Original Communication

Thiamine pyrophosphate diminishes nitric oxide synthesis in endothelial cells

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Abstract: Although thiamine pyrophosphate (TPP) is considered a protective agent for endothelial cells, it is still unknown if this is associated with nitric oxide (NO) synthesis. Our aim was to evaluate the synthesis of NO in endothelial cells incubated with TPP and high glucose concentrations. Endothelial cells from the umbilical cord vein from newborns (n = 20), were incubated with 5, 15 or 30 mmol/L glucose, in absence or presence of 0.625 mg/ml of TPP. Our results showed a significant increase in cell proliferation (>40%; P < 0.05), and cell viability (>90%; P < 0.001) after 48 h in endothelial cells cultured with glucose plus TPP. Likewise, in the presence of glucose and TPP an important rise in the consumption of glucose by the endothelial cells was observed after 24 h (>7%; P < 0.001) and 48 h (>10%; P < 0.05). Additionally, the levels of lactate after incubation with glucose and TPP showed only slight variations after 48 h (P < 0.05). However, these changes were clearly different from those observed in the absence of TPP. Interestingly, we found that the changes mentioned were linked with reduced levels of nitrites both at 24 h (< 171 pmol/µg protein; P < 0.001), and 48 h (< 250 pmol/µg protein; P < 0.05), which was associated with a reduced expression of mRNA of eNOS in endothelial cells incubated with TPP and high glucose. In conclusion, the presence of TPP regulates the consumption of glucose and the synthesis of NO, which would explain its protective effect in the endothelium of diabetic patients.

Keywords: Thiamine, thiamine pyrophosphate, endothelial cell, diabetes mellitus

Introduction

Thiamine (vitamin B1) which was identified in 1926 by Jansen et al. [1], plays roles as coenzyme and non-coenzyme within the body [2]. Inadequate levels of thiamine have been associated with impaired oxidative and energy metabolism, which can lead to neurological damage or death in severe cases [3]. Its active form, thiamine pyrophosphate (TPP) intervenes at different stages of the anabolic and catabolic metabolism [4, 5]. In particular, TPP is very important to the metabolism of glucose, since it is involved in the glycolysis, Krebs cycle and pentose-phosphate cycle [2, 4, 5]. Recently, the role of both thiamine and TPP in critically ill patients or in metabolic diseases has been re-evaluated [2, 3]. In patients with diabetes mellitus, it was reported that thiamine or TPP levels are diminished, a fact that was associated with a diminished activity of the transketolase enzyme due to the reduced levels of TPP [6, 7]. In comparison, it has been reported that thiamine supplementation for six months has numerous benefits for the diabetic patient since it improves the lipid profile, and creatinine and thiamine serum levels [8]. Therefore, the utility of continuous thiamine supplementation to the diabetic patient has been suggested [7]. It is relevant to mention, that these beneficial effects could derive from an increase in the TPP levels, as it was observed in healthy subjects that received infusions of thiamine [8, 9].

In the patient with diabetes mellitus, the endothelial cell is an important target of the damage induced by the increase in glucose levels [10, 11]. Previous evidences have shown that high glucose impaired the synthesis of nitric oxide (NO), an important mediator produced by endothelial cell [12]. Some reports have shown that thiamine corrects the delayed replication, and diminishes the synthesis of lactate and of advanced glycation end products (AGE products) in endothelial cell incubated with high glucose [13]. Additionally, it has been reported that thiamine inhibits the endothelial activation as well as negative effects of hyperglycemia on endothelial cell migration [14]. On the other hand, Gioda et al. [15], after evaluating the effects of thiamine deficiency on NO synthesis, found that an insufficient quantity of this vitamin diminishes the NO synthesis leading to decreased vasorelaxation of rat aorta.
Thiamine pyrophosphate (TPP) is an essential cofactor for three enzymes essential for glucose metabolism: the transketolase (TK), α-ketoglutarate dehydrogenase (αKDH), and pyruvate dehydrogenase (PDH) [16], therefore its use is considered as beneficial. However, and in spite of these evidences, information about the effects of TPP on the NO synthesis by the endothelial cell is scarce. Recently, it has been reported that TPP significantly reduced the degree of hyperglycemia-induced retinopathy in rats treated with alloxan, as well as the retinal sugar-induced neovascularization in Wistar rats, in addition to having the capability to inhibit the formation of AGE products [17–19]. TPP relevance in cellular metabolism, as well as its protective effect in retinopathy, suggests that this coenzyme may play a role as a protective agent in the endothelium. Therefore, the objective of this work was to evaluate the effect of TPP on the NO synthesis in endothelial cells obtained from the umbilical cord (HUVECs) vein and cultured in high glucose conditions.

### Material and methods

#### Isolation and culture of human umbilical vein endothelial cells (HUVECs)

Umbilical cords were obtained from newborn (n = 20) with young non-diabetic mothers (20–25 years old), without a family history of type 2 diabetes, nor gestational diabetes at the time of delivery (Table 1). Human umbilical vein endothelial cells (HUVECs) were isolated of each cord using type II collagenase (0.2 mg/mL, Gibco/BRL, Grand Island, NY, USA), these were cultured individually in T-75 culture plates with M-199 medium (Gibco/BRL), which contained 5 mmol/L glucose (medium control), and supplemented with 20% Fetal Bovine Serum (FBS) (HyClone, Logan, UT, USA), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 37.5 μg/mL of endothelial cell growth factor (Roche, Mannheim, Germany), at 37 °C in a humid atmosphere containing 7% CO2 [20]. In some cultures, the M-199 medium was complemented with additional glucose to obtain a final glucose concentration of 15 or 30 mmol/L (experimental medium), and with or without 0.625 mg/ml of TPP (gift from Investigaciones Filosóficas y Científicas, S.A de C.V., HYPATIA, Mexico). HUVECs were used within passages 5–10 and identified as endothelial by its positive stain to CD105 surface antigen (BD Pharmingen, San Diego, California, USA), and by their morphology. This protocol was approved by the Institutional Scientific and Bioethics Committees of the National Institute of Respiratory Diseases “Ismael Cosío Villegas”. All patients signed a written informed consent.

#### Cell proliferation and cell viability

Isolated HUVECs (5 × 10³ cells) were cultured for 24 h in 96-multiwell tissue culture plates with M-199 culture medium (without phenol red). After this period, the medium was discarded and 100 μL of fresh medium with 5, 15 or 30 mmol/L of glucose and with or without TPP were added to the cultures for a further 24 or 48 h cultivation period. After this time, the plates were washed with saline phosphate buffer (PBS) (PBS: 0.01 M NaH₂PO₄ • H₂O, 0.01 M NaHPO₄, 0.15 M NaCl, pH 7.2) and stained with 100 μL from 0.1% crystal violet solution (w/v) (Sigma, St. Louis, MO, USA) dissolved in 70% ethanol for 20 min. Later, the plates were washed with deionized water and air-dried. The bound dye was dissolved with 100 μL of a 10% acetic acid. Optical density was measured at 595 nm in a 96-multiwell-plate spectrophotometer (Biotek Instruments, Inc. Highland Park, Winooski, VT, USA). Crystal violet stain allows the evaluation of cellular proliferation in relation to the culture time [20]. Additionally, to evaluate cell viability we determined mitochondrial activity. Different cultures of endothelial cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL, Sigma) dissolved in sterile PBS [20]. Endothelial cells were incubated during the times mentioned with medium alone or supplemented with either 15 or 30 mmol/L of glucose, and with or without TPP. Cells were stained with 20 μL of PBS-MTT for the last 4 h of the culture period. The MTT reduced end-product, formazan, was dissolved with 100 μL of 0.04 N-HCl in isopropyl alcohol, and the absorbance was recorded at 570 nm.

#### Determination of glucose consumption, and levels of lactate and nitrites

HUVECs (10 × 10³) were cultured in 96-multiwell tissue culture plates with M-199 culture medium without phenol...
red, with 5, 15 or 30 mmol/L of glucose and with or without TPP for further 24 or 48 h. Glucose consumption was determined in the culture medium before and after incubating the cells during the times mentioned using Amplex® red glucose/glucose oxidase assay kit (Molecular Probes, Eugene, OR, USA), and the difference in the culture medium was determined. The levels of lactate were obtained using a Lactate reagent (Sigma, St. Louis, MO, USA) after growing HUVECs in our experimental conditions. All results were normalized and expressed as μg Glc/μg of protein, and μg lactate/μg of protein, which was determined by the micro Lowry method. To evaluate the synthesis of nitric oxide (NO), the levels of nitrites in our cultures were determined. Before determining the concentration of nitrites in the culture medium using the Griess reagent (Sigma), the culture media were incubated with nitrate reductase. Later, 100 μL of the culture medium obtained from the different experimental conditions were mixed with 100 μL of the reagent and incubated for 15 min. Afterwards, the absorbance was evaluated at 540 nm in a 96-multiwell plate spectrophotometer (BIO-TEK, Winooski, VT, USA). The concentration of nitrites was obtained by comparison with a standard curve of sodium nitrite [21]. The results are expressed as pmol of nitrites/μg of protein.

RT-PCR for endothelial nitric oxide synthase

Total RNA was extracted from 1 × 10⁶ HUVECs cells incubated for 48 h in the glucose concentrations previously mentioned, and in presence or absence of TPP, using 1 mL of Trizol (Life Technologies, USA). The RNA pellet was re-suspended in diethyl pyrocarbonate-treated water and its concentration was determined in a Lambda 18 Spectrophotometer (Perkin Elmer, Germany) at 260 nm. RNA (1 μg) was mixed with 1 μL of oligo-d[T], 4 μL of 5 × reverse transcriptase first strand buffer, and 10 μL of diethyl pyrocarbonate-treated water. Reverse transcription was performed using 20 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The RT mixture was incubated at 42 °C for 50 min and then at 70 °C for 15 min. The resulting cDNA was amplified by PCR. The primers for eNOS were 5'-CCA GCT AGC CAA AGT CAC CAT-3' (upstream) and 5'-GTC TCG GAG CCA TAC AGG ATT-3' (downstream), the amplified product was of 350 pb [21]. The primers for β-actin, used as internal control, were 5'-CGT TCA CCT TGA GCC CAT T-3' (upstream) and 5'-TCC AAG GGT CCG CTG CAG GTC-3' (downstream), with a final amplified product of 230 pb [21]. The conditions for PCR were 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 2 min (extension) in a P2 Thermal Cycler (Thermo Electron Corporation, USA). After 35 cycles, PCR products were detected by electrophoresis on 2% agarose gel and stained with ethidium bromide. Each band was analyzed and its optical density was determined in an UVP image analyzer (Ultraviolet Products, CA, USA), and finally the ratio of eNOS/beta actin mRNA expression was obtained.

Statistical analysis

For the analysis of the data, the mean and standard deviation were used as summary measures. To determine the type of distribution, the Shapiro-Wilk test and the Levene’s test were used to verify the homogeneity of variances. For the analysis between more than two groups we use the Kruskal-Wallis test and the Mann-Whitney U test between two groups. The level of significance for the P value was < 0.05. The statistical analysis was made using software Stata 13.

Results

Cell proliferation and cell viability

First, we evaluated the effect of different concentrations of TPP on proliferation and cell viability of HUVEC cells (Figure 1). Results showed that cell viability (using MTT), was significantly modified in the presence of 2.5 and 5 mg/mL of TPP (P < 0.05 and P < 0.0001, respectively). In comparison, the cell proliferation (crystal violet) was affected only in the presence of 5 mg/mL of TPP (P < 0.001). Based on these results, we decided to use 0.625 mg/mL in all experiments.

It has been established that high glucose affects the metabolism, proliferation, and migration of endothelial cell [13, 14]. Therefore, we consider important to evaluate the viability and cell proliferation of endothelial cells incubated with 5, 15 and 30 mmol/L of glucose, in absence or presence of 0.625 mg/mL of TPP (Figure 2). Our results showed a minimal increase in the cell viability percentage after 24 h of culture with glucose only, an increase that was significant when compared with the results observed in endothelial cells incubated with 5 mmol/L of glucose (P < 0.05; Figure 2A). A similar response was observed in HUVECs after 48 h of culture with glucose only, our results showed an increase in the cell viability of 98.7 ± 7.6 in the presence of 15 mmol/L, and of 87 ± 12.7% in the presence of 30 mmol of glucose (P < 0.001) (Figure 2A). However, the presence of 0.625 mg/mL of TPP plus 15 or 30 mmol/L of glucose induces a drastic increase of 88.4 ± 6.4 and 83.3 ± 19.2% in the cell viability in HUVEC cells after 24 h of culture (P < 0.001; Figure 2B). In addition, the incubation of endothelial cells with glucose plus TPP during 48 h showed
the next results: in 15 mmol of glucose the increase was of 90 ± 12.7%, while that of the incubation with 30 mmol of glucose showed a rise of 91.3 ± 12.2% (P < 0.001; Figure 2B). The results for proliferation after 24 h in endothelial cells incubated with glucose only, showed an increase of 21.7 ± 7.3% in the cell proliferation in presence of 15 mmol/L of glucose, while culturing with 30 mmol/L glucose induced an increase of 13.8 ± 5.5% (P < 0.05; Figure 2C). Results showed that after incubation during 48 h with 15 mmol of glucose an increase of 74 ± 4.4% was observed; in comparison, this rise was of 86.5 ± 2.6% in the presence of 30 mmol/L glucose (P < 0.001; Figure 2C). Meanwhile, the incubation with glucose plus TPP during 48 h induced an increase in the cell proliferation in the presence of 15 mmol (49.6 ± 18.9%), and 30 mmol of glucose (46.1 ± 17.6%). Statistical analysis showed significant differences in comparison with endothelial cells incubated with 5 mmol glucose (P < 0.05; Figure 2D). Here, it is relevant to mention that incubation with glucose plus TPP for 48 h, was also associated with an increase in cell proliferation. Our results showed an increase of 49.4 ± 8.9% in HUVEC cells incubated with 15 mmol glucose plus TPP; while the endothelial cells incubated with 30 mmol glucose plus TPP showed a rise of 44.3 ± 1.1% of cell proliferation (P < 0.05; Figure 2D).

**Determination of glucose consumption, and levels of lactate and nitrites**

In previous reports, an increase in the levels of thiamine pyrophosphate (TPP) in healthy subjects that received infusions of thiamine has been observed; and also, it is well known that TPP is an essential cofactor for three enzymes essential for glucose metabolism [8, 9, 16]. Therefore, we considered important to determinate the effect of TPP in the consumption of glucose, as well as on the levels of lactate and nitric oxide which are associated with the metabolism of glucose. Determination of glucose uptake by HUVEC cells after 24 h of culture was 8.05 ± 1.7 μg; and 31.5 ± 3.5 μg, and 45.7 ± 9.8 μg Glc/μg of protein in the presence of 5 mmol, 15 mmol and 30 mmol/L of glucose, respectively (P < 0.0001, this after comparing with the values of cells incubated with 5 mmol of glucose). In comparison, the uptake of glucose was 12.3 ± 0.9 μg; 25.2 ± 3.1 μg, and 50.7 ± 5.6 μg Glc/μg of protein after 48 h culture of endothelial cells with 5 mmol, 15 mmol and 30 mmol/L of glucose (P < 0.0001) (Figure 3A). The incubation during 24 h of HUVECs with glucose plus TPP showed that the intake of glucose was 7.9 ± 1 μg; 14.9 ± 3.2 μg, and 21.7 ± 6.7 μg Glc/μg of protein after incubation with 5, 15 and 30 mmol/L of glucose (P < 0.001). Results obtained after 48 h of culture with glucose (5, 15 and 30 mmol/L) plus TPP showed that these were 13.6 ± 2.1 μg; 15.2 ± 1.8 μg, and 19.7 ± 8.9 μg Glc/μg of protein (P < 0.05) (Figure 3B). After comparing results in the absence or presence of TPP, a diminution in the consumption of glucose in the cultures where the TPP was present both at 24 h and at 48 hours was observed.

About the levels of lactate these did not show drastic changes, however, they were significantly different in some conditions. After 24 h, the levels of lactate showed significant differences between 5 and 30 mmol of glucose vs 15 mmol of glucose (P < 0.05). The incubation for 48 h only showed difference between 15 and 30 mmol/L of glucose (P < 0.01) (Figure 3C). Interestingly, the presence of TPP was not associated with changes in the levels of lactate after 24 h (P > 0.05); and although slight changes after 48 h of culture with glucose plus TPP were observed, this was only observed between the cultures with 15 and 30 mmol/L glucose (P < 0.05) (Figure 3D).
In relation to nitrites levels, they showed an increase linked to glucose concentration. After 24 h of culture in the presence of glucose, our results showed significant differences between 5 mmol vs 15 mmol of Glc (P < 0.05), but not when compared to 30 mmol glucose. These changes in the levels of nitrites were noticeable after 48 h of culture; the levels determined were 298.7 ± 41.5 pmol for 5 mmol of glucose, and were different in comparison to the values obtained for 15 mmol (782.5 ± 20.5 pmol) (P < 0.001), and 30 mmol of Glc (580.7 ± 18.6 pmol) (P < 0.003) (Figure 4A).

Here, it is very important to highlight that the presence of TPP had an inhibitory effect on the levels of nitrites, which was associated moreover with reduced expression of the mRNA of endothelial nitric oxide synthase (eNOS). The levels of nitrites after 24 h of culture with glucose plus TPP were 171 ± 81.1 ± 10, and 80.2 ± 9 pmol nitrites/μg of protein in the presence of 5, 15 and 30 mmol of glucose, respectively (P < 0.001). Although an increase in the levels of nitrites was observed after 48 h of culture, these increases were lower than those observed in the cultures without TPP. In the cultures with 5 mmol of Glc plus TPP the levels of nitrites were 265 ± 50 pmol; with 15 mmol of Glc they were 360 ± 20 pmol, and finally with 30 mmol of glucose they were 350 ± 75 pmol of nitrites per μg of protein (P < 0.05) (Figure 4B).

Although some evidences suggest that TPP has the capability to protect the endothelium of side effects derived from high glucose, a fact probably linked with the synthesis of NO [17–19], the reality is that the mechanism associated with these facts is still unknown. Therefore, we evaluated after 48 h of culture the levels of expression of eNOS mRNA, taking into consideration that in our study the effects of TPP were observed principally at this time. Our results showed similar expression levels after 48 h of
culture, in HUVECs cultured with different glucose concentration only (Figure 4C), however and interestingly, the levels of expression of eNOS mRNA diminished in the presence of TPP, but moreover, this event was principally linked to the high glucose concentrations (Figure 4D).

**Discussion**

Thiamine pyrophosphate (TPP, the active form of thiamine) has an important role in both cellular and glucose metabolism since it intervenes in glycolysis, Krebs cycle and pentose-phosphate cycle [2-5]. Our results showed that 0.625 mg/ml of TPP in the presence of 5 mmol/L of glucose has no effect on viability or proliferation of endothelial cells. However, the incubation of endothelial cells with TPP plus a high glucose concentration resulted in an increase in viability and cellular proliferation. Previous reports showed that deficiency of thiamine inhibits the mitochondrial activity in brain endothelial cell through its action on both pyruvate dehydrogenase complex and α-ketoglutarate mitochondrial complex [22]. Therefore, in our study the presence of TPP could be inducing an increase in the cell proliferation, through its effect on this mitochondrial complex in the endothelial cells incubated with high glucose concentrations. Probably, our results are similar to those previously observed in endothelial cells incubated with high glucose and thiamine [13]. It is important to mention, that these results can be very relevant

![Figure 3](https://econtent.hogrefe.com/doi/pdf/10.1024/0300-9831/a000650)
since it has been reported that high glucose \textit{per se}, inhibits the cell proliferation and induces the apoptosis of endothelial cells [20, 22, 23]. Consequently, the increase in glucose levels has been considered as the initial event to damage endothelial cells.

Our results showed that consumption of glucose was directly proportional to its concentration. Interestingly, the presence of TPP diminished this effect. Previous reports showed that a deficient diet or the inhibition of thiamine transport using pyrithiamine stimulates glucose consumption, as well as a rise in lactate levels [22]. However, in our study, we observed a minor consumption of glucose, as well as a diminution in the lactate levels after culturing cells with the coenzyme. These results could be explained by the effect of the TPP on the metabolism of endothelial cells, especially at the mitochondrial level associated with a better utilization of glucose [24, 25]. Results obtained in animal models showed that high doses of thiamine inhibit...
the hexosamine pathway, improve cell proliferation and decrease the lactate levels [25]. Additionally, alternate pathways of glucose metabolism as for instance the pentose pathway are activated in the presence of TPP; the glucose levels and percentage of glycated HbA1c are reduced also after a short treatment with thiamine [25]. Likewise, the development of lactic acidosis in the absence of thiamine has been reported [26], which is rapidly reversed in critically ill patients treated with thiamine [27]. Altogether, these results support the important role of the of thiamine active form in stimulating cellular and bodily metabolisms. Here, it is important to remember that we used TPP, which directly intervenes with different pathways of cellular metabolism, and that it does no longer needed any further phosphorylation to fulfill its function.

The endothelial cell is one of the main sites damaged by high glucose concentrations [10, 11], and, as a side effect, induces the impaired synthesis of nitric oxide (NO) [11, 12]. Gioda et al. [15], reported that a thiamine deficiency was linked with a deficient synthesis of NO, however, they did not determine the mechanism implicated in this alteration. In our work, we found that high glucose levels induce a significant increase in NO; in comparison, the presence of the coenzyme reduces them. Currently, the evidences regarding the effect of higher glucose levels on NO synthesis are divergent. Some evidences reported a deficient synthesis of NO [12], while other described that increased glucose levels initially augment drastically the NO synthesis [28]. The results of our work showed a strong increase in the levels of NO (nitrites) associated with glucose concentration and with the time of incubation. However, the expression levels of eNOS transcripts were stable after 48 h of incubation. In comparison, the culture with glucose and TPP for 48 h had as an effect a reduced nitrites synthesis which was linked with a lower expression of eNOS mRNA. Although more experiments are still necessary to find out the mechanism that explains the differences observed in the synthesis of nitrites and transcripts of eNOS, our results suggest the importance of TPP in activating cellular metabolism therefore stimulating NO synthesis. In this regard, a similar association between nitrites levels with the expression of eNOS mRNA in the presence of high glucose has been reported [28]. Likewise, some works show that using benfotiamine (synthetic analogous of thiamine) favors the glucose metabolism, and the activation of eNOS which increases the levels of NO, avoiding consequently the endothelial dysfunction [25, 29].

Although, our work has some limitations as the number of samples evaluated, they were carefully selected especially in relation to their family history, since the relevance of the history of diabetes or hypertension for the endothelium response has been reported [20, 21]. Additionally, it is important to mention that each sample of HUVEC was cultured individually, this fact is important since it has been reported that by using pools of HUVECs the response of endothelial cell is modified [30]. This fact gives additional support to our conclusions. Also, it is important to mention that HUVECs are more sensitive to changes in the culture conditions than other cell types therefore they were used only between the passes 5 to 10 a fact that unfortunately limits the number of questions what we can answer with these cell cultures.

Conclusions

Our results support the importance of TPP in cellular metabolism activation, which was associated with a regulation of eNOS mRNA expression, as well as with the synthesis of NO. These results, allow us to suggest that this is the mechanism activated by TPP, which inhibits the development of endothelial dysfunction. However, more experiments are still necessary to determine the mechanisms associated with this activation of endothelial nitric oxide synthase in the presence of TPP.

References


History
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Conflict of interest
The authors declare that there are no conflicts of interest.

Ethical approval
This protocol was approved by the Institutional Scientific and Bioethics Committees of the National Institute of Respiratory Diseases “Ismael Cosío Villegas”. All patients signed a written informed consent.

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