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Vitamin B12 does not increase cell viability after hydrogen peroxide induced damage in mouse kidney proximal tubular cells and brain endothelial cells

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Abstract

Vitamin B12 (B12) is an essential co-factor for two enzymes in mammalian metabolism and can also act as a mimetic of superoxide dismutase (SOD) converting superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). High oral dose B12 decreases renal $O_2^{\bullet-}$ and post-ischemia/reperfusion injury in mice and protects against damage induced by hypoxia/reperfusion in mouse kidney proximal tubular cells (BU.MPT). $O_2^{\bullet-}$ is unstable and rapidly converted to H_2O_2. H_2O_2 mediates oxidative stress associated with $O_2^{\bullet-}$. Whether B12 protects against damage induced by H_2O_2 is unknown. Both BU.MPT cells and mouse brain endothelial cells (bEdn.3) were applied to test the effects of B12 on H_2O_2-induced cytotoxicity. Both types of cells were treated with different doses of H_2O_2 with or without different doses of B12. Cell viability was analyzed 24 h later. H_2O_2 caused cell death only at a very high dose, and high pharmacological dose of B12 did not prevent this detrimental effect in either cell type. In bEnd.3 cells, transcriptional levels of heme oxygenase-1 (HO-1) increased, while nuclear factor erythroid 2-related factor 2 (Nrf2) decreased by H_2O_2. The levels of transcripts were not affected by the B12 treatment. We conclude that the cytotoxic effects of exogenous H_2O_2 in BU.MPT and bEdn.3 cells are not prevented by B12.

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Disclosures

None.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Azraa Ayesha: Investigation. **Edward M Bahnson:** Data curation, Formal analysis, Writing – review & editing. **Yukako Kayashima:** Investigation, Data curation, Formal analysis, Writing – review & editing. **Jennifer Wilder:** Investigation, Data curation, Formal analysis, Writing – review & editing. **Phillip K Huynh:** Investigation, Writing – review & editing. **Sylvia Hiller:** Investigation. **Nobuyo Maeda-Smithies:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Feng Li:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing.

Keywords

Vitamin B12; Hydrogen peroxide; BU.MPT cells; bEdn.3 cells; Cell viability

Introduction

At a normal physiological range (approximately 1–10 nM), hydrogen peroxide (H_2O_2) plays an important role in intracellular signaling as a mediator of multiple physiological processes including cell differentiation and proliferation, cellular metabolism, and survival [1]. In addition, H_2O_2 produced by immune cells has an anti-virulence feature through disrupting the tyrosine phosphorylation network in pathogens [2].

In both human and mice, one of the main processes producing H_2O_2 is the dismutation of superoxide ($O_2^{\bullet-}$) catalyzed by superoxide dismutases (SOD) ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) [3]. Superoxide can be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) located in the plasma membrane or phagosomes [4], through complex I and III of the respiratory chain in the mitochondria [5], and cytochrome P450-monooxygenases in the endoplasmic reticulum (ER) [6]. SOD1 (Cu/Zn-SOD) is present in the cytoplasm, SOD2 (Mn-SOD) is within the mitochondria, and SOD3 (Cu/Zn-SOD) is present in extracellular space. H_2O_2 can cross the cellular membrane through the chloride channels and specific aquaporins (AQP3 and AQP8) [7].

In order to maintain the intracellular homeostatic concentration ranges of H_2O_2, there is a well-orchestrated balance between formation and removal of H_2O_2. There are three major types of enzymes to defend against H_2O_2 : (1) Catalase (CAT); [8] (2) glutathione peroxidases (GPXs); [9] (3) peroxiredoxins (PRXs), reducing H_2O_2 and lipid hydroperoxides at the expense of thioredoxin [10].

If the delicate balance is disturbed by either excess production of H_2O_2 and/or impaired defense mechanisms leading to an increase in H_2O_2 concentration, it could act as a damaging oxidant causing cell growth arrest and cell death. H_2O_2 has been shown to induce apoptosis of cultured rat mesangial cells [11] and act as one of the mediators in cisplatin-induced apoptosis in primary cultures of mouse proximal tubule cells [12]. Takeda et al. further reported that H_2O_2 induced necrosis, apoptosis, oncosis and apoptotic oncosis of mouse proximal tubule cells [13]. The detrimental effects of H_2O_2 are also observed in endothelial cells [14,15] Accordingly, it is important to explore compounds that protect cellular functions against H_2O_2 -induced cellular damage.

Very recently, we have demonstrated that vitamin B12 (B12 – cyanocobalamin) at a high dose protects from post-ischemia/reperfusion injury in mice and in immortalized mouse kidney proximal tubular cells (BU.MPT) in culture [16]. In ischemia/reperfusion kidneys B12 treatment limits the increase of $O_2^{\bullet-}$ concentration, increases mRNA levels of *Sod1*, *Sod2* and *Gpx1*, while decreasing *Nox2* mRNA levels. Excess $O_2^{\bullet-}$ plays an important role in damage caused by ischemia/reperfusion. During the ischemic phase, the absence of oxygen leads to the accumulation of metabolic intermediates. During reperfusion, these metabolic intermediates react with oxygen to produce a sudden increase in oxygen radicals

and H_2O_2, resulting in the uncontrolled oxidation of cellular components [17]. B12 is SOD mimetic with a reaction rate close to that of SOD [18]. Additionally, the reduced form of B12, cob(II)alamin is capable of directly reacting with H_2O_2, though at a much slower rate [18]. However, whether B12 can directly protect against H_2O_2-induced damage is unknown. Therefore, in the current study, we investigated whether B12 protects against cell death induced by exogenously supplied H_2O_2 in mouse proximal tubular cells and brain endothelial cells. Our results demonstrate that B12 has no beneficial effect either in the BU.MPT cells or in brain endothelial cells (bEdn.3, CRL2299) treated with H_2O_2 at the concentrations tested.

Materials and methods

BU. MPT cell culture

BU.MPT cells, a conditionally immortalized mouse kidney proximal tubular epithelial cell (PTEC) line, was derived from a transgenic mouse bearing a temperature-sensitive (ts) mutation (tsA58) of the SV40 large tumor antigen (TAg) under the control of the mouse major histocompatibility complex (MHC) H-2Kb class I promoter [19]. Cells were maintained at 37 °C with humidified 5% (v/v) CO2 atmosphere in the presence of interferon-γ (IFN-γ) and 100 U/mL penicillin/streptomycin in Dulbecco's Modified Eagle Medium (DMEM) to enable stable expression of the tsA58-mutated TAg transgene. For experiments, cells were grown to ≥90% confluence at 39 °C, in the absence of IFN-γ. Under this condition, expression of the tsA58-mutated TAg transgene is inhibited by >95%, and BU.MPT cells behave like primary cultures of mouse kidney PTEC as described elsewhere [19]. At confluence, cells were starved for approximately 18 h without fetal bovine serum (FBS). Cells were then enrolled into different treatment groups. After 24 h with/without different doses of H_2O_2 and of B12 in FBS-free medium, medium and cells were collected for analysis. The doses of B12 used here was based on our previous study [16], and doses of H_2O_2 was in the range based on the study published by other investigators [13,15]

Endothelial cell culture

Mouse brain microvascular endothelial cells (the bEnd.3, ATCC® CRL2299, Gaithersburg, MD, United States) were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin at 37 °C and 5% CO2 in a humidified incubator. At confluence, cells were starved for 18 h without FBS. Cells were then enrolled into different treatment groups. After 24 h, medium and cells were collected for analysis.

Cell viability assay

BU.MPT or bEdn.3 cells were seeded in separate transparent 96-well (Falcon, 35307) plates at 1×10^4 cells per well in 100 μL of respective media and allowed to attach overnight. After confluence, cells were starved with 0% FBS for approximatly18 h (approximately average of 5.5×10^4 cells for BU.MPT cells and 5.0×10^4 cells for bEdn.3 cells). Media were then discarded, and cells were rinsed and replaced with 100 μL of fresh FBS-free media added with the different concentrations of H_2O_2 and/or B12. After 24 h treatment, CCK-8 solution (Sigma, #96992) was added to each well (final 1/10 dilution) and incubated for 1 h. The absorbance was measured at 450 nm using a microplate reader (SpectraMax M5 Microplate

Reader, Molecular Devices). The percent cell viability in individual treatment groups was determined from the optical density (OD) relative to the OD of control cells, whose mean was set as one. Experiments were repeated twice with each experimental group consisting of 6–8 replicates.

Quantitative RT-PCR

bEdn.3 cells were cultured in 100 mm plates. After confluence, cells were starved for approximately 18 h (approximately average of 5×10^6 cells) were enrolled into four groups: (1) control: without any treatment; (2) control + B12: treated with 10 mL of 5 μM B12 (equivalent to 0.1 nmole/10^4 cells); (3) H_2O_2: treated with 10 mL of 0.8 mM H_2O_2 (0.16 μmole/10^4 cells); and (4) H_2O_2 + B12: treated with 10 mL of 5 μM B12 plus 0.8 mM H_2O_2. After 24 h treatment, cells were collected and total RNA from cells was extracted using Trizol (Life Technologies, St. Paul, MN) following the manufacturer's instruction. Experiments were repeated twice with each experimental group consisted of 3 replicates. mRNA was quantified with TaqMan real-time quantitative RT-PCR (7500 real time PCR system, Applied Biosystems, Foster City, CA) by using the one-step RT-PCR Kit (Bio Rad, Hercules, CA) with *Hprt* as the reference gene in each reaction. $2^{-\Delta\Delta Ct}$ method was used for comparing the data [16,20,21]. Sequences of primes and probes are shown in Table 1.

Statistical analysis

Data are presented as mean ± SEM. Multifactorial ANOVA test was used to assess statistical significance with the program JMP 15.0 (SAS Institute Inc. Cary, NC). Post hoc analyses were done using the Tukey–Kramer Honest Significant Difference test. A p value less than 0.05 is recognized as statistically significant.

Results

High dose hydrogen peroxide (H_2O_2) has detrimental effects on mouse kidney proximal tubular cells (BU.MPT)

H_2O_2 has been shown to have different effects at different doses; therefore, we tested the effects of different doses of H_2O_2 on BU.MPT cells. First, we tested the effects of H_2O_2 at 0.001, 0.01, 0.1 mM (equivalent to 0.000018, 0.00018, 0.0018 μmole/10^4 cells) because Takeda et al. have reported that H_2O_2 resulted in a time- and dose-dependent decrease in viability in cultured primary mouse proximal tubular cells at doses of 0.05 and 0.1 mM [13]. We found that none of these doses of H_2O_2 reduced viability of BU.MPT cells (Fig. 1A). Instead, viability of H_2O_2 treated cells was slightly higher. Then, we tested the effects of higher doses (1 and 10 mM, equivalent to 0.018, 0.18 μmole/10^4 cells) of H_2O_2. At 10 mM, H_2O_2 significantly decreased the viability of BU.MPT cells; in contrast, 1 mM of H_2O_2 increased cell viability (Fig. 1B).

B12 is not able to protect H_2O_2-induced cell death at a high dose

We have demonstrated that B12 at a dose of 0.3 μM increases cell survival in BU.MPT cells under the hypoxia/reperfusion setting [16]. We, therefore, investigated the role of B12 in H_2O_2-induced cytotoxic effects in BUM.PT cells.

BU.MPT cells were treated with 0, 1 and 10 mM H_2O_2 alone or combined with either 0.5 or 5 μM B12 (equivalent to 0.0091 or 0.091 nmole/10^4 cell) and cell viability was determined. B12 at doses of 0.5 and 5 μM increased the cell viability of control cells approximately two-fold. There is no difference between the effects of 0.5 and 5 μM B12. B12 at doses of 0.5 and 5 μM tended to increase the cell viability of BU.MPT cells treated with 1 mM H_2O_2 but the effects did not reach statistical significance. However, B12 did not increase cell survival of cells treated with 10 mM H_2O_2 (Fig. 2).

In order to determine which dose of H_2O_2 between 1 and 10 mM may cause cell death, another batch of BU.MPT cells were treated with 1, 2, 4, 8, 10 mM H_2O_2 (equivalent to 0.018, 0,036, 0.072, 0.144, 0.18 μmole/10^4 cells). At dose of 2 mM, H_2O_2 increased cell viability. At dose of 4 mM, H_2O_2 decreased cell viability, and H_2O_2 at dose of 8 mM decreased cell viability to a greater extent, which is the same as effects of 10 mM H_2O_2 (Fig. 3A). Next, we tested whether B12 had any beneficial effects on cells treated with 4 mM H_2O_2 by adding B12 2 h before or simultaneously with H_2O_2. In either condition, B12 did not increase cell survival in this treatment regimen (Fig. 3B).

Effects of H_2O_2 at different doses on mouse brain endothelial cells (bEnd.3)

Because H_2O_2 is known to show different effects on different types of cells, and it influences cell survival in mouse brain endothelial cells [15]. We tested the effects of H_2O_2 and B12 on bEnd.3 cells. First, we tested the effects of different doses of H_2O_2 on the viability of bEdn.3 cells. At 0.4 μM (equivalent to 0.008 μmole/10^4 cells), H_2O_2 did not decrease the survival rate of bEnd.3 cells. However, at dose of 0.8, 1.6 mM (equivalent to 0.016, 0.032 μmole/10^4 cells), H_2O_2 markedly decreased the survival rate of bEnd.3 cells. There was no difference between the effects of 0.8 and 1.6 mM of H_2O_2 (Fig. 4).

B12 does not have effects on the cell death induced by H_2O_2 in bEnd.3 cells

We tested whether B12 could protect against damage caused by H_2O_2. bEnd.3 cells treated with 0, 0.4, 0.8 and 1.6 mM H_2O_2 alone or combined with either 0.5 or 5 μM of B12 (equivalent to 0.01 or 0.1 nmole/10^4 cells). B12 at both doses increased cell viability to approximately 1.6x when cells treated with 0 or 0.4 mM H_2O_2. However, B12 did not increase cell viability when cells treated with 0.8 or 1.6 mM H_2O_2 (Fig. 4).

B12 does not affect the transcriptional levels of heme oxygen 1 (HO-1) or nuclear factor erythroid 2-related factor 2 (Nrf2)

HO-1 plays an important role in defensing against oxidative stress and H_2O_2 is shown to increase HO-1 expression about 2x [22]. Consistent with this report, the mRNA levels of *Hmox-1* (coding HO-1) was about 2x higher in cells treated with 0.8 mM H_2O_2 (equivalent to 0.016 μmole/10^4 cells) than those in non-treated cells (0 μmole H_2O_2), and B12 at dose of 5 μM (equivalent to 0.1 nmole/10^4 cells) did not alter the elevated mRNA levels of *Hmox-1* treated with H_2O_2 (Fig. 5A).

Nrf2 is involved in regulating several antioxidant genes including *Hmox-1,* therefore, the mRNA levels of *Nfe2l2* (coding Nrf2) were also determined. The mRNA levels of *Nfe2l2* was about 0.4x in cells treated with 0.8 mM H_2O_2 compared to those in non-treated cells

(0 μmole H_2O_2), and B12 at dose of 5 μM did not prevent the reduction of mRNA levels of *Nfe2l2* in the cells treated with H_2O_2 (Fig. 5B).

Chen et al. have reported that H_2O_2 increased mRNA levels of toll-like receptor 4 (TLR4) in MS1 microvascular endothelial cells [23]. Accordingly, we measured mRNA levels of *Tlr4* and found that neither H_2O_2 nor B12 altered the mRNA levels of *Tlr4* under our experimental condition (Fig. 4C).

Discussion

Among many different types of cells in the kidneys, the proximal tubular cells are most susceptible to ischemia/reperfusion. Previously, we have demonstrated that B12 protects against injury caused by hypoxia/reperfusion in the immortalized mouse proximal tubular cells (BU.MPT). In the current study, we evaluated whether B12 has any protective effects against H_2O_2-induced cell damage in BU.MPT and brain endothelial cells (bEdn.3). We showed that B12 does not protect against cell death induced by H_2O_2 in either cell type, and it does not modify the effect of H_2O_2 on Nrf2 and HO-1 gene expression in bEdn.3 cells at the concentrations tested.

Different types of cells have different sensitivity to H_2O_2. We found that approximately 10x higher dose of H_2O_2 to induce cell death for BU.MPT cells than that for bEdn.3 cells. Anasooya Shaji et al. reported that the similar dose was required to induce cell death of rat brain microvascular endothelial cells [15].

Vitamin B12 (B12 – cyanocobalamin), the last vitamin characterized, is the largest (MW1355) of all vitamins and has several unique features. All cobalamins (vitamin B12 derivatives) contain the rare transition metal, cobalt (Co) positioned in the center of a corrin ring. While cobalamins are synthesized only in certain bacteria and archaea [24], they are co-enzymes essential for all life, except in plants. In higher vertebrates, methyl-cobalamin and 5'-deoxyadenosyl-cobalamin are essential for the function of methionine synthase and methylmalonyl CoA mutase, respectively [25]. Containing a transition metal makes cobalamins capable of participating in redox reactions. Once inside the cell, the oxidation state of the cobalt atom is reduced from Co(III) to Co(II) and/or to Co(I). Reduced forms can readily react with superoxide ($O_2^{\bullet -}$). Importantly, Cob(II)alamin is a highly effective intracellular $O_2^{\bullet -}$ scavenger with a reaction rate close to that of superoxide dismutases (SOD) [18]. Indeed, we have reported that B12 protects against damage induce by ischemia/reperfusion in kidneys in mice by decreasing superoxide [16]. In addition, B12 normalizes the mRNA levels of *Gpx1*, *Sod1 and Sod2* suppressed by ischemia/reperfusion and decreases the mRNA levels of *Nox2* elevated by ischemia/reperfusion [16]. After $O_2^{\bullet -}$ is produced by NOXs and/or mitochondria, it is rapidly converted to H_2O_2 by SODs. Hence, H_2O_2 can mediate many of the effects associated with increased $O_2^{\bullet -}$ levels. B12 converts $O_2^{\bullet -}$ produced by ischemia/reperfusion to H_2O_2, which could in turn increase the level of H_2O_2. On the other hand, B12 also increases Gpx1, which could decrease the level of H_2O_2. Additionally, cob(II)alamin is also capable of reacting with H_2O_2; however, at a much slower rate [18]. Hence it is of interest to assess the possible effect of B12 on H_2O_2-mediated damage. Studies have shown that H_2O_2 has dual functions. Ohguro et al.

have reported that high levels (1 mM) of H_2O_2 killed rabbit lens epithelia cells (TOTL-86) and sublethal levels (0.1mM) suppressed their proliferation. From 1 nM to 1 μM of H_2O_2, there was a dose dependent increase in the cell numbers [26]. In our current study, we treated BU.MPT cells with 4 mM H_2O_2, which led to cell death comparable to death rate related to hypoxia/reperfusion [16,27]. We showed that B12 at high physiological doses does not protect against H_2O_2-induced cell death. The level of H_2O_2 might have been limited in the B12 treatment setting of ischemia (hypoxia)/reperfusion due to increased GPX, leading to the beneficial effects of B12 we observed in our previous study. The role of GPX levels on B12-mediated protection needs to be further explored. We also note that compartmentalization plays an important role. We added H_2O_2 extracellularly and if gradients formed across membranes it could affect the effects of H_2O_2 [28]. Cob(II)alamin is mostly mitochondrial [29] and one of us has shown that B12 can protect specifically against mitochondrial $O_2^{\bullet -}$ [30]. Hence, the lack of an effect could be due to H_2O_2 and B12 acting in different sub-cellular compartments. Further experiments are needed to test the role of compartmentalization on B12-mediated protection against oxidative stress. Although the effects of $O_2^{\cdot -}$ are generally thought to be mediated by H_2O_2, a possibility remains that $O_2^{\cdot -}$ directly mediated cytotoxic effects in our hypoxia/reperfusion model, which would explain why B12 is protective in our hypoxia/reperfusion model and not in the current study. Finally, it is possible that in the hypoxia/reperfusion model, the cytotoxic effects could be mediated by $ONOO^-$. By decreasing $O_2^{\cdot -}$, B12 would indirectly decrease $ONOO^-$ production and hence protect against $ONOO^-$ —dependent damage.

In our current experimental setting, the amount of H_2O_2 used is much higher than that of B12 (μmole/10^4 cells vs. nmole/10^4 cells), which could be the reason we did not observe the beneficial effects of B12. If we increase B12 amount to μmole/10^4 cells, that dose will be a thousand times higher than even pharmacological doses applied. Whether such high dose of B12 leads to potential toxic effects to biological systems is not known. We considered a possibility that the uptake of B12 by the cells might not have been optimal when they are given simultaneously with H_2O_2. However, pre-treatment with B12 for 2 h did not show protective effects.

HO-1 expression is upregulated by H_2O_2 and protects against oxidative stress [22]. In the current study, we show that mouse brain endothelial cells treated with H_2O_2 have elevated transcriptional levels of HO-1, which is consistent with previous reports [22,31]. Antioxidant genes such as NAD(P)H-qui-none oxidoreductase 1 (NQO1), HO-1, etc. are regulated by the transcription factor Nrf2. Nrf2 signaling pathway plays an important role in cell survival responses to oxidative stresses [32]. Under normal physiological conditions, Nrf2 is bound with Kelch-like ECH-associated protein 1 (Keap1). Keap1 facilitates ubiquitination and degradation of Nrf2. However, under the oxidative stress conditions, Nrf2 cannot be ubiquitinated and degraded. Hence, it accumulates and translocates to the nucleus where it binds to conserved antioxidant response element sequence to activate transcription of antioxidant genes like HO-1 [33]. In particular, Cys226/613/622/624 in Keap1 are responsible of sensing H_2O_2 and activate the Nrf2 pathway [34]. In the current study, we show that B12 does not alter the H_2O_2-induced increase in HO-1 gene expression at the condition we tested. Additionally, we show that H_2O_2 treatment decreases the transcriptional level of Nrf2. Song et al. have shown that H_2O_2 treatment at dose of 0.5 mM for 24 h

decreases Nrf2 levels in bEnd.3 cells and glutathione (GSH) co-treatment attenuated this decrease, however, the effects on HO-1 is not mentioned in their report [35]. Although B12 also functions as antioxidant, our current study show that B12 does not influence the effects of H_2O_2 on Nrf2 expression.

In summary, although B12 effectively reduces $O_2^{\bullet -}$ and protects against injury induced by ischemia/reperfusion *in vivo* [16], it does not protect against exogenous H_2O_2-induced injury in cultured mouse proximal tubular cells or brain endothelial cells.

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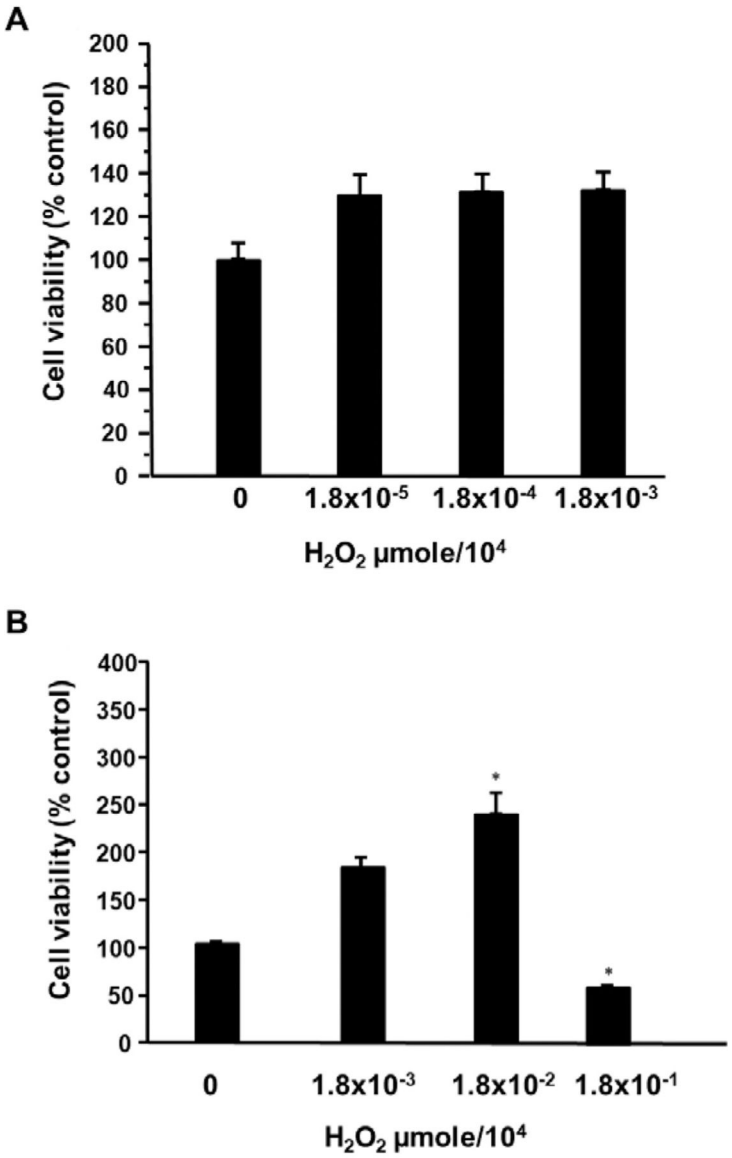

Fig. 1. Dose effects of H_2O_2 on the viability of mouse kidney proximal tubular cells (BU.MPT).
BU.MPT cells were treated with different doses of H_2O_2 as described in the Materials and Methods section. A. H_2O_2 did not change the cell viability at doses less than 0.0018 µmole/10^4 cells. B. 0.018 µmole/10^4 cells of H_2O_2 increased cell viability while 0.18 µmole/10^4 cells of H_2O_2 decreased cell viability. * $p < 0.05$ vs. 0 µmole group. $n \geq 14$.

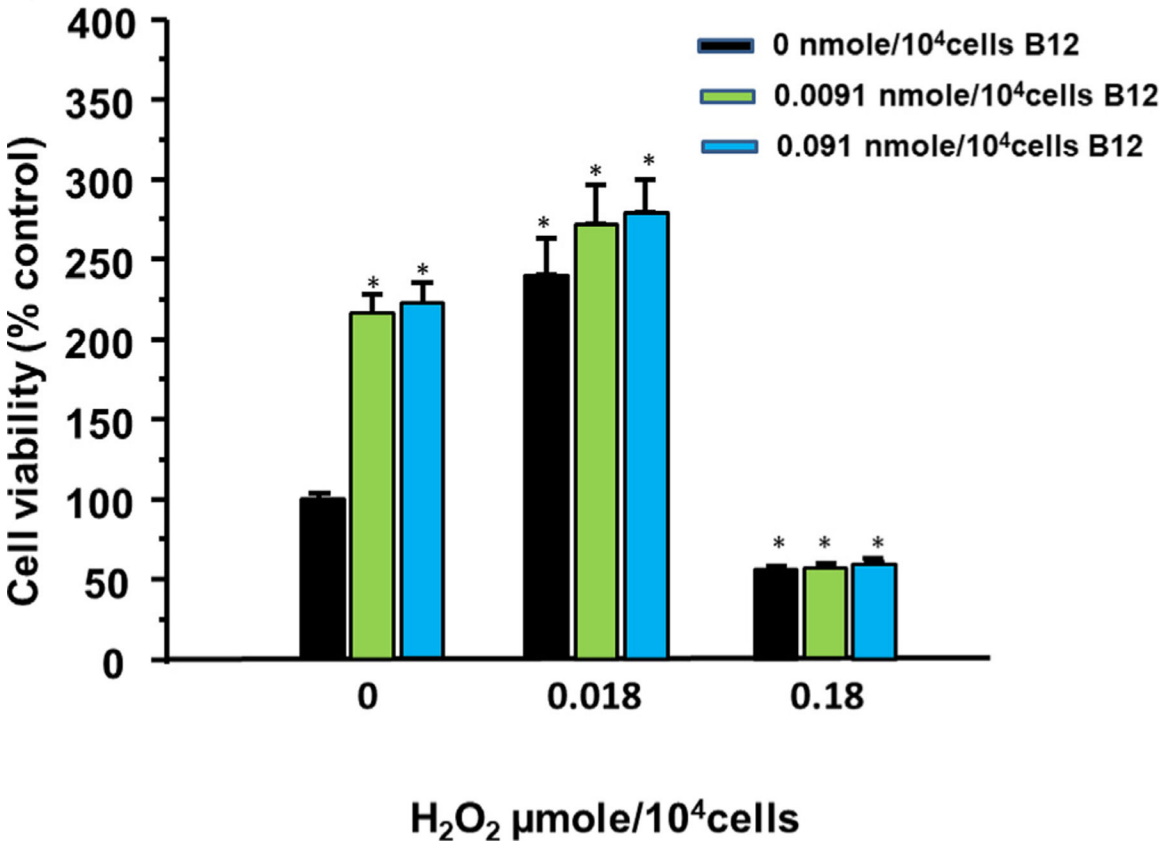

Fig. 2. Dose effects of B12 on the viability of mouse kidney proximal tubular cells (BU.MPT) treated with H_2O_2.

BU.MPT cells were treated with different doses of H_2O_2 and B12 as described in the Materials and Methods section. B12 at dose of 0.0091 and 0.091 nmole/10^4 cells increased cell viability in 0 μmole/10^4 cells of H_2O_2 treated cells, but had no effects on the cells treated with 0.18 μmole/10^4 cells of H_2O_2. * $p < 0.05$ vs. cells with 0 μmole/10^4 cells of H_2O_2 and 0 nmole/10^4 cells of B12. $n \geq 14$.

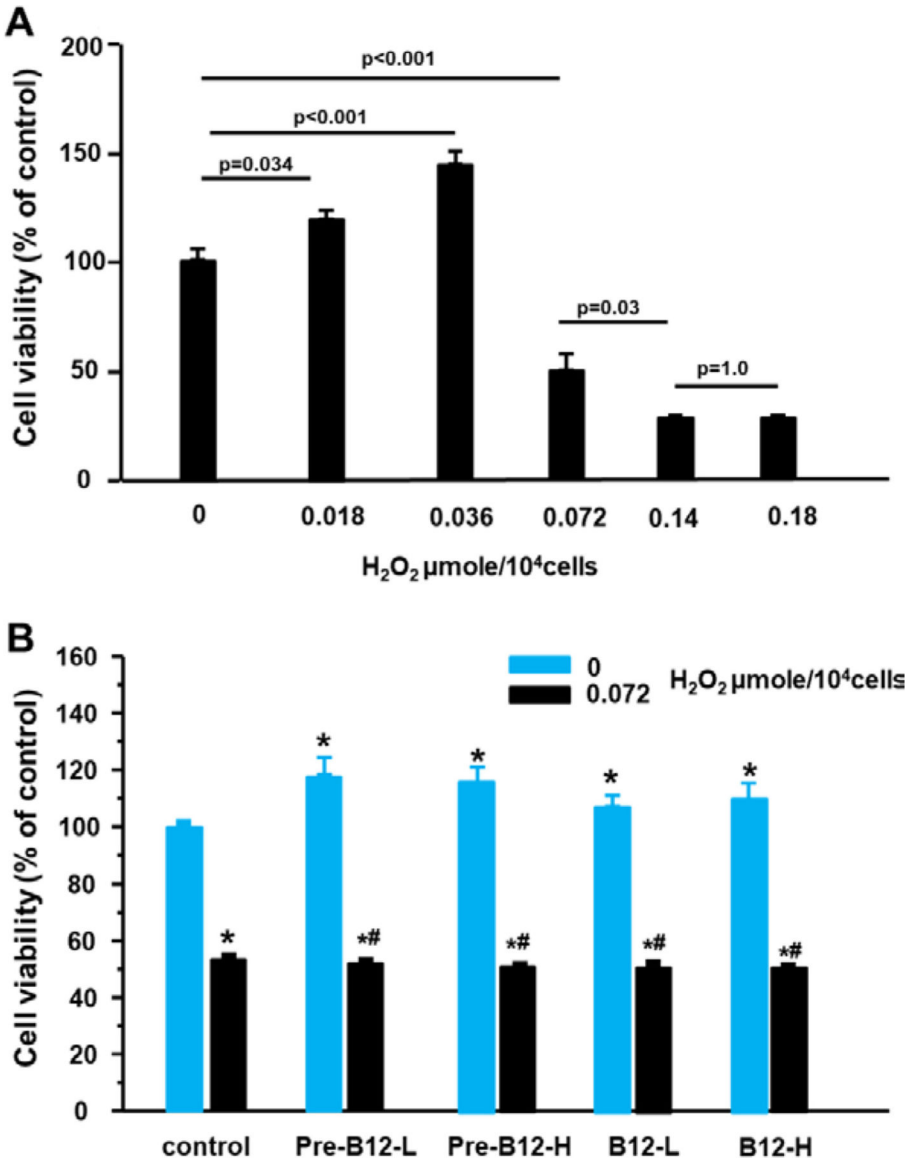

Fig. 3. Pre-treated with B12 does not increase the viability of BU.MPT cells treated with H_2O_2.

Cells were treated with different doses of H_2O_2 and B12 as described in the Materials and Methods section. A. H_2O_2 at doses of 0.018, 0.036 µmole/10^4 cells increased viability of cells, but H_2O_2 at doses of 0.072 and higher decreased the cell viability. B. B12 at dose of 0.0091 and 0.0911 nmole/10^4 cells increased the cell viability of cells treated with 0 µmole/10^4 cells of H_2O_2, but had no effects on the cells treated with 0.072 µmole/10^4 cells of H_2O_2. * $p < 0.05$ vs. cells with 0 µmole H_2O_2 and 0 nmole of B12, # $p < 0.05$ vs. cells treated with 0 µmole H_2O_2 but with B12 as designed. Pre-B12-L: pretreated with B12 at dose of 0.0091 nmole/10^4 cells for 2 h, Pre-B12-H: pre-treated with B12 at dose of 0.091 nmole/10^4 cells for 2 h, B12-L: treated with B12 at dose of 0.0091 nmole/10^4 cells simultaneously with H_2O_2, B12-H: treated with B12 at dose of 0.091 nmole/10^4 cells simultaneously with H_2O_2. $n \geq 14$.

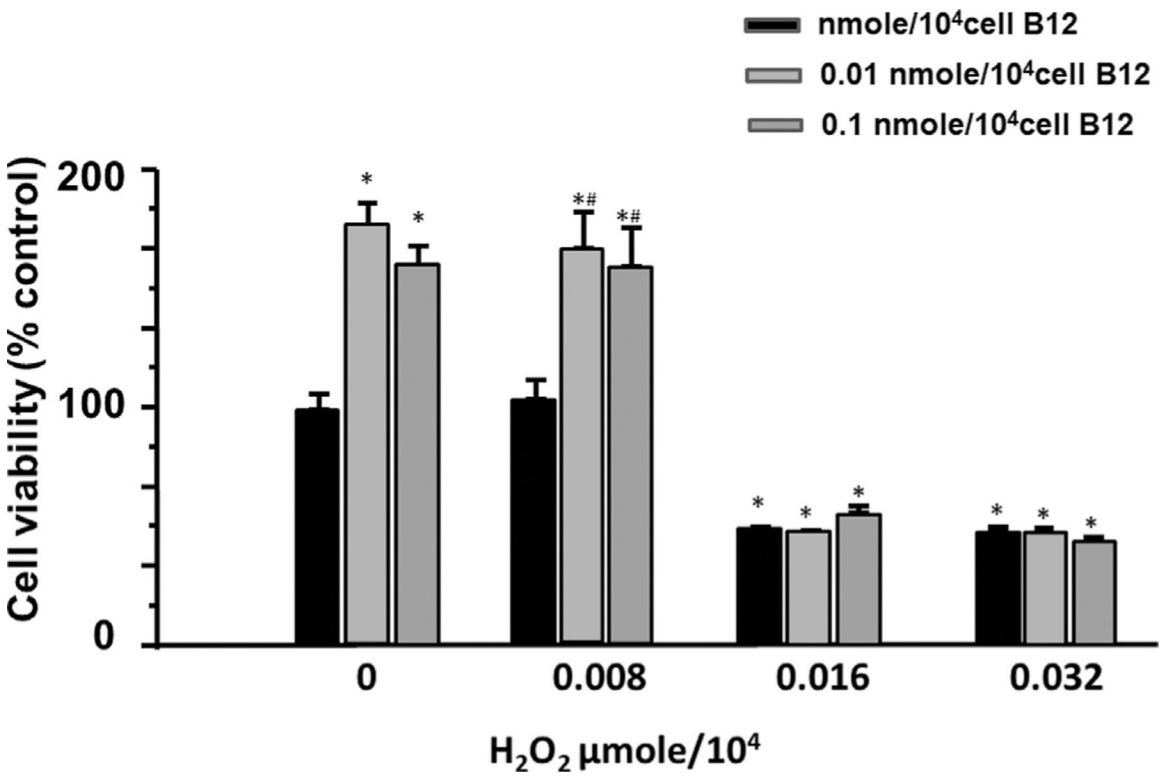

Fig. 4. Dose effects of H_2O_2 and B12 on the viability of mouse brain endothelial cells (bEdn.3).

bEdn.3 cells were treated with different doses of H_2O_2 and B12 as described in the Materials and Methods section. H_2O_2 at doses of 0.008 µmole/10^4 cells had no effects on cell viability of bEdn.3 cells, but H_2O_2 at doses of 0.016 and 0.032 µmole/10^4 cells decreased the cell viability. B12 at dose of 0.01 and 0.1 nmole/10^4 cells increased the viability of cells treated with 0 or 0.008 µmole/10^4 cells of H_2O_2, but had no effects on the cells treated with 0.016, 0.032 µmole/10^4 cells of H_2O_2. * $p < 0.05$ vs. cells with 0 µmole H_2O_2 and 0 nmole of B12, # $p < 0.05$ vs. cells treated with 0.008 µmole/10^4 cells of H_2O_2 and 0 nmole of B12. $n \geq 14$.

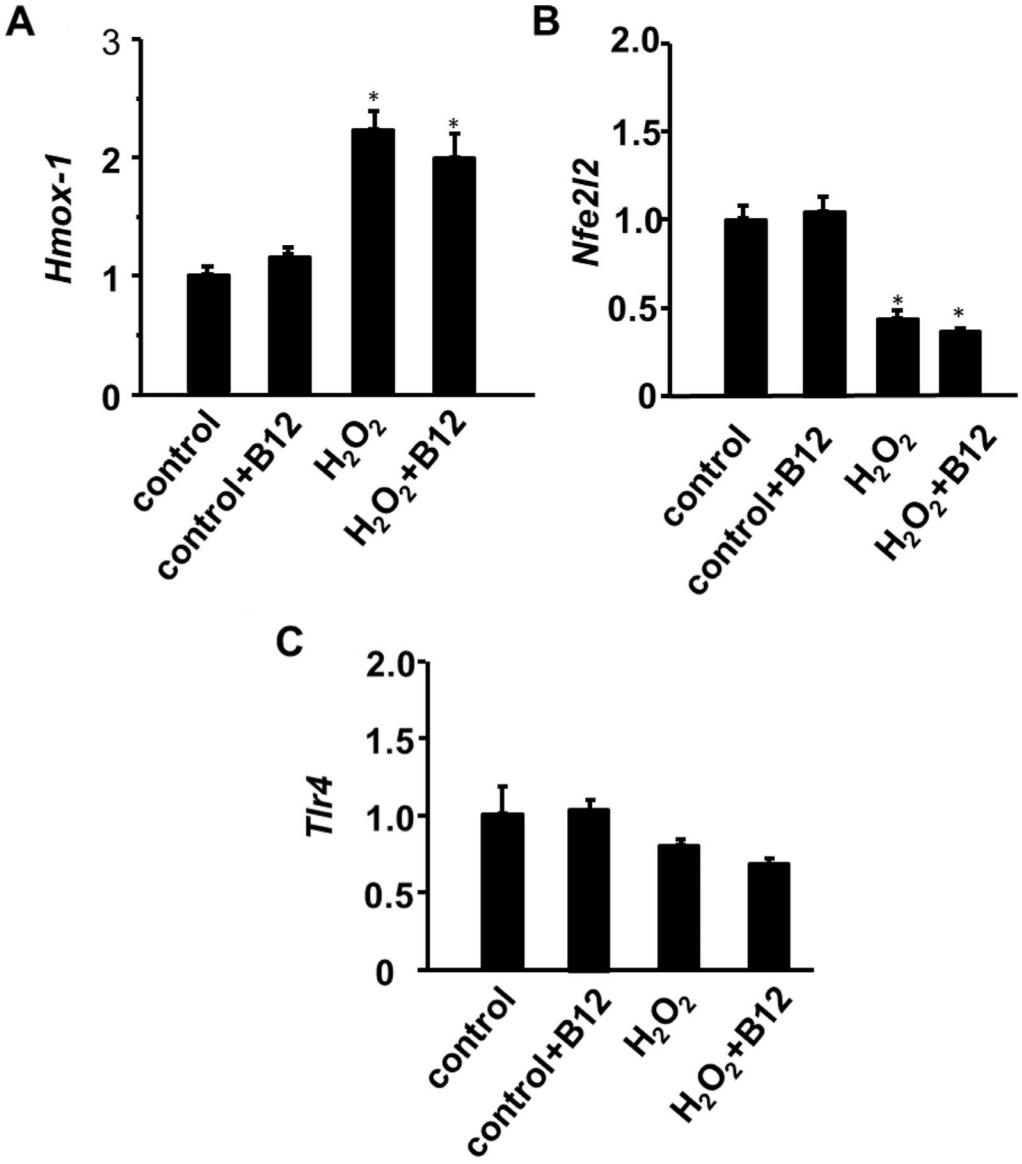

Fig. 5. B12 did not affect the expression of genes which altered by H_2O_2.

bEdn.3 cells were treated with different reagents as described in the Materials and Methods section. Gene expression is determined by qRT-PCR. A. The mRNA levels of *Hmox-1* (coding HO-1) were increased by 0.16 μmole/10^4 cells of H_2O_2, which was not affected by 0.1 nmole/10^4 cells of B12. B. The mRNA levels of *Nfe2l2* (coding Nrf2) were decreased by H_2O_2, which was not affected by B12. C. Neither H_2O_2 nor B12 had effect on the mRNA levels of *Tlr4*. * $p < 0.05$ vs. control. $n = 6$.

Table 1

Primers and probes for qRT-PCR

Gene	Type	Sequence (5'-3')
Hmox-1	Forward	ATG GCG TCA CTT CGT CAG AG
	Reverse	TGT CTG GGA TGA GCT AGT GC
	Probe	*FAM*-AG ACA CCC CGA GGG AAA CCC CA—*TAMRA*
Nfe2l2	Forward	CAT GAG TCG CTT GCC CTG GA
	Reverse	ATT GAG GGA CTG GGC CTG AT
	Probe	*FMA*-TC CCC AGC CAC GTC GAA AGT TCA G- *TAMRA*
Tlr4	Forward	GGT GAG AAA TGA GCT GGT AAA G
	Reverse	GCA ATG GCT ACA CCA GGA AT
	Probe	*FAM*-TG CCC CGC TTT CAC CTC TGC CTT CA—*TAMRA*
Hprt	Forward	GGA CTG ATT ATG GAC AGG AC
	Reverse	CAG AGG GCC ACA ATG TGA T
	Probe	*FAM*- CC TCC CAT CTC CTT CAT GAC ATC TCG —*TAMRA*