VITAMINS PROVOKE THE RELEASE OF NITRIC OXIDE BY SODIUM NITROPRUSSIDE: IN VITRO STUDY

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Abstract

It is plausible to assume that vitamins act as nitric oxide scavengers or donors like cobalamine derivatives. Therefore, this study aimed to screen the effect of folic acid, hydroxycobalamine and phytomenadione on nitrogen species. The effects of hydroxyl cobalamine, folic acid and phytomenadione were studied on the synthesized peroxynitrite as well as on their ability to generate peroxynitrite. It is ability to donate nitric oxide or to scavenge released nitric oxide by sodium nitroprusside (10mM) also investigated *in vitro* experimental model. Synthetic peroxynitrite was scavenged by folic acid, hydroxycobalamine and phytomenadione. Their effects varied with their concentrations. These vitamins *per se* failed to release nitric oxide while they improved the bioavailability of nitric oxide released by 10 mM sodium nitroprusside. The beneficial effects of folic acid and to less extent hydroxycobalamine and phytomenadione in improving the nitric oxide bioavailability raise the idea of using these substances as adjunct therapy in diseases associated with endothelial dysfunction.

Keywords: Hydroxycobalamine, folic acid, phytomenadione, sodium nitroprusside.

Introduction

Several substances are known to act as donors or scavengers for reactive nitrogen species [1,2]. There is cumulative evidence that cobalamin or its derivative have dual effects on nitric oxide. Broderick *et al* [3] found that nitrosyl-cobinamide (NO-Cbi) a structural analog of cobalamin (vitamin B12) released nitric oxide that is in aqueous solution. While *in vitro,* cobinamide itself, a vitamin B12 analog, is an effective NO scavenger [4]. Cobinamide improved the survival of fly in a well established model of bacterial sepsis associated with high nitric oxide level [4].

5-methyltetrahydrofolate, a folic acid derivative, is indirectly suppressed the activity of nitrogen species via its activity on superoxide anion. It improved NO-mediated endothelium-dependent vasomotor responses and reduced vascular superoxide, both *ex vivo and in vivo* [5]. Folate derivatives had the most prominent peroxynitrite scavenging activity via donating the electron [6].

On the other side, phytomenadione strongly inhibited the generation of nitrite and it interfered with nitric oxide pathway by producing high level production of superoxide anion [7].

Therefore, it is worth trial to investigate the effects of hydroxycobalamine, folic acid and phytomenadione on the scavenging or releasing nitric oxide and peroxynitrite *in vitro* experimental model.

Experimental

This study was conducted in Department of Biochemistry, College of Medicine, Al-Mustansiriyia University in Baghdad, Iraq from April to June 2008.

Peroxynitrite ($ONOO^-$) was prepared by mixing 1 volume cooled hydrogen peroxide (1 M) and 1 volume cooled sodium nitrite (1 M) in dark room, then 2 volumes cooled sodium hydroxide (1.5 M) was added to the mixture as prescribed by Whiteman and Halliwell [8]. Peroxynitrite was quantified spectro-photometrically (ε = 1670 $M^{-1}.cm^{-1}$ at 412 nm), and 1 molar of peroxynitrite was obtained. From this yield, 180µM $ONOO^-$ was incubated with 100 µl of each drug; hydroxycobalamine [6.25-50µg] or folic acid [62.5-500µg] or phytomenadione (vitamin K1) [62.5-500µg] in phosphate buffer saline for 15 minutes and the absorbance at 302 nm of the samples was recorded.

Peroxynitrite mediated nitration of phenol was measured for hydroxycobalamine, folic

acid and phytomenadione as described by Beckman et al [9] cited VanUffelen et al [10]. Briefly, 50 µg hydroxycobalamine or 500 µg folic acid or 500µg phytomenadione was added to 5mM phenol in 50 mM sodium phosphate buffer pH 7.4 in a final volume of 3 ml. After incubation for 2 hours at 37ºC, 50 µl 0.1 M sodium hydroxide was added, and the absorbance at 412 nm of the samples was immediately recorded. The yield of nitrophenol was calculated from $\varepsilon = 4400\ M^{-1}.cm^{-1}$.

Nitric oxide donating activity was determined as described by Newaz et al [11]. Briefly hydroxycobalamine [25-125 µg] or folic acid [0.25 mg-1.25 mg] or phylloquinone [0.25 mg-1.25 mg] to 50 µl HCl (6.5M) and 50 µl sulfunalic acid (37.5mM). After incubation for 10 min, 50µl naphthylethylenediamine dihydro-chloride (12.5mM) was added and incubated for further 30 min, centrifuged for 10 min at 1000g. The absorbance at 540 nm was immediately recorded. All experiments were performed in duplicate.

Nitric oxide radical scavenging was estimated by the use of Greiss reaction [12]. Greiss reagent was modified by using naphthylethylenediamine dihydrochloride (0.1 % w/v). The reaction mixture was (3ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer (0.5 ml) and each drug with above concentrations or distilled water as negative control (final volume 0.5 ml) was incubated in dark room at 25ºC for 150 min. After incubation, 0.5 ml of the reaction mixture mixed with 1 ml of sulfunalic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then 1 ml of naphthylethylenediamine dihydrochloride was added, mixed and allowed to stand for 30 min in dark place at 25ºC. A pink colored chromophore is formed. The absorbance of these solutions was measured at 540 nm against corresponding blank. All experiments were performed in duplicate.

Hydroxycobalamine ampoule (1 mg/ml), folic acid ampoule (10 mg/ml) and ampoule (vitamin K1, 10 mg/ml) were purchased from local sources. All chemicals used in the study were of Analr grade. All the drugs and chemicals were prepared freshly prior to the experiment.

Results

None of the tested compounds generate peroxynitrite as detected as nitrophenol at 412nm, while these compounds showed variable effect on the exogenous $ONOO^-$ when they are incubated with $ONOO^-$ (180 µmol).

Folic acid at concentrations lower than 250 µg scavenged the presence of $ONOO^-$ Fig.(1). Both hydroxycobalamine and phytomenadione exerted stepwise decline in the level of $ONOO^-$ and they showed dual effect i.e. at concentration < 200 µg they potentiate the release of ONOO while at high concentration scavenged $ONOO^-$. None of these compounds released NO *per se* as detected by Greiss reaction Fig.(2). In presence sodium nitroprusside, none of these compounds scavenged NO in fact they augmented the action of sodium nitroprusside in releasing NO. Folic acid show one small hump curve in potentiation of NO release while hydroxycobalamine showed only one peak and phytomenadione showed one hump but took minor range of folic acid.

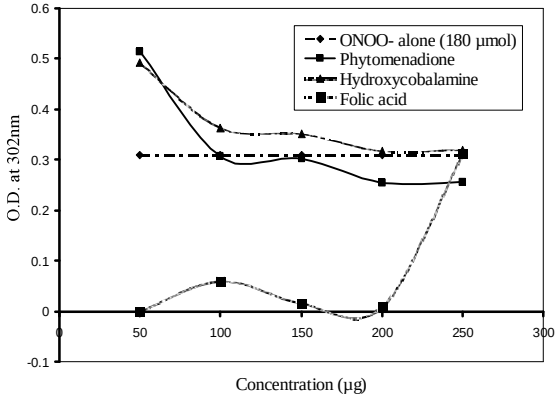

Fig. (1) : Effect of phytomenadion, hydroxycobalamine and folic acid on the exogenous peroxynitrite (ONOO-).

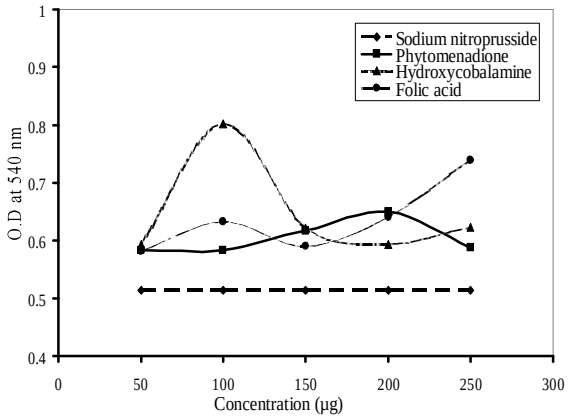

***Fig. (2) : Effect of phytomenadion, hydroxycobalamine and folic acid on the nitric oxide released by 10 mMol sodium nitroprusside*.**

Discussion

A common trial of the folic acid, vitamin B12 and vitamin K_1, in the nitric oxide-peroxynitrite cycle biochemistry is improving the bioavailability of NO. This effect is similar to that reported with ascorbic acid which facilitated the decomposition of SNP and thereby improved NO bioavailability [(13)]. Recently, Tirapethiet *et al* [(14)] and Shukala *et al* [(15)] found that folic acid improved the endothelium function via conservation of nitric oxide. In isolated perfuse rat heart, low concentration of folic acid (100 µmol) significantly increased coronary flow accompanied by significant increase in nitrite out flow as well as reduced the superoxide production [(16)]. Moreover, low dose folic acid (400µg/day) reduced superoxide production in patients with coronary artery disease [(17)]. The results of folic acid in this study documented the above previous observations and add further finding that folic acid improved the bioavailability of nitric oxide from sodium nitroprusside by mechanism unrelated to regulation of endothelial nitric oxide synthase [(18)]. Previous study found that hydroxycobalamin itself, provoked a rapid accumulation of extracellular hydrogen peroxide [(19)], and in this study showed dual effect on $ONOO^-$ level. The results of vitamin K_1 in this work shared that of Tirapelli et al who found the antagonistic effect of vitamin K_1 to nitric oxide synthase inhibitors (14).

In conclusion, the beneficial effects of nutrient substances in improving the nitric oxide bioavailability raise the idea of using these substances as adjunct therapy to ameliorate nitrate tolerance in patients with coronary artery disease.

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الخلاصة

من المقبول افتراضا ان الفيتامينات تعمل على وهب او كسح اوكسيد النتريك كما هو عليه في مشتقات كوبالامين ، لذا هدفت الدراسة الى التحري عن تأثير حمض فوليك ، هيدروكسي كوبالامين، و فايتومينادايون في الانواع النتروجينية. تم دراسة تأثير كل من حمض فوليك ، هيدروكسي كوبالامين، و فايتومينادايون على بيروكسي ناترايت المصنع وكذلك في قابليتهم على توليد بيروكسي ناترايت. كما ودرست قابليتهم على وهب اوكسيد النتريك او كسح اوكسيد النتريك المحرر بفعل نتروبروساید صوديوم (10 ملي مول) في أنموذج مختبري.

اظهرت فيتامينات حمض فوليك ، هيدروكسي كوبالامين، و فايتومينادايون فعل كسح بيروكسي نانترايت المصنع وأختلفت التاثيرات باختلاف التراكيز المستعملة. هذه الفيتامينات بذاتها لا تحرراوكسيد النتريك ولكنها تزيد من التوافر الحيوي لاوكسيد النتريك المحرر بفعل نتروبروسايد صوديوم (10 ملي مول).

يستنتج من ذلك ان التأثيرات النافعة لحمض فوليك وبدرجة اقل مع هيدروكسي كوبالامين و فايتومينادايون في تحسين التوافر الحيوي لاوكسيد النتريك تطرح فكرة استعمالها كعلاج مساعد في الامراض االمتعلقة بسوء وظيفة بطانة الاوعية الدموية.