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Full Length Article

Protective Effect of *Bacillus subtilis* B10 against Hydrogen Peroxide-Induced Oxidative Stress in a Murine Macrophage Cell Line

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Abstract

The present study was designed to determine, whether *Bacillus subtilis* B10 could enhance the antioxidant activity of RAW 264.7 cells and attenuate the oxidative stress induced by hydrogen peroxide (H_2O_2). In addition, the responses were contrasted with a powerful antioxidant, vitamin E. RAW 264.7 cells were pretreated for 12 h with 100 mM vitamin E or *B. subtilis* B10 (1×10^8 c.f.u. well^{-1}), respectively and consecutively exposed to 500 μM H_2O_2 for an additional 6 h of incubation. Additional control group was included with exposure to *B. subtilis* B10 (1×10^8 c.f.u. well^{-1}) in the absence of H_2O_2. After incubation, cells were collected for further analysis of anti-oxidative indicators. The results showed that, cells pre-treated with *B. subtilis* B10 manifested remarkable increase in total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), catalase (CAT), anti-superoxide activity (ASOA), glutathione peroxidase (GPX) as well as glutathione reductase (GR) levels and significant drop in myeloperoxidase (MPO) and malondialdehyde (MDA) content. The activity of inducible NO synthase (iNOS) was also elevated in *B. subtilis* group, while NO content decreased markedly. Additionally, exposure to H_2O_2 with prior *B. subtilis* B10 treatment induced high gene expression levels of glutathione S-transferases (GST) and γ-glutamylcysteine synthetase (γ-GCS) in RAW 264.7 cells. However, vitamin E showed a limited protective effect when compared with *B. subtilis* B10 in our study. The current findings concluded that, *B. subtilis* B10 protected cells from H_2O_2 induced oxidative damage, presumably by enhancing enzymatic antioxidant defense systems of RAW264.7 cells.

Keywords: *Bacillus subtilis*; Vitamin E; Oxidative stress; RAW264.7 cells

Introduction

Oxidative damage, mediated by reactive oxygen species (ROS), has been implicated in initiation or progression of numerous disorders, and closely relates to animal production, reproductive performance and general welfare of the farm animals (Lykkesfeldt *et al*., 2007). Mammalian cells possess inherent antioxidant mechanisms to scavenge or neutralize ROS (Wijeratne *et al*., 2005); however there is insufficient antioxidant capacity to prevent ROS-mediated injury when animals are fed in high stress condition, and exposed to invading pathogens or some chemicals. Therefore, antioxidant therapy may provide a potentially important alternative treatment to reduce the oxidative damage (Lykkesfeldt *et al*., 2007).

Due to the potential toxic effect of synthetic antioxidants, much attention then has been focused on the use of natural antioxidants (e.g., vitamin E, carotenoids, GSH, probiotics) to improve animal health and performance (Wongputtisin *et al*., 2007). Applications of probiotics have been revealed numerous health benefits since its application to exert, beneficiary effects particularly in animals and human (Huang *et al*., 2012; Rajput and Li, 2012). The anti-oxidative effect of probiotics has been reported recently (Koller *et al*., 2008; Martarelli *et al*., 2011). *Bacillus subtilis* is one of the direct-fed microbial products that have been safely and commonly used as human food and animal feed (Qin *et al*., 2013). Application of *B. subtilis* was beneficial to improve antioxidant capacity of ducks (Rajput *et al*., 2013) and broilers (Li *et al*., 2011). Previously, we have reported that *B. subtilis* B10 possessed antioxidant properties and were able to protect human derived colon cells against oxidative damage induced by H_2O_2 (Cui *et al*., 2011). Intestinal macrophages are essential for local homeostasis, and little information is presently available about the effects of *B. subtilis* on anti-oxidant activity of macrophages under oxidative stress. Therefore, evidences inspired us to focus our research on the protective effect of *B. subtilis* B10 against hydrogen peroxide-induced oxidative stress in RAW264.7 cells.

Materials and Methods

Bacteria

Bacillus subtilis B10 (*B. subtilis* B10) used during

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experiment was isolated and identified by Institute of Feed Science, Zhejiang University. The bacterial strain was cultured in Luria-Bertani (LB) (Oxoid, England) medium at 30°C till log phase (16 h). Bacterial cells were harvested by centrifugation (10 min at 6000 rpm), washed twice with sterile Phosphate-Buffered Saline (PBS) buffer (pH 7.4) and heated at 100°C for 30 min.

Cell Culture

RAW 264.7 (ATCC TIB71), a murine macrophage cell line, was obtained from the Cell Resource Center of Shanghai Institute of Biological (Shanghai, China). The cells were cultured according to our previous procedure (Li *et al*., 2012).

Experimental Design

RAW 264.7 cells with different treatments were designated as G I, G II, G III, G IV and G V respectively. G I, with no hydrogen peroxide (H_2O_2) treatment, served as control group. Oxidation was induced by exposing RAW 264.7 cells to 500 µM H_2O_2 in DMEM for 6 h (group II, H_2O_2). In group III (G III, VE+H_2O_2) and IV (G IV, B10+H_2O_2), RAW 264.7 cells were pretreated for 12 h with 100 mM vitamin E or *B. subtilis* B10 (1×10^8 c.f.u. well^{-1}, heat-killed) respectively, and consecutively exposed to 500 µM H_2O_2 for an additional 6 h of incubation. Group V (G V, B10) with exposure to *B. subtilis* B10 (1×10^8 c.f.u. well^{-1}, heat-killed) in the absence of H_2O_2 acted as additional control group. Vitamin E was dissolved in absolute ethanol and then diluted in DMEM to a final concentration (100 mM). Each group had six replicated wells. After the 18-h incubation, cells were collected separately for further analysis.

Anti-oxidative Indicators Measurement

The activities of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), glutathione peroxidase (GPX), glutathione reductase (GR), anti-superoxide activity (ASOA), inducible NO synthase (iNOS) and concentrations of glutathione (GSH), glutathione disulfide (GSSG), malondialdehyde (MDA), nitric oxide (NO) were determined using the detection kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Hu *et al*., 2012; Tong *et al*., 2012).

Quantitative Real-time PCR Studies

Total RNA extraction was extracted by using RNAiso Plus reagent (Takara, Japan). RT-PCR was performed as previously described (Li *et al*., 2013). Change in gene expression was determined using the $2^{-\Delta\Delta CT}$ method. The primer sequences used for qRT-PCR are described in Table 1. At least 3 independent experiments performed in triplicate.

Statistical Analysis

Data were analyzed using the one-way analysis of variance procedure of SPSS 16.0 for Windows. All data were represented as mean ± S.D. P-values of (<0.05) were considered to be statistically significant.

Results

Antioxidant Enzyme Activity

The results showed (Table 2) that, treatment with H_2O_2 (G II) significantly increased MPO activity while the activities of GPX and GR were obviously decreased. Additionally, no change was found in T-AOC, T-SOD, GSH and GSSG levels as compared to control (G I).

Results analysis revealed that VE prevention group (G III, VE+H_2O_2) had high levels of GPX and GR while, MPO showed remarkable decrease (P<0.05) when compared with H_2O_2 group (G II). Besides, VE group (G III) showed a non-significant effect on the other antioxidant indexes.

The activities of T-AOC, T-SOD, CAT, ASOA, GPX and GR were observed remarkable higher whereas the levels of MPO and MDA were markedly lower in *B. subtilis* B10 group (G IV, B10+H_2O_2) as compared to G II and G I. In addition, antioxidant enzyme activities of cells pretreated with B10 (G IV, B10+H_2O_2) were higher (P<0.05) than that of VE group (G III, VE+H_2O_2). GSH concentrations showed a slight increase in (G IV), while this increase was not statistically significant. Additionally, cells treated with *B. subtilis* B10 in the absence of H_2O_2 exhibited elevated levels of T-SOD, GR as well as MPO in (G V, B10).

Nitric Oxide and Inducible Nitric Oxide Synthase

After incubation with H_2O_2 for 6 h, significant reduction in NO levels was noted in the cells of group (II, III, IV) as compared to non-treated cells in (G I). The activity of iNOS showed notable decrease (P <0.05) in (G II) and (G III) when compared with control (G I). Whilst, *B. subtilis* B10 (G IV, B10+H_2O_2) was found able to restore iNOS activity to the normal level. Besides, remarkably elevated levels of NO and iNOS were observed in (G V, B10) (Fig. 1).

Expression of Genes Involved in Oxidative Stress

The results (Fig. 2) showed that GCS, XO and NOX1 mRNA expressions were significantly enhanced in H_2O_2 group (G II), while no change was found in GR, GST, SOD and TRX2 mRNA expressions levels as compared to control (G I). The expression levels of relevant oxidant-related genes of cells pretreated with VE were not significantly different from that of H_2O_2 group (G II). *B. subtilis* B10 (G IV, B10+H_2O_2) promoted GST and GCS mRNA expressions (P<0.05), whereas expression of SOD was down-regulated markedly by B10 when compared with H_2O_2 group (G II). While, changes in transcription levels of GR, TRX2, XO and NOX1 were not observed between G IV and G II.

Table 1: Sequences of forward and reverse primers used for qRT-PCR

Target	Primer sequence	Size (bp)
β-actin	F:5'CCTGTACGCCAACACAGTGC3'	211
	R:5'ATACTCCTGCTTGCTGATCC3'	
GAPDH	F:5'GTTGTCTCCTGCGACTTCA3'	293
	R:5'GCCCCTCCTGTTATTATGG3'	
GSR	F:5'GTAGGAAGCCCACCACGAC3'	173
	R:5'AGCATAGACGCCTTTGACATT3'	
GST	F:5'GCCCTGTGGCATAAGGTGAT3'	129
	R:5'TTTGTCCTGGAGGAACTTGTCT3'	
γ-GCS	F:5'AACTCTGCCTATGTGGTATTCG3'	269
	R:5'TTCCCATTGATGATGGTGTCTA3'	
SOD	F:5'GAAGCATGGCGATGAAAGC3'	273
	R:5'CAGTCACATTGCCCAGGTCTC3'	
XO	F:5'CTGTCTTTGCGAAGGATGAG3'	140
	R:5'CTGGATTGTGATAATGGCTGG3'	
TRX2	F:5'GGAAACCAGAAGCCGAACA3'	151
	R:5'GACAAGGAATAGAAGGGACAGAT3'	
NOX1	F:5'CCGTTCATATTCGAGCAGC3'	81
	R:5'TCCTGGGCATTGGTGAGT3'	

Table 2: Antioxidant enzyme activities of cells in different treatment groups

Target	G I (control)	G II (H_2O_2)	G III (VE+H_2O_2)	G IV (B10+H_2O_2)	G V (B10)
T-AOC	0.49 ± 0.11^{c}	1.04 ± 0.20^{bc}	1.41 ± 0.25^{b}	4.45 ± 0.83^{a}	0.638 ± 0.32^{c}
T-SOD	27.5 ± 0.72^{c}	26.7 ± 0.46^{c}	27.5 ± 0.79^{c}	48 ± 0.54^{a}	39.5 ± 0.77^{b}
CAT	ND	ND	ND	28.93±1.48	ND
MPO	0.44 ± 0.01^{c}	0.75 ± 0.03^{a}	0.63 ± 0.05^{b}	0.59 ± 0.04^{b}	0.63 ± 0.01^{b}
ASOA	ND	ND	ND	144.7±1.04	ND
MDA	1.38 ± 0.18^{a}	1.50 ± 0.60^{a}	1.27 ± 0.06^{a}	1.03 ± 0.17^{b}	1.40 ± 0.21^{a}
GPX	1.9 ± 0.8^{c}	ND	3.64 ± 0.11^{b}	6.52 ± 0.21^{a}	1.44 ± 0.07^{c}
GR	9.89 ± 0.4^{c}	8 ± 0.27^{d}	10.3 ± 0.74^{c}	14.1 ± 1.88^{a}	11.9 ± 0.74^{b}
GSH	4.46±0.78	4.32±0.47	3.97±1.01	5.43±0.47	5.45±0.63
GSSG	0.2±0.02	0.21±0.02	0.19±0.02	0.23±0.05	0.22±0.03

Data were expressed as mean ± SD. Different letters indicate a statistically significant difference between groups (P<0.05). ND stands for Non-Detectable. Units: T-AOC (U/mgprot), T-SOD (U/mgprot), CAT (U/mgprot), MPO (U/gprot), ASOA (U/gprot), MDA (nmol/mgprot), GPX (U/mgprot), GR (U/gprot), GSH (μmol/gprot), GSSG (μmol/gprot)

Discussion

Antioxidant Enzyme Activity

Body endures oxidative stress, when natural antioxidant defense mechanism fails to protect cells against damage caused by high level of ROS (De Bont and van Larebeke, 2004). The mammalian cells have evolved an elaborative and protective antioxidant defense system to prevent oxidative damage by scavenging free radicals. Evidences have been shown that the antioxidant triad comprising SOD, CAT, GPX constitutes the first line of defense against the adverse effects of ROS (Koziorowka-Gilun *et al*., 2011). Besides, GSH redox system appears to be the main non-enzymatic antioxidant against ROS-mediated damage. GSH can be converted to GSSG through GPX, and converted back to GSH by GR (Yao *et al*., 2006). Thus, changes in the GSH, GSSG and its related enzymatic reactions (GPX and GR) may sensibly reflect the antioxidant status.

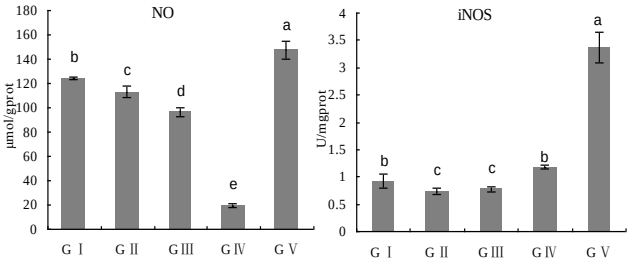

Fig. 1: Nitric oxide content and inducible NO synthase activity of cells in different treatment groups

Data were expressed as mean ± SD. Different letters indicate a statistically significant difference between groups (P<0.05). The error bars indicate standard deviations. G I (control), G II (H_2O_2), G III (VE+H_2O_2), G IV (B10+H_2O_2), G V (B10)

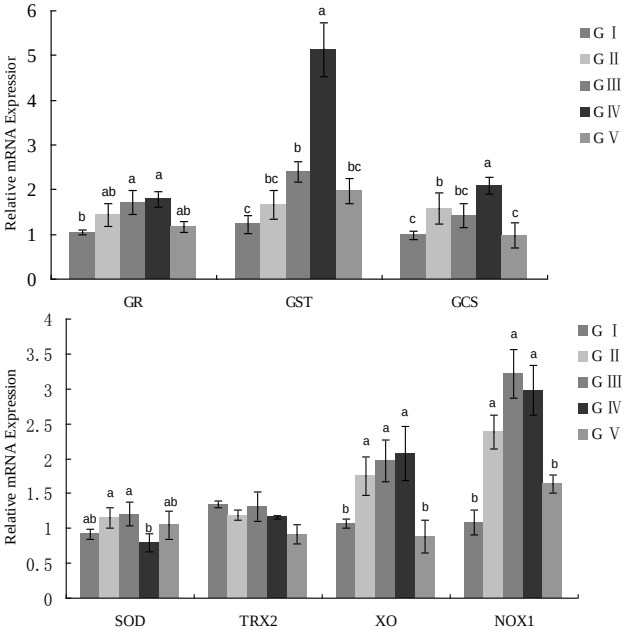

Fig. 2: Expression of genes involved in oxidative stress of cells in different treatment groups

Data were expressed as mean ± SD. Different letters indicate a statistically significant difference between groups (P<0.05). The error bars indicate standard deviations. G I (control), G II (H_2O_2), G III (VE+H_2O_2), G IV (B10+H_2O_2), G V (B10)

In the present study, we found remarkable reduction in GPX and GR activities in cells after exposure to H_2O_2, indicating that oxidative stress might have occurred. Conversely, *B. subtilis* B10 showed a potent protective effect in our study. Cells pre-incubated with *B. subtilis* B10 exhibited elevated levels of T-AOC, T-SOD, CAT, ASOA, GPX as well as GR. These findings coincided with Li *et al.* (2012) who demonstrated that, *Enterococcus faecium* EF1 could enhance the activity of enzymatic antioxidant defense systems of Caco-2 cells under oxidative stress. Another study described that lactic acid bacteria were also protective

towards H_2O_2 induced oxidative damages in colon cells (Koller *et al*., 2008). Our results revealed beneficial effects of *B. subtilis* B10 against oxidative stress by increasing the scavenging rate of free radicals via enhancing enzymatic defense.

Generally, it is accepted that MPO converts H_2O_2 to highly reactive hypochlorous acid with potent cytotoxic properties (Mütze *et al*., 2003) and it also serves as a reliable marker of the degree of oxidative stress during haemodialysis (Wu *et al*., 2005). In our study, activity of MPO showed remarkable increase in macrophage cells incubated with H_2O_2, while pre-incubation with *B. subtilis* B10 reduced the level of MPO. Previously, Westman *et al*. (2006) reported that activated macrophages produced and released MPO during inflammation. The current findings suggested that *B. subtilis* B10 may possess antioxidant properties to prevent H_2O_2-induced macrophages activation and the ensuing damage. Meanwhile, the molecular mechanisms that initiate these changes in MPO activity will require additional study.

MDA is a main product of lipid peroxidation, and the most commonly indicator for the extent of stress-induced damage (Weismann *et al*., 2011). In the current findings, significantly lower MDA content was noted in *B. subtilis* B10 pre-treated group, and the reduction in MDA content was inversely proportionate in coordination to increase in T-AOC, T-SOD, CAT, GPX and GR levels. Therefore, the ensemble of results suggested that *B. subtilis* B10 could ameliorate oxidative stress on RAW264.7 cells.

Vitamin E is a powerful lipid-soluble antioxidant vital for the maintenance of oxidant-antioxidant homeostasis. Therefore, in the current study, vitamin E revealed greater activities of GPX and GR, while lower MPO levels in vitamin E pre-treated cells, suggesting that vitamin E may play a limited role to down-regulate the H_2O_2 induced oxidative stress in RAW 264.7 cells. So, as pointed out by van Haaften *et al*. (2003), vitamin E might have the protective effects on GSH-dependent enzymes. Our data indicated that, although vitamin E is a vital chain-breaking antioxidant, it may have a limited capacity to enhance the antioxidant defense system of RAW 264.7 cells.

Nitric Oxide and Inducible Nitric Oxide Synthase

Nitric oxide (NO) has been proposed to possess both anti-oxidant and pro-oxidant properties (Borniquel *et al*., 2006). NO could react with peroxyl radicals as a sacrificial chain-breaking antioxidant (Hummel *et al*., 2006). This might account for the reduced NO levels observed in our study after treatment with H_2O_2. Conversely, NO also acts as a pro-oxidant when it forms highly reactive peroxynitrite with superoxide, to enhance antibacterial activity of macrophages (Hummel *et al*., 2006). In our study, NO level was lower in *B. subtilis* B10 pre-treated macrophage amongst groups. This finding may indicate that pre-treatment with *B. subtilis* B10 may result in amounts of NO consumption in macrophages, and may promote the reaction of NO with peroxyl radicals, thereby decreasing the NO content in macrophages.

Bacterial infection generally leads to activation of iNOS in macrophages, thus producing large amounts of NO to kill invading bacteria (Chiou *et al*., 2000). As observed here, significant reduction in iNOS levels was noted in cells treated with H_2O_2. This finding was in line with a previous study, which showed that oxidative stress induced by pro-oxidants inhibited the expression of iNOS in macrophages (Cho *et al*., 2005). However, we found a discrepancy between NO concentration and levels of iNOS. In the current study, elevated iNOS activity was manifested in *B. subtilis* B10 group, and this might be consistent with a greater rate of NO consumption in macrophages, thus a higher level of iNOS may be required.

Expression of Genes Involved in Oxidative Stress

Glutathione S-transferases (GST), a superfamily of phase II detoxification enzymes, catalyze conjugation of GSH to a wide variety of electrophilic chemicals (Townsend and Tew, 2003). While, γ-glutamylcysteine synthetase (γ-GCS) is a rate-limiting enzyme in de novo synthesis of GSH and it plays a pivotal role in GSH homeostasis (Hibi *et al*., 2004). It was observed in our report that, exposure to H_2O_2 with prior *B. subtilis* B10 treatment induced high gene expression levels of GST and γ-GCS in RAW 264.7 cells. Previous studies have shown that, exposure cells to oxidants such as H_2O_2, caused depletion of GSH, which was concomitant with increased mRNA expression for γ-GCS gene (Tian *et al*., 1997). Moreover, Shukla *et al*. (2000) also illustrated that transcriptional levels of GST and γ-GCS were up-regulated to help maintain redox homeostasis and to cope with oxidative stress in alveolar epithelial cells. Our data may indicate that, prior *B. subtilis* B10 treatment enhanced capacity of RAW 264.7 cells to counteract the oxidative stress caused by H_2O_2 treatment. These adaptive responses to H_2O_2-induced stress were probably due to the increased rates of glutathione utilization. Transcriptional activation of γ-GCS might be responsible for the enhanced GSH synthesis, while GST induction might provide a more efficient means for the elimination of lipid peroxidation products (Shukla *et al*., 2000).

It is known that NADPH oxidase system (NOXs) is a family of superoxide-generating enzymes that can catalyze the regulated formation of ROS (Gianni *et al*., 2010). Furthermore, Dikalov *et al*. (2008) reported that NADPH oxidase-1 (NOX1) was responsible for angiotensin II-induced superoxide production and the increased oxidant stress and vascular disorders. Here we demonstrated that, NOX1 mRNA expression was up-regulated in RAW 264.7 cells challenged with H_2O_2. This finding was in keeping with previous studies by Qian *et al*. (2011) who reported that exposure of cells to H_2O_2 could lead to the activation of NOX activity in retinal pigment epithelium cell.

Moreover, it was reported that ROS derived from NADPH oxidases could modulate endothelial cell xanthine oxidase (XO) levels (McNally *et al*., 2003). XO is a ubiquitous enzyme, widely known for its production of ROS and, has been implicated in many inflammatory diseases (Kanczler *et al*., 2003). Our data showed that the transcription level of XO in RAW 264.7 cells was significantly augmented after exposure to H_2O_2 and, this might be associated with the increased expression of NOX1 mRNA.

Thioredoxin (TRX) is an important antioxidant present in all types of organisms (Takatsume *et al*., 2005) and mitochondrial thioredoxin-2 was commonly used as a source of reducing equivalents to scavenge H_2O_2 (Nonn *et al*., 2003). TRX2, the gene encoding thioredoxin-2, is already known to be induced by several chemicals that cause oxidative stress (Takatsume *et al*., 2005). It has previously been described that over-expression of thioredoxin gene TRX2 resulted in reduction of oxidative cellular damage (Gómez-Pastor *et al*., 2010). However, in the current study, no change was found in TRX2 mRNA expression level in cells following different treatment. Additionally, we found a discrepancy between mRNA levels and enzymatic activity of SOD. This discrepancy was probably due to differences in mRNA and protein turnover rates, as well as the posttranscriptional regulation.

Taken together, *B. subtilis* B10 showed a potent protective effect in our study and we found that *B. subtilis* B10 could increase the scavenging rate of free radicals by enhancing enzymatic defense, thereby ameliorating oxidative stress on RAW264.7 cells induced by H_2O_2. However, the precise mechanism whereby *B. subtilis* increased the antioxidant activity of RAW264.7 cells remained unknown, therefore, their signaling and transduction pathways need further investigation.

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