Original Communication

Vitamin B_6 Compounds are Capable of Reducing the Superoxide Radical and Lipid Peroxide Levels Induced by H_2O_2 in Vascular Endothelial Cells in Culture

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Abstract: Pyridoxamine, pyridoxine, and pyridoxal phosphate were tested to examine if they have antioxidant properties. Endothelial cells exposed to 0.5 mM H_2O_2 for 2 hours increased the superoxide anion and lipid peroxide levels as biomarkers of oxidative stress. The increase of superoxide was mainly due to the activation of NADPH-oxidase by H_2O_2. Preincubation of the endothelial cells with 0.1 or 1.0 mM of pyridoxamine or pyridoxal phosphate for one-half hour before H_2O_2 exposure significantly reduced the superoxide and lipid peroxide compared to the cells exposed to H_2O_2 only. Preincubation of the cells with 0.1 or 1.0 mM of pyridoxine also significantly reduced the lipid peroxide but did not significantly affect the superoxide level unless the preincubation time was extended to 24 hours. The prostacyclin release by endothelial cells was also significantly inhibited by H_2O_2. However, the preincubation of endothelial cells with 1.0 mM of pyridoxamine, pyridoxine, or pyridoxal phosphate did not prevent that inhibition. These results indicate that pyridoxamine, pyridoxine, and pyridoxal phosphate acted as antioxidants and reduced the superoxide and lipid peroxides induced by H_2O_2, but did not protect the cells from the effects directly related to H_2O_2 itself.

Key words: Vitamin B_6 Compounds, hydrogen peroxide, superoxide anion, lipid peroxide, NADPH-oxidase, antioxidative effect, endothelial cells

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Introduction

A considerable body of evidence implicates oxidative stress as an important pathogenic element in endothelial dysfunction [1–3] and cell injury, which contribute to atherosclerosis [4] and other cardiovascular diseases [5]. Oxidative stress, defined as an increase in the steady state levels of reactive oxygen species (ROS), may occur as a result of increased free-radical generation and/or to a decline in the antioxidant defense mechanism. ROS are constantly formed in the human body and must be removed by antioxidants. A lack of balance in the oxidant-antioxidant activity is involved in many free radical mediated pathologies such as atherosclerosis and cardiovascular disease (CVD).

Recent biochemical studies have shown that an NADPH-oxidase is the major source of superoxide anions ($O_2^{\bullet -}$) in vascular wall cells [6,7] and has been implicated in endothelial dysfunction [8,9]. Vascular NADPH-oxidase-dependent overproduction of superoxide contributes to the pathogenesis of cardiovascular disease [6,10–13] and its activity is associated with endothelial dysfunction and with clinical risk factors [9]. NADPH-oxidase is present in human vascular smooth muscle cells and endothelial cells in culture [14,15].

Vitamin B_6, also called pyridoxine, is one of eight water-soluble B vitamins. It is the precursor of the biologically active derivatives pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate, with functional roles in a number of enzymatic reactions, especially those involved in amino acid metabolism [16]. Pyridoxine, although not classified as an antioxidant, has recently been shown to have highly effective antioxidant properties [17].

Ehrenshaft *et al.* [18] reported a novel gene, SOR1, involved in *de novo* vitamin B_6 biosynthesis, and showed that pyridoxine quenched singlet oxygen at a rate comparable to those of vitamins C and E. In addition, Chen *et al.* [19] reported that pyridoxine was required for plant development and stress tolerance. They revealed that vitamin B_6 protected membranes from lipid peroxidation; using pdx1 knockout mutants treated with UV, they showed that PDX-1 genes were expressed in all plant parts and played a role in pyridoxine synthesis. Furthermore, Chumnantana *et al.* [20] reported that vitamin B_6 compounds prevented the death of yeast cells due to menadione, a reactive oxygen generator. Satchithanandam [21] also showed that vitamin B_6 has a protective effect against chromium-induced oxidative stress in rat livers, demonstrating the antioxidant potential of vitamin B_6. While vitamin B_6 deficiency increased oxidative stress [22], vitamin B_6 supplementation reduced oxidative stress related to complications in diabetes and neurodegenerative disease [23].

The antioxidant properties of vitamin B_6 compounds have been shown in different systems. Pyridoxamine inhibits oxidative propagation of protein damage by scavenging hydroxyl radical [24]. Pyridoxine also prevented the increase of lipid peroxidation and the inhibition of NO synthase in endothelial cells (EC) induced by oxidized low-density lipoprotein [25]. Pyridoxine and its derivatives acted as strong quencher of singlet molecular oxygen and as a potential fungal antioxidant [26]. Pyridoxine and pyridoxamine significantly decreased protein oxidation in lens cells and crystalline protein solution [27], and inhibited $O_2^{\bullet -}$ and lipid peroxidation in high glucose-treated erythrocytes [28].

The objective of the present study was to examine if vitamin B_6 and its derivatives, pyridoxal-5′-phosphate or pyridoxamine, can ameliorate the oxidative stress induced in EC in culture by H_2O_2. It is known that H_2O_2 is capable of feeding forward and activating NADPH-oxidase in vascular cells [29] and inducing oxidative stress in EC by increasing intracellular $O_2^{\bullet -}$ levels through NADPH oxidase [30]. H_2O_2 is also capable of inhibiting superoxide dismutase (SOD) activity [31].

Materials and methods

Cell culture

Fetal bovine serum (FBS), Eagle's minimum essential medium (MEM), pyridoxamine dihydrochloride, pyridoxine (Vitamin B_6), pyridoxal 5′-phosphate, and diphenylene iodonium chloride (DPI) were purchased from Sigma (St. Louis, MO). Lucigenin (bis-N-methylacridinium nitrate) was obtained from Molecular Probes (Eugene, OR). DPI was dissolved in dimethyl sulfoxide (DMSO) at a level of 4 mM and kept at -20°C. The concentrations of pyridoxamine (PM), pyridoxine (P), or pyridoxal phosphate (PP) were used at levels of 1 mM or 0.1 mM while DPI was used at 20 μM in the culture medium.

Human endothelial cells (EC) were obtained from American Type Culture Collection (ATCC, Rockville, MD) The cells at passage 6 from the time it was received were cultured in MEM supplemented with 20% FBS in a 5% CO_2 incubator at 37°C. The confluent EC were seeded into 12-well plates, 25- or 75-cm^2 flasks (Corning Medical and Scientific Co., Park

Ridge, IL), and grown to 80 % confluence. The medium was replaced with FBS-free MEM with or without the desired concentrations of PM, P, or PP and pre-incubated for one-half hour or 24 hours. The medium was then removed and fresh medium containing the same concentrations of PM, P, or PP plus 500 μM H_2O_2 was added. After 2 hours of cell exposure to H_2O_2, the following experiments were performed.

Measurement of cellular superoxide anion production

Human EC were grown in MEM cell culture medium in 25-cm^2 flasks until they reached 80–90 % confluence. The medium was replaced by a fresh FBS-free medium containing either 1 mM or 0.1 mM of PM, P, or PP and preincubated for one-half or 24 hours at 37°C, after which the medium was replaced with fresh medium containing the same concentrations of PM, P, or PP plus 500 μM H_2O_2. After 2 hours incubation with H_2O_2 the cells were washed, trypsinized, and resuspended in 0.4 mL Krebs buffer (pH 7.4) and kept on ice until use. Before measuring the superoxide production, cell suspensions were kept at room temperature for 3 minutes and then added to a scintillation vial containing dark-adapted lucigenin (5 μM) [32] in 2 mL of 100 mM phosphate buffer (pH 7.4). Photon emission was measured every minute for 10 minutes in a Beckman LS 6500 scintillation counter (Fullerton, CA) in out-of-coincidence mode. A buffer blank was subtracted from each reading. Protein content was determined by the Bio-Rad reagent (Hercules, CA) according to the Bradford method [33] using bovine serum albumin (BSA) as standard.

Measurement of lipid peroxides

The lipid peroxide content of EC was determined after control or H_2O_2 exposures by measurement of thiobarbituric acid reactive substances (TBARS) as described by Ohkawa *et al.* [34]. The relative volume of the assay was proportionally reduced, and fluorescent spectrophotometry, rather than absorbance at 532 nm, employed to increase the sensitivity of the assay [35]. These modifications permitted detection of subnanomolar quantities of malondialdehyde (MDA) produced from external standard 1,1,3,3-tetramethoxypropane (Sigma Co.). The TBA reaction mixture consisted of 0.1 mL disrupted cells in 1.15 % KCl, 0.1 mL 8.1 % sodium dodecyl sulfate (SDS), 0.75 mL 20 % acetic acid solution (pH 3.5), 0.75 mL 0.8 % aqueous TBA solution (Sigma Co., St. Louis, MO), and 0.3 mL distilled water. The mixture was heated at 95°C for 60 minutes in tightly capped tubes. After cooling, 0.1 mL of distilled water was added. The samples were then extracted with 2.5 mL n-butanol:pyridine (15:1 both from Fisher) and centrifuged (1,000 x g for 20 minutes) to separate the phases and removal of cellular debris. The organic phase was analyzed (excitation: 515 nm; emission: 553 nm) with a Perkin-Elmer 650–10 S fluorescence spectrophotometer. TBA-reactive substances were expressed as nmol MDA/mg cell protein by extrapolation from an external standard curve.

NADP(H) oxidase assay

Control cultures or cultures that had been incubated with H_2O_2 with or without PM, P, or PP were washed five times with 5 mL ice-cold phosphate buffer saline (PBS). The cells were trypsinized and transferred to 15-mL centrifuge tubes, and the flasks were washed twice with an additional 3 mL of PBS. Cells were then centrifuged at 750 x g at 4°C for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 500 μL of lysis buffer containing protease inhibitors (20 mM potassium phosphate, 1 mM EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was then doused 100 times on ice and the homogenate was stored on ice until use. Protein content of the homogenate was measured by the Bradford method [33].

NADPH-oxidase activity was measured by chemiluminescence assay in a 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as electron acceptor, and 100 μM NADPH as the substrate in a final volume of 1mL [36]. In some experiments DPI (20 μM) was added 5 minutes before reading the lucigenin buffer solution. The reaction was started by the addition of 100 μL of cell homogenates (50–300 μg protein). Chemiluminescence was monitored every minute for 10 minutes.

Measurement of cellular prostacyclin production

The control cells, or the cells after being exposed to 500 μM H_2O_2 for 2 hours in absence or presence of PM, P, or PP for 24 hours, were washed with Tris-HCl buffer, pH 7.4 (150 mM Tris-HCl, 150 mM NaCl, 5.5 mM glucose) then 3 mL of fresh buffer were added to the intact cell monolayer and incubated for 30 minutes at 37°C in a 5% CO_2 incubator. The supernatant was centrifuged for 5 minutes at 1000 x g and retained for the assay of 6-Keto $PGF_1\alpha$; the stable metabolite of prostacyclin (PGI_2). The PGI_2 content of the Tris-buffer was determined by radioimmunoassay (RIA) measured as 6-Ketoprostaglandin $PGF_1\alpha$ with a commercially available kit (Amersham, Piscataway, NJ). PGI_2 production is expressed as picogram 6-Keto- $PGF_1\alpha$ per mg cell proteins.

Fatty acid analysis of cellular phospholipid

After preincubation of the cells with 0.1 mM PM, P, or PP for the designated time and exposure to H_2O_2 for 2 hours the cells were rinsed three times with ice-cold PBS, trypsinized, collected, and suspended in 2 mL of methanol, then sonicated for 30 seconds, then another 3 mL of methanol and 10 mL of $CHCl_3$ were added for lipid extraction by the Folch method [37]. The phospholipids (PL) fraction of the cell lipid was separated from other portions of the lipid extract with a polysilisic acid-impregnated glass fiber sheet (Gelman Science, Ann Arbor, MI) using a solvent system of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). The PL fractions were then transesterified using a BF_3/MeOH complex [38].

The fatty acids were separated by gas chromatography (GC) using a Hewlett Packard Model 5890 Series II gas chromatograph (Hewlett Packard, Chicago, IL) equipped with an all-glass splitter and flame ionization detector (FID) to separate methyl esters on a Varian CP-Select CB 200 m x 0.25 mm film thickness, fused silica capillary column (Varian, Walnut Creek, CA Part # 7421). Retention times, peak areas, and peak relative-area percentages were determined electronically using a Hewlett-Packard 3390 Reporting Integrator. Methyl esters of fatty acids were identified by comparing relative retention times with authentic standards (Nu.Chek.Prep, Elysian, MN).

Hydrogen peroxide measurement

Hydrogen peroxide content in the culture medium and the cell lysate was measured in the cells exposed to 500 μM H_2O_2 for up to 2 hours using the peroxide assay kit (DIOX-250) obtained from Bioassay System (Hayward, CA).

After H_2O_2 exposure the medium was collected and the cells were washed, trypsinized, and then lysed in 0.5 mL of 0.1% Triton X-100. Using a 96-well plate and microplate reader at 585 nm, small aliquots (20 μL) were used from the medium and the cell lysate for H_2O_2 measurement following the manual accompanying the kits. The amounts of H_2O_2 were obtained by extrapolation of the standard curve. The cell protein was measured by the Bradford method [33]. The H_2O_2 concentration was expressed as μM in the medium and as ng/mg cell protein in the cells.

Endothelial cell injury assay

EC injury was estimated by the release of ^{51}chromium as previously described [39]. Confluent monolayer cells in 12-well plates were pre-labeled with 2μ Ci of [^{51}Cr] sodium chromate (Perkin-Elmer, Bellerica, MA) for 6 hours in the growth medium. Cells were then washed twice with MEM and incubated with 3 mL of MEM medium containing 500 μM H_2O_2 for 2 hours. A 500-μL aliquot of the medium was removed in duplicates and the radioactivity due to ^{51}Cr released by the injured cells was measured by a Packard-Cobra II gamma-counter. Results were expressed as percentages of specific ^{51}Cr release, calculated as follows: (A-B)/(C-B) x 100, where A represents ^{51}Cr release due to H_2O_2, B represents the spontaneous ^{51}Cr release, and C represents the maximum release ^{51}Cr. Spontaneous release was determined in cells incubated with medium only while maximum release was measured in cells treated with H_2O_2 and lysed in 0.1% TritonX-100.

Statistical analysis

All data are given as means ± SE and analyzed by one-way ANOVA and Dunnet's method. Differences with $p<0.05$ were considered significant.

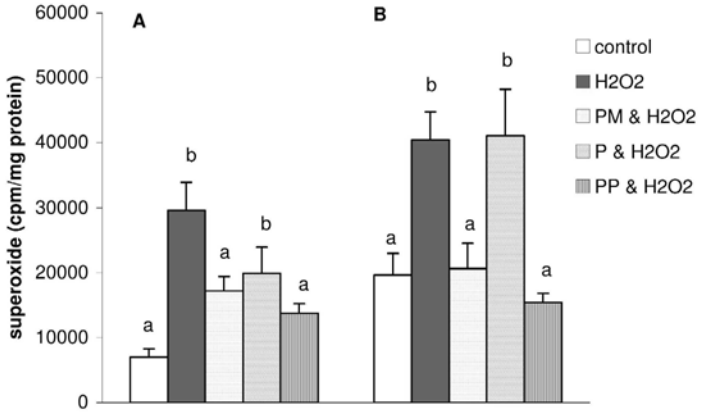

Figure 1: Superoxide anion levels in EC exposed for 2 hours to 0.5 mM H_2O_2 without or with preincubation with 0.1 mM (A) or 1 mM (B) of PM, P, or PP for $\frac{1}{2}$ hour. Values are mean ± SE of twelve (A) or six (B) different experiments. Bars with different superscript letters are significantly different at level of $p<0.05$.

Results

The superoxide anion level in EC incubated with 0.25 mM or 0.5 mM H_2O_2 was significantly increased (98 %) as compared to the control cells as measured by lucigenin-enhanced chemiluminescence. The superoxide continued to increase as the H_2O_2 increased to 1.0 mM (129 %) and 1.5 mM (165 %) in the culture medium. There was no further increase in the superoxide as the H_2O_2 increased from 1.5 to 2.5 mM. At 0.5 mM H_2O_2 the superoxide anion significantly increased after a one-hour incubation compared to the control cells, and there was no further increase as the incubation time increased from 1 to 2.5 hours. Based on the dose-response curve we used 0.5 mM concentration of H_2O_2 and 2 hours incubation to study the protective effect of B_6 vitamins.

Pretreatment of EC with 1 mM of PM, P, or PP for 30 minutes before their exposure to 0.5 mM H_2O_2 for 2 hours caused a significant decrease of superoxide production by PM (49 %) and PP (62 %), but no significant change was observed for P compared to the cells treated with 0.5 mM H_2O_2 only (Figure 1B). Under the same conditions, using 0.1 mM of PM, P, or PP also caused a significant decrease in superoxide level by PM (42 %) and PP (53 %), but no significant change was observed by P compared to the cells treated with 0.5 mM H_2O_2 only (Figure 1A). These results indicate that increasing the concentrations of PM and PP from 0.1 mM to 1.0 mM slightly enhanced the protective effects of PM (from 42 to 49 %) and PP (from 53 to 65 %), but P had no significant effect on the superoxide level in EC either at 0.1 or 1.0 mM concentration. By extending the preincubation time with B_6 vitamins from one-half hour to 24 hours, then with 0.5 mM of H_2O_2 for 2 hours, Figure 2 shows that at 1 mM the PM, P, and PP were all capable of significantly reducing the superoxide level by 66, 56, and 61 %, respectively, compared to the cells treated with 0.5 mM H_2O_2 only. This indicates that by extending the preincubation time, pyridoxine (P) became capable of reducing the superoxide significantly, and the protective effects of PM and PP were also enhanced by extending the preincubation time. Figure 3 also shows that the superoxide level was significantly reduced in the cells preincubated for 24 hours with 0.1 mM of P (31.5 %) compared to the cells treated with H_2O_2 only, indicating that by extending the preincubation time, P became effective in reducing the superoxide anion even at 0.1 mM concentration.

TBARS content in the EC was also measured to estimate the lipid peroxidation level measured as malondialdehyde (MDA). The TBARS content in the cells treated with 0.5 mM H_2O_2 significantly increased compared to the control cells (Figures 4A and 4B). Preincubation of the cells with 1 mM of PM, P, or PP for one-half hour, then with 0.5 mM H_2O_2 for 2 hours, significantly decreased the TBARS content by 29, 27, and 33 % respectively, compared to the cells treated with 0.5 mM H_2O_2 only (Figure 4B). At 0.1 mM of PM, P, or PP, the TBARS content decreased by 28, 13, and 35 %, respectively, compared to the cells treated with 0.5 mM H_2O_2 only (Figure 4A). It is obvious that the maximum inhibition of TBARS was almost reached by 0.1 mM of PM and PP and no further inhibition was observed at 1 mM, but the decreasing effect of P on TBARS was enhanced by increasing the concentration from 0.1 mM (13 %) to 1.0 mM (27 %). These results indicate that PM and PP are more effective in reducing lipid peroxidation than P.

Since MDA as the end product of fatty acid peroxidation was reduced by PM, P, or PP, we measured

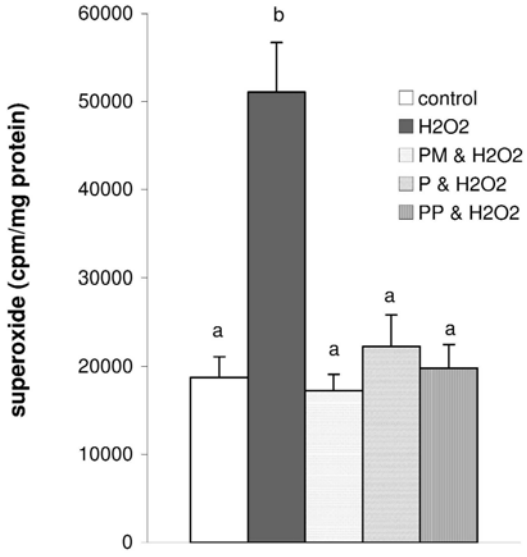

Figure 2: Superoxide anion levels in EC exposed for 2 hours to 0.5 mM H_2O_2 without or with preincubation with 1 mM of PM, P, or PP for 24 hours. Values are mean ± SE of three different experiments. Bars with different superscript letters are significantly different at level of $p<0.05$.

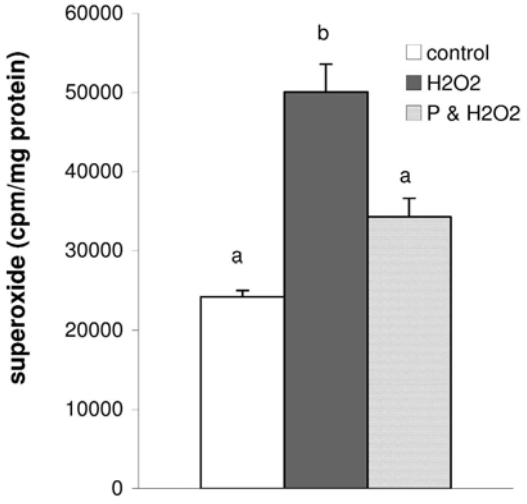

Figure 3: Superoxide anion levels in EC exposed for 2 hours to 0.5 mM H_2O_2 without or with preincubation with 0.1 mM of P for 24 hours. Values are mean ± SE of three different experiments. Bars with different superscript letters are significantly different at level of $p<0.05$.

the change of the polyunsaturated fatty acid (PUFA) content of the cellular phospholipid, which represents the membrane lipid fatty acids considered as the main target for oxidation. Table I shows that the PUFA (with two or more double bonds) content of the EC treated with 0.5 mM H_2O_2 for 2 hours decreased by 20 % compared to the control cell. In the EC preincubated with 0.1 mM of PM, P, or PP for one-half hour, then with 0.5 mM H_2O_2 for 2 hours, the PUFA decrease was 0.0, 11.8, and 7.2 %, respectively, indicating that PM and PP were more protective against PUFA oxidation while P was the weakest, almost paralleling their effects on the cellular TBARS content (Figure 4A).

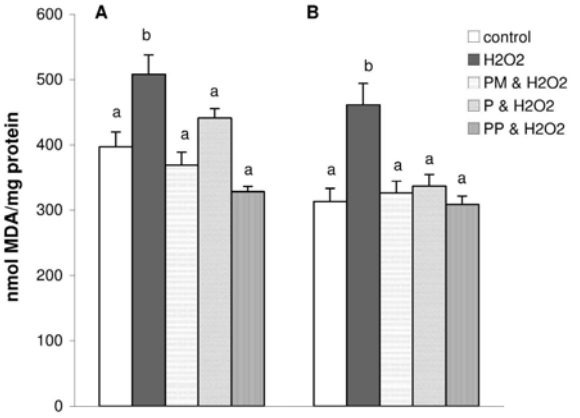

Figure 4: Lipid peroxide levels measured as malondialdehyde (MDA) in EC exposed for 2 hours to 0.5 mM H_2O_2 without or with preincubation with 0.1 mM (A) or 1 mM (B) of PM, P, or PP for $\frac{1}{2}$ hour. Values are mean ± SE of six (A) or nine (B) different experiments. Bars with different superscript letters are significantly different at level of $p<0.05$.

The role of NADPH-oxidase in H_2O_2 induction of $O_2^{\bullet}$ was confirmed by measuring the activity of this enzyme in the control and the H_2O_2-treated cells in the presence of 100 μM of NADPH as substrate. Preincubation of the cells with 1 mM of PM, P, or PP for 24 hours, then with 0.5 mM H_2O_2 for 2 hours, resulted in significantly more $O_2^{\bullet}$ production in cells treated with 0.5 mM H_2O_2 (140069 ± 17712 cpm/μg protein), with 0.5 mM H_2O_2 + 1mM PM (101395 ± 9457 cpm/μg protein), with 0.5 mM H_2O_2 + 1 mM P (107861 ± 8123 cpm/μg protein), or 0.5 mM H_2O_2 + 1 mM PP (179056 ± 13214 cpm/μg protein) compared to the control cells (75334 ± 3406 cpm/μg protein). The superoxide was almost abolished in the H_2O_2-treated cells preincubated for 5 minutes with 20 μM DPI before reading lucigenin chemiluminescence (2480 ± 237 cpm/μg protein) (Figure 5), indicating complete inhibition of NADPH-oxidase as the main source of $O_2^{\bullet}$. These data indicate that H_2O_2 enhanced NADPH-oxidase in EC. It also showed that although NADPH-oxidase activity was decreased in

Table I: Change of total polyunsaturated fatty acids (PUFA) content in the phospholipids of the endothelial cells incubated with 500 μM H_2O_2 for 2 hours in absence or presence of 0.1mM of pyridoxamine, pyridoxine, or pyridoxal phosphate

	Total PUFA %	PUFA as % of control cells	% decrease of PUFA
Control	15.2 ± 0.4	100	0.0
H_2O_2	12.1 ± 0.6	79.6	20.4
PM + H_2O_2	15.2 ± 0.35	100	0.0
P + H_2O_2	13.4 ± 0.2	88.2	11.8
PP + H_2O_2	14.1 ± 0.2	92.8	7.2

Cells were incubated with a medium containing 0.1mM of PM, P, or PP for $\frac{1}{2}$ hour, then with fresh medium containing the same concentration of vitamins plus 500 μM H_2O_2 for 2 hours. The cells were harvested and lipids were extracted as in the method section and the fatty acids of the phospholipid fraction were analyzed using GC.

Results are expressed as mean ± SE of three different incubations.

the cells preincubated with 1 mM of PM or P, but that decrease was not significantly different compared to the cells exposed to H_2O_2 only.

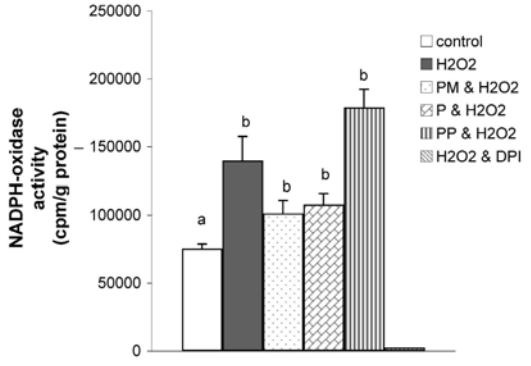

Figure 5: NADPH-oxidase activity in EC exposed for 2 hours to 0.5 mM H_2O_2 without or with preincubation with 1 mM of PM, P, or PP for 24 hours. Notice the complete inhibition of the enzyme in presence of 20 μM DPI added to lucigenin buffer 5 minutes before reading the chemiluminescence. Values are mean ± SE of nine different experiments. Bars with different superscript letters are significantly different at level of $p<0.05$.

Table II shows that the amount of PGI_2 released and measured as 6-keto$PGF_{1\alpha}$ as pg/mg cell protein was significantly reduced by more than 90 % in all the cells treated with 0.5 mM H_2O_2 for 2 hours in the absence or presence of 1 mM of PM, P, or PP. This indicates that reducing the superoxide anion induced by H_2O_2 by PM, P, or PP did not prevent the inhibition of PGI_2 formation in the EC cells.

The time-course change of the concentration of H_2O_2 in the culture medium as well as in the cells was followed by measuring the H_2O_2 concentration in both the medium and the cells at different intervals

Table II: 6-Ketoprostaglandin $F_1\alpha$ released from endothelial cells

Treatment	6-Keto $PGF_1\alpha$ (pg/mg protein)
Control	2433 ± 259 [a]
H_2O_2	82.0 ± 13.4 [b]
PM + H_2O_2	59.8 ± 4.8 [b]
P + H_2O_2	78.8 ± 4.7 [b]
PP + H_2O_2	69.1 ± 6.8 [b]

Cells were preincubated with 1mM pyridoxamine, pyridoxine, or pyridoxal phosphate for 24 hours then a fresh medium added containing the same vitamin concentrations plus 500 μM H_2O_2 and incubated for 2 hours. The medium was removed and 3 mL of fresh Tris buffer (pH 7.4) was added and incubated for 30 minutes. The 6-keto $PGF_1\alpha$ released was measured using RIA kit as described in the method.

Results are expressed as mean ± SE of three different incubations

Mean values with different superscript letters are significantly different at $p<0.05$

during the 2-hour incubation. Figure 6 shows that H_2O_2 concentration in the medium decreased from 526 μM to 150 μM during the first 10 minutes then decreased to 63, 50, 42, and 47 μM at 20, 30, 60, and 120 minutes, respectively. In the cells, H_2O_2 content was 8.8 ng/mg protein after 10 minutes which then increased to 8.9, 9.0, 11.9, and 12.5 ng/mg protein at 20, 30, 60, and 120 minutes, respectively. It is obvious that H_2O_2 rapidly disappeared (90 %) from the medium during the first twenty minutes and slightly increased in the cells, indicating that the amount of H_2O_2 taken up by the cells was immediately degraded by the cells.

Using a ^{51}Cr release method, the cell injury by the 0.5 mM H_2O_2 for 2 hours did not increase beyond

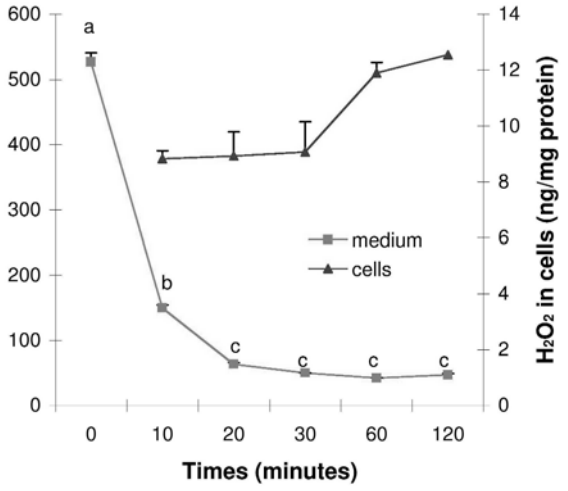

Figure 6: Change of H_2O_2 concentration in the medium (μM) and in the cells (ng/mg protein) during 2 hours incubation of the cells with 0.5 mM H_2O_2. Points with different superscript letters are significantly different at a level of $p<0.05$.

6.2%. All the cell monolayers we used were intact and no cell lysis was observed.

Discussion

Exposure of EC to H_2O_2 increased the level of $O_2^{\bullet -}$, which was mainly attributed to the enhancement of NADPH-oxidase activity by H_2O_2, in agreement with previous reports [29,30]. PM and PP at 0.1 or 1.0 mM in the culture medium were capable of significantly reducing the $O_2^{\bullet -}$ level in the cells when preincubated for one-half hour before H_2O_2 exposure. However, P at 0.1 or 1.0 mM was capable of reducing $O_2^{\bullet -}$ levels in EC only when the preincubation time was extended to 24 hours before exposure to H_2O_2.

The vitamin B6 group consists of six interconvertible compounds in mammalian cells: P, pyridoxal, PM, pyridoxine-5′-phosphate, PP, and pyridoxamine-5′-phosphate. Therefore, the longer time needed for P to be effective in reducing the $O_2^{\bullet -}$ in the EC may indicate that during the 24-hour preincubation, more uptake or P may be converted to one of the other active metabolites such as PM or PP. P also can participate in the maintenance of glutathione (GSH) levels by acting as a cofactor in the synthesis of cysteine [40]. Thus it is possible that pyridoxine increases the intracellular level of GSH, which plays an important role in the protection against reactive O_2 species and free radicals. Vitamin B_6 also increases glutathione peroxidase (GPx) activity by enhancing the incorporation of Se in GPx [41]. Therefore, through one or more of these mechanisms P may be able to protect the cells from the increased $O_2^{\bullet -}$ induced by H_2O_2. A recent *in vitro* study [42] showed that P does not react with $O_2^{\bullet -}$ at all while displaying very low reactivity with $^{\bullet}OOH$, but it is most reactive with $^{\bullet}OH$, which confirms that P cannot be effective in reducing $O_2^{\bullet -}$ through direct interaction with $O_2^{\bullet -}$.

Pyridoxine was capable of reducing the level of lipid peroxide (measured as TBARS) in the EC preincubated for one-half hour with 0.1 or 1.0 mM of P, indicating a more reactive role of P toward the free radicals ($^{\bullet}OH$ and $^{\bullet}OOH$), as previously reported [42]. PM and PP also were effective in reducing lipid peroxides more efficiently than P. These data are in agreement with others who found that Vitamin B_6 compounds can prevent lipid peroxidation caused by high glucose in human erythrocytes [28] and by H_2O_2 in U937 monocytes [43]. P also protected the membrane from lipid peroxidation [39] and prevented the death of yeast cells due to menadione, a reactive oxygen generator [20]. P also prevented the oxidative stress and endothelial dysfunction induced by oxidized low-density lipoprotein (oxLDL) [25]. The order of effectiveness in our study was PP≥PM>P.

The antioxidant potency in vitamin B_6 compounds has been suggested due to the presence of –OH and $–NH_2$ groups and the pyridine ring, because groups such as hydroxyls and amines can scavenge oxygen radicals [44]. PM has been shown to inhibit the accumulation of $^{\bullet}OH$ by the Fenton reaction [45]. The PM structural analog 3-hydroxypyridine was as effective as PM at inhibiting $^{\bullet}OH$ accumulation, indicating that the phenolic hydroxyl ring substituent of PM is sufficient for this activity [46].

The present study showed that $O_2^{\bullet -}$ level increase by H_2O_2 was due to NADPH-oxidase activation. In the cells preincubated with 1 mM of PP, the NADPH-oxidase activity remained highly similar to the cells treated with H_2O_2 only, which suggests that PP reduced the $O_2^{\bullet -}$ level by direct interaction with $O_2^{\bullet -}$. In the cells preincubated with 1 mM of PM or P, the NADPH-oxidase activity was inhibited compared to the cells treated with H_2O_2 only, but that inhibition did not reach significance. This may indicate that PM and P decrease the $O_2^{\bullet -}$ partly by regulating the enzyme, especially since P [42] and PM [24] have been shown to not directly interact with $O_2^{\bullet -}$, but can nonetheless scavenge the hydroxyl radicals.

It is well known that inactivation of NO by a superoxide generates a peroxynitrite ($ONOO^-$) radical [47,48] that interferes with arachidonic acid metabolism and inhibits PGI_2 formation through inac-

tivation of PGI_2 synthase [49]. Therefore, one would expect that activation of NADPH-oxidase by H_2O_2 would increase the $O_2^{\bullet-}$, which then interacts with endothelial NO and produces peroxynitrite, inhibiting the PGI_2 formation by EC through a nitration mechanism [49]. Therefore, the significant decrease of $O_2^{\bullet-}$ by PM, P, or PP should be expected to prevent the inhibition of PGI_2 synthesis by H_2O_2. In contrast, the PGI_2 formation was inhibited by more than 90 % in all the cells exposed to H_2O_2 without or with preincubation with PM, P, or PP.

Previous studies have shown that EC exposed to H_2O_2 underwent potently blocked PGI_2 formation, and that the maximum inhibition occurred within 1 minute after H_2O_2 exposure due to the inhibition of cyclooxygenase [50]. In the same study, a similar inhibition of PGI_2 formation was observed when EC were incubated with the superoxide-generating system, xanthine plus xanthine oxidase. In both conditions the damaging species appeared to be H_2O_2 and not superoxide itself, in that catalase, but not superoxide dismutase (SOD), was protective [50]. In another study H_2O_2 inhibited the cyclooxygenase activity in the EC and the effect was prevented by catalase, not SOD, indicating that H_2O_2 was responsible for that inhibition, not the superoxide [39]. In that regard, it has been shown that cyclooxygenase loses activity after exposure to H_2O_2 or lipid peroxides [51].

The above information explains why, despite a significant decrease of the $O_2^{\bullet-}$ and lipid peroxides (TBARS) in the cells preincubated with PM, P, or PP, their PGI_2 synthesis remained very low because the inhibition of PGI_2 formation is caused by H_2O_2, not by $O_2^{\bullet-}$ or lipid peroxides.

H_2O_2 is an uncharged molecule, relatively longer-lived and freely diffusible, which differs from $O_2^{\bullet-}$ which is charged, hardly permeable, and extremely short-lived [52]. In EC H_2O_2 can cross membranes almost as readily as can water [53]. The present study showed that the H_2O_2 concentration in the medium was rapidly decreased more than 90 % during the first 20 minutes after H_2O_2 exposure, with a small increase of H_2O_2 in the cells, indicating that H_2O_2 was rapidly taken up by the cells and degraded. We also noticed that the increase of $O_2^{\bullet-}$ and lipid peroxides in the cells exposed to H_2O_2 was maintained for at least two hours after the exposure, at the time when H_2O_2 was the lowest in the medium.

Therefore, H_2O_2 has two different effects on EC; 1) an effect that includes the inhibition of PGI_2 formation due to cyclooxygenase inhibition, which occurs within one minute from the exposure to H_2O_2 [50], at the time when H_2O_2 is at the maximum concentrations. This effect was due to H_2O_2 not due to $O_2^{\bullet-}$ because previous studies showed that catalase was able to prevent it, but not SOD [39,50]. Furthermore, 2) another effect included the increase of $O_2^{\bullet-}$ and lipid peroxide, which was maintained for at least 2 hours after H_2O_2 exposure, at a point at which H_2O_2 concentration was the lowest and caused by NADPH-oxidase activation, free-radical generation, and chain reaction. It is obvious that PM, PP, and P were able to reduce the levels of $O_2^{\bullet-}$ and TBARS in the cells but did not prevent the effect of H_2O_2 itself.

Under our experimental conditions, the percentage of injured EC as indicated by measuring the ^{51}Cr release was 6 % after exposure to 0.5 mM H_2O_2 for 2 hours. This is comparable to others who reported that EC exposed to 1 mM of H_2O_2 for 6 hours induced 5 % injury as measured by ^{51}Cr release, while after incubation with 2 mM H_2O_2 for 2 hours, cell injury was not observed [39]. Another study found that EC exposed to 1 mM H_2O_2 did not significantly increase ^{51}Cr release until after 60 minutes [50].

In conclusion, we have shown that PM, P, and PP were effective as antioxidants and reduced the levels of $O_2^{\bullet-}$ and lipid peroxides induced by H_2O_2 in the EC, but did not prevent the inhibition of PGI_2 formation caused by H_2O_2. Based on our findings we believe that PM and PP can be used as efficient antioxidants in conditions that enhance NADPH-oxidase activity and increase vascular superoxide generation, such as high-glucose [54,55] and high-angiotensin conditions [56,57].

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