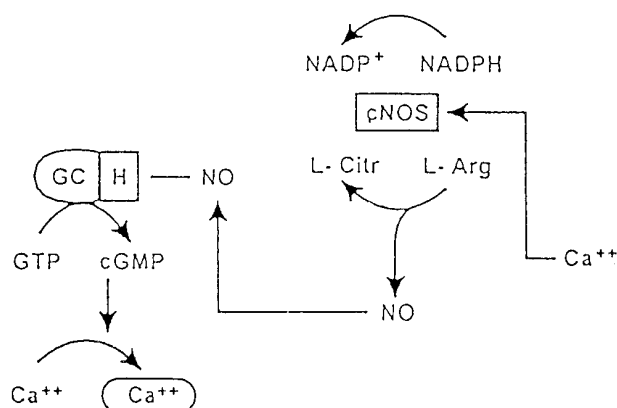


Figure 1 Scheme demonstrating cyclic guanosine monophosphate production. Ca^{++} , free calcium; H, heme; L-Citr, L-citrulline; L-Arg, L-arginine; NADP(H), nicotinamide adenine dinucleotide phosphate. Other abbreviations as in the text.

platelets of subjects affected by H-PTH before and after removal of adenoma. We evaluated platelet cGMP in three different conditions: (1) with 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase, to make cGMP measurable, (2) with IBMX and IONO that induces a maximum calcium influx from the outside and hence a maximal stimulation of the Ca^{++} -dependent cNOS activity, and (3) with IBMX and SNP, an NO donor, that maximally stimulates sGC and can therefore be used as an indirect measurement of active sGC (Fig. 2).

Subjects and Methods

Eight normotensive (WHO criteria) patients (four females and four males, aged 61.4 ± 2.5 years, body mass index (BMI) $24.8 \pm 0.9 \text{ kg/m}^2$, mean \pm S.E.M.) with H-PTH were studied after giving informed consent. The clinical suspicion of H-PTH was confirmed by the laboratory data showing elevated blood levels of intact parathyroid hormone (i-PTH), alkaline phosphatase (ALP) and total calcium (t-Ca), while phosphate blood levels (P) tended to be low (see Table 1). The localization of parathyroid adenomas was obtained by scintiscan and ultrasonography studies. Six patients presented with adenoma and two with hyperplasia. Surgical correction of H-PTH was obtained in all cases and the typical histological features of parathyroid adenoma were demonstrated. In all patients blood samples were withdrawn at 0800 h for determination of platelet cGMP, i-PTH, ALP, t-Ca and P and sampling was repeated 45 days after removing the parathyroid adenoma. cGMP was also evaluated in seven normal subjects (four females and three males, aged 58.9 ± 1.2 years, BMI 23.9 ± 0.5). Plasma i-PTH (1–84) was measured by the Incstar SP (Incstar Corporation, Stillwater, Minnesota, USA) immunoradiometric assay (IRMA) which utilizes

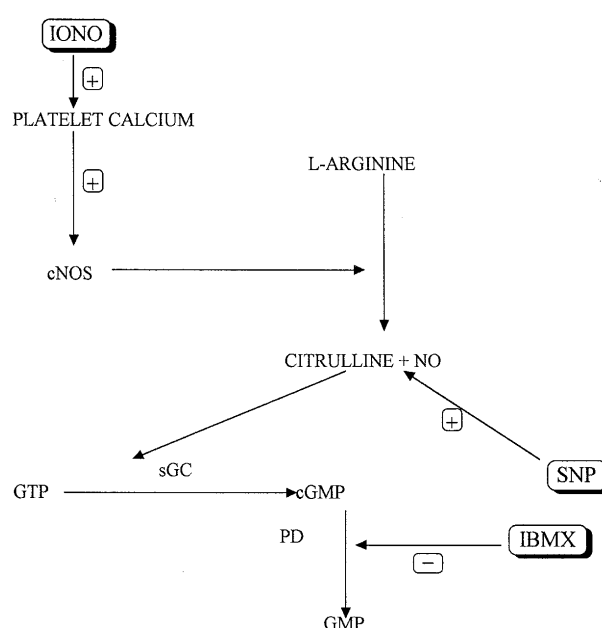


Figure 2 Simplified scheme of drug influence on cGMP production. PD, phosphodiesterase. Other abbreviations as in the text.

two different polyclonal antibodies purified by affinity chromatography. These purified antibodies are specific for two different regions of the PTH molecule. The interassay variation was 4.9%.

Platelet cGMP has been evaluated in three different conditions: (1) with IBMX (Sigma Chemical Co., St Louis, MO, USA), (2) with IBMX and IONO (Calbiochem, San Diego, CA, USA), and (3) with IBMX and SNP (Sigma Chemical Co.). For these measurements the blood samples were collected in one-tenth volume of 3.8% trisodium citrate in plastic tubes. Platelet-rich plasma (PRP) (pH 7.6) was prepared by centrifugation at room temperature for 15 min at 200 *g*. The platelet PRP quantification was performed by an impedance method with Coulter S.T.S. Microdiff 18. For the determination of platelet cGMP content, PRP was preincubated at 37 °C (30 min) in the presence of 0.5 mM IBMX, then three aliquots were incubated for 15 min at 37 °C, one as control (IBMX alone) and the other two after the addition of 1 mM SNP or 2 μM IONO. After incubation, ice-cold trisodium citrate was added to PRP, platelets were quickly pelleted by centrifugation and the plasma was aspirated; then 0.3 ml 96% ethanol was added to the tubes. The ethanol was evaporated and the samples were frozen and kept at -80°C until analysis. The amount of platelet cGMP was measured by a cGMP [^3H] assay kit provided by Amersham International plc (Amersham, Bucks, UK) and the results were expressed as fmoles cGMP/ 10^6 platelets. The sensitivity of the assay was 0.5 pmol/ $100 \mu\text{l}$ and the intra- and interassay coefficients of variation were

Table 1 Blood levels (means \pm S.E.M.) of i-PTH, ALP, total calcium and phosphate in patients with hyperparathyroidism before and after surgery. Normal values are shown in parentheses

	i-PTH (10–65 pg/ml)	ALP (40–145 U/l)	Total calcium (4.4 \pm 5.2 mEq/l)	Phosphate (0.6–2.1 mmol/l)
Before surgery	177.5 \pm 23.9	173.4 \pm 28.8	6.5 \pm 0.5	0.8 \pm 0.1
After surgery	45.0 \pm 8.8	116.0 \pm 14.5	4.6 \pm 0.1	1.3 \pm 0.2
P value	<0.005	<0.005	<0.005	<0.005

between 6.3 and 13.1% and between 6.2 and 19.4% respectively.

All data in text, tables and figures are presented as means \pm S.E.M. Statistical analyses were carried out using non parametric two-way analysis of variance and Spearman's rank correlation coefficient when appropriate.

Results

Forty-five days after parathyroidectomy levels of i-PTH, ALP and t-Ca had decreased compared with basal values (177.5 \pm 23.9 vs 45.0 \pm 8.8 pg/ml (P <0.005), 173.4 \pm 28.8 vs 116.0 \pm 14.5 UI/l (P <0.005), 6.5 \pm 0.5 vs 4.6 \pm 0.1 mEq/l (P <0.005) respectively) while P levels had increased (0.8 \pm 0.1 vs 1.3 \pm 0.2 mmol/l (P <0.005)) (Table 1).

The concentrations of platelet IBMXcGMP and IONOCcGMP were elevated in patients affected by H-PTH compared with normal subjects (1.9 \pm 0.3 vs 0.8 \pm 0.2 fmol/10⁶ platelets (P <0.02) and 2.7 \pm 0.4 vs 1.4 \pm 0.3 (P <0.05) respectively), but SNPcGMP was not modified (3.9 \pm 0.6 vs 2.5 \pm 0.5) (Fig. 3).

The concentrations of platelet IBMXcGMP and IONOCcGMP decreased after surgical correction of parathyroid adenomas (1.9 \pm 0.3 fmol/10⁶ platelets vs 0.8 \pm 0.2 (P <0.005) and 2.7 \pm 0.4 vs 1.8 \pm 0.3 (P <0.05) respectively) while the SNPcGMP concentration did not change after adenoma removal (3.9 \pm 0.6 vs 4.3 \pm 0.9) (Fig. 3).

cGMP increase after SNP addition (SNPcGMP – IBMXcGMP) was higher compared with the increase after IONO addition (IONOCcGMP – IBMXcGMP) in normal (1.7 \pm 0.4 vs 0.6 \pm 0.1 (P <0.01)) and in H-PTH patients before and after surgery (3.5 \pm 1.0 vs 0.9 \pm 0.3 (P <0.05) and 1.9 \pm 0.7 vs 0.8 \pm 0.2 (P <0.01) respectively).

Total Ca was correlated with basal cGMP (IBMXcGMP; P <0.02, r_s =0.613) in the whole population. i-PTH was also correlated with IBMXcGMP (P <0.02, r_s =0.576) and with t-Ca (P <0.001, r_s =0.840).

Discussion

This study demonstrates that platelet cGMP, both basal (IBMXcGMP) and after stimulation with ionomycin

(IONOCcGMP), is increased in subjects affected by H-PTH compared with normal subjects and it decreases to normal values after surgery of adenoma. cGMP in the platelet is produced by sGC, the activity of which is modulated by the NO levels; hence, the increase in IBMXcGMP could be due to an increase in sGC activity and/or to an increase in cNOS activity (i.e. by an increase in NO production). SNPcGMP levels, before and after surgery, are superimposable suggesting that the sGC activity is not affected by H-PTH, while the elevated IONOCcGMP levels suggest that H-PTH is able to increase cNOS activity. Furthermore, the cGMP increase after SNP addition, both in normal subjects and in patients with H-PTH, is significantly higher than the cGMP increase after IONO addition, excluding a limiting role of sGC in the regulation of cGMP level in the platelets.

It is well known that a diet rich in calcium content is able to reduce the blood pressure in young people with mild hypertension (Grobbee & Hofman 1986). This finding was confirmed in spontaneously hypertensive rats, with a significant positive correlation between systolic blood pressure and PTH (Rao *et al.* 1994). On the other hand some evidence indicates that in H-PTH the intraplatelet calcium levels are increased. Calcium may activate calcium/calmodulin-dependent cNOS, increasing NO production. Furthermore, PTH has an acute hypotensive effect (Mok *et al.* 1989) in spontaneously hypertensive Wistar-Kyoto rats, which persists in the presence of an inhibitor of NO synthesis, indicating that the hypotensive response to PTH is NO-independent (Schleiffer *et al.* 1995). Certainly H-PTH affects platelet calcium by increasing extracellular calcium, the increased gradient between extracellular and intracellular calcium eliciting a greater calcium entry (Kawashima 1990). Actually, it has been demonstrated that normotensive patients with H-PTH have elevated concentrations of intraplatelet calcium that correlate well with blood PTH concentrations and return to normal after correction of the H-PTH condition (Fardella & Rodriguez-Portales 1995). Assuming a high basal intraplatelet calcium in our H-PTH patients, we can explain the higher levels of IBMXcGMP in resting platelets and the correlation of these values with blood calcium and PTH. Taken together these data suggest that the hypotensive action of calcium could be mediated by an enhancement of NO production. On this basis we suggest that the increased levels of IBMXcGMP

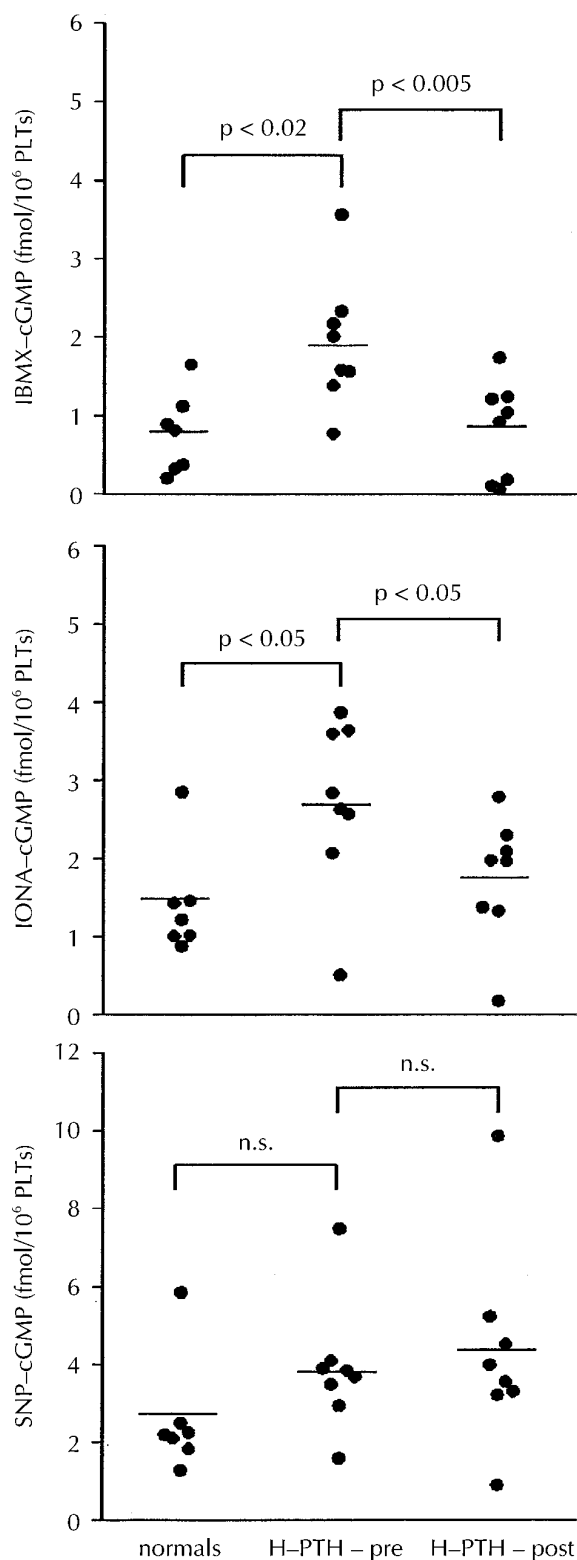


Figure 3 Platelet cGMP levels in normal subjects and in patients affected by hyperparathyroidism before (H-PTH-pre) and after (H-PTH-post) surgery. PLTs, platelets; n.s., not significant.

and IONOcGMP in H-PTH normotensive patients counteract calcium action on the contractility of the smooth muscle preventing the development of arterial hypertension. In agreement with this hypothesis it has been demonstrated that in essential hypertension the intracellular calcium is elevated (Fardella & Rodriguez-Portales 1995) but that the endothelium-dependent NO-induced cGMP production is decreased (Otsuka *et al.* 1985, Shirasaki *et al.* 1988). Finally, since arterial hypertension has been reported in up to 30–40% of patients with primary hyperparathyroidism (Lafferty 1986), we may suppose that in these patients, as in patients with essential hypertension, NO production is not sufficient to counteract the contraction of the smooth muscle cells due to the increase in Ca⁺⁺ cell content. On the other hand the presence of a parathyroid hypertensive factor in H-PTH patients with high blood pressure cannot be ruled out.

In conclusion, our data has demonstrated for the first time that: (1) cGMP is higher in normotensive subjects with H-PTH, (2) cGMP, both basal and following stimulation by ionomycin, decreases after surgery. As our H-PTH patients were normotensive and assuming that platelet cGMP levels are an expression of cNOS activity it can be speculated that the increase in NO production (by platelets and possibly also by endothelial cells) is a mechanism able to counteract the vasoconstriction caused by the high calcium levels in the smooth muscle cells. After surgery, together with normalization of PTH and t-Ca levels, cGMP and hence NO production returned to normal values suggesting a role of calcium and PTH in the regulation of cNOS activity. Further studies are necessary to understand the mechanism by which patients with H-PTH develop arterial hypertension.

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