Barley beta-glucan promotes MnSOD expression and enhances angiogenesis under oxidative microenvironment

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Abstract

Manganese superoxide dismutase (MnSOD), a foremost antioxidant enzyme, plays a key role in angiogenesis. Barley-derived (1.3) β-D-glucan (β-glucan) is a natural water-soluble polysaccharide with antioxidant properties. To explore the effects of β-glucan on MnSOD-related angiogenesis under oxidative stress, we tested epigenetic mechanisms underlying modulation of MnSOD level in human umbilical vein endothelial cells (HUVECs) and angiogenesis in vitro and in vivo. Long-term treatment of HUVECs with 3% w/v β-glucan significantly increased the level of MnSOD by 200% compared to control and by 50% compared to untreated H2O2-stressed cells. β-glucan-treated HUVECs displayed greater angiogenic ability. In vivo, 24 hrs-treatment with 3% w/v β-glucan rescued vasculogenesis in Tg (kdrl: EGFP) s843Tg zebrafish embryos exposed to oxidative microenvironment. HUVECs overexpressing MnSOD demonstrated an increased activity of endothelial nitric oxide synthase (eNOS), reduced load of superoxide anion (O2−) and an increased survival under oxidative stress. In addition, β-glucan prevented the rise of hypoxia inducible factor (HIF)1α under oxidative stress. The level of histone H4 acetylation was significantly increased by β-glucan. Increasing histone acetylation by sodium butyrate, an inhibitor of class I histone deacetylases (HDACs I), did not activate MnSOD-related angiogenesis and did not impair β-glucan effects. In conclusion, 3% w/v β-glucan activates endothelial expression of MnSOD independent of histone acetylation level, thereby leading to adequate removal of O2−, cell survival and angiogenic response to oxidative stress. The identification of dietary β-glucan as activator of MnSOD-related angiogenesis might lead to the development of nutritional approaches for the prevention of ischemic remodelling and heart failure.

Keywords: beta-glucan → angiogenesis → endothelial cells → antioxidants → histone deacetylases

Introduction

In humans, the chronic exposure to reactive oxygen species (ROS) is a major risk factor for the impairment of endothelial function [1] and angiogenesis [2], which are both essential in self-repair ability of ischemic heart [3]. Even if ROS activates angiogenesis [2], compelling evidence indicates that the endothelial dysfunction because of superoxide anion (O2−) overload represents a mechanism for deregulated capillary formation after ischemia [4]. The safe and effective modulation of ROS-driven angiogenesis is still a challenging achievement, despite significant advances in the past two decades. High levels of the manganese superoxide dismutase (MnSOD), a major antioxidant enzyme, enhance the endothelial resistance to oxidative stress [5, 6] and angiogenesis via the scavenging mitochondrial O2− [7, 8]. Thus, MnSOD is a promising candidate target for modulation of ROS-induced angiogenesis. To date, cellular MnSOD levels are significantly increased by a gene therapy approach [9, 10] or via treatment with histone deacetylases (HDAC) inhibitors leading to cardioprotection through histone acetylation [11], which allows the expression of anti-apoptotic and angiogenic paracrine factors in vitro and in vivo [12]. However, a natural activator of MnSOD-related angiogenesis under oxidative stress is more desirable.

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Barley-derived (1.3) beta-D-glucan (β-D-glucan), a natural water-soluble chain of D-glucose monomers linked by β-glycosidic bonds [13], lowers either oxidative stress [14] or serum cholesterol level in a dose-dependent manner [15]. The daily intake of β-D-glucan (1.5–4%) similarly induces the above-mentioned benefits in animal models and humans [16, 17]. To date, it is unknown whether β-D-glucan promotes the MnSOD expression and the angiogenic ability of mature endothelial cells. Despite some controversial preliminary evidence [18], it has been suggested that long-term treatment with β-D-glucan enhances MnSOD-related angiogenesis under oxidative stress. For this purpose, we used the combination of two gold standard angiogenic tests, such as the in vitro human umbilical vein endothelial cells (HUVECs) matrigel assay [12, 19, 20] and the in vivo zebrafish angiogenesis assay [21, 22].

Materials and methods

Chemicals

Barley-derived (1.3) β-D-glucan, hydrogen peroxide (H2O2) and sodium butyrate (NaBu) were purchased from Sigma-Aldrich Chemical Co (MO, USA).

Endothelial cell cultures

Human umbilical vein endothelial cells (Cambrex Bio Science Inc, Walkersville, MD, USA) and human cardiac microvascular endothelial cells (HMVEC-C, Lonza, Milan, Italy) were cultured in endothelial growth medium (EGM-2 medium from Lonza) at 37°C in a humidified atmosphere of 5% CO2. Cells were exposed to the culture medium with 10% foetal bovine serum for at least 1 day before experiments. All assays were conducted using low cell passage cells (2–5 passages).

Cell viability assay

Low-dose treatment with H2O2 (50 μM) was used to induce chronic oxidative stress on HUVECs [23, 24]. After 24 hrs of exposure to H2O2 with or without 3% w/v β-D-glucan, cell viability was assessed by the Trypan blue dye exclusion test, as previously described [25]. When indicated, H2O2-stressed HUVECs were cultured in the presence of NaBu, a specific inhibitor of class I HDAC (reviewed in [26]), at rising concentrations (5–500 μM). All measurements were performed in triplicate.

Dihydroethidium staining

Endothelial superoxide anion generation was determined by staining of HUVECs with fluorescent-labelled dihydroethidium (DHE; Invitrogen, CA, USA), according to manufacturer’s instructions.

Superoxide anion assay

Superoxide anion levels were assessed using a Superoxide Anion Assay Kit (Sigma-Aldrich Chemical Co), according to manufacturer’s instruction relative to testing changes in superoxide anion production directly on intact cells.

Fig. 1 β-D-glucan treatment promotes endothelial cell survival and MnSOD expression under chronic oxidative stress. (A) HUVECs survival after 24 hrs treatment with 50 μM H2O2, alone or in combination with 3% β-D-glucan. Untreated and unstressed cells were used as control. (B) Quantification of the relative intensity of fluorescence in DHE-positive cells, compared to control condition. (C) Quantification of the relative intensity of luminescence in cells producing superoxide anion, compared with control condition. (D) Representative western blot bands for MnSOD and GAPDH. (E) Measurement of the level of MnSOD expression normalized over loading control (GAPDH). (mean ± SD; n = 4) *P < 0.05 versus control; $P < 0.05 versus H2O2.
Western blotting

Equal amounts of protein extracted with RIPA buffer from cell pellets were processed for western blotting assay. The ratio of phospho-Ser1177-eNOS (p-eNOS) and total eNOS, a hallmark of eNOS activity [27], was then determined as previously described [28]. Protein bands on immunoblots were quantified using ImageJ software.

Nitric oxide detection

DAF-FM staining for the determination of intracellular nitric oxide bioavailability in human endothelial cells was performed as described elsewhere [29].

In vitro angiogenesis assay

The in vitro angiogenesis assay was performed as described previously [12, 19, 20]. Analysis of capillary-like tube formation was performed using gel precoated wells (Cultrex® Basement Membrane Extract, BME, Therna). Image analysis was performed by ImageJ software.

Zebrafish lines, imaging and stages

Zebrafish transgenic embryos Tg (kdrl:EGFP) s843Tg (expressing EGFP in the vascular system) were raised and maintained under standard laboratory conditions [30] as described elsewhere [31].
oxidative stress was induced in vivo by treatment with PMA, an agonist of protein kinase C [32]. Imaging was performed on zebrafish embryos at 24 hpf (hours post-fertilization). Investigation was approved by the Animal Care Committee of the Italian Ministry of Health in accordance with the European law (EU 63-2010) and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Statistical analysis

The statistical analysis was performed using GraphPad Prism ver. 5. All results are presented as mean ± SD. Statistical comparisons were made by ANOVA and Dunnett’s Multiple Comparison Test was used as the post-hoc test. \( P < 0.05 \) was considered statistically significant.

**Results**

\( \beta\)-d-glucan treatment promotes endothelial cell survival and MnSOD expression under chronic oxidative stress

As shown in Figure 1A, the treatment with 3% \( \beta\)-d-glucan significantly increased the number of viable cells under oxidative stress.

The measure of fluorescence in the nuclei of cells positive for dEDT staining showed that \( \beta\)-d-glucan normalized the ROS load of stressed cells (Fig. 1B) while such amount was significantly increased in the absence of treatment. In addition, we confirmed that \( \beta\)-d-glucan reduced the total \( O_2^- \) in HUVECs (Fig. 1C) and HMVEC-C (Fig. S1). \( \beta\)-d-glucan significantly
enhanced the protein expression of MnSOD in viable cells exposed to normal microenvironment. The level of MnSOD in \( \beta \)-D-glucan-treated cells exposed to \( \text{H}_2\text{O}_2 \) was tendentially higher compared to untreated stressed cells (Fig. 1C, quantified in 1D).

\( \beta \)-D-glucan treatment increases eNOS phosphorylation and histone H4 acetylation in viable endothelial cells

\( \beta \)-D-glucan treatment of stressed cells caused a significant increase in p-eNOS/eNOS ratio without affecting the level of p-AKT, AKT, and heat shock protein-70 (HSP-70; Fig. 2A, quantified in 2B). We detected a significant increase in nitric oxide production in stressed \( \beta \)-D-glucan-treated HUVECs (Fig. 3) and HMVEC-C (Fig. S1). Interestingly, the rise of hypoxia inducible factor (HIF)-1\( \alpha \) level was prevented in \( \text{H}_2\text{O}_2 \)-stressed cells treated with \( \beta \)-o-glucan (representative blots shown in Fig. 2A, quantified in 2B). As shown in Figure 4A, the level of H4 acetylation in \( \beta \)-o-glucan-treated cells was significantly higher than untreated cells under normal and oxidative microenvironment.

\( \beta \)-o-glucan treatment promotes human capillary formation in vitro

\( \beta \)-o-glucan increased the tube formation activity of normal endothelial cells cultured without exogenous endothelial growth factors (Fig. 4B). As shown in Figure 4B (right panel), \( \beta \)-o-glucan induced a significant slight increase in ROS-driven angiogenesis.

In the presence of exogenous endothelial growth factors (Fig. 4C), the formation of capillaries from normal or \( \text{H}_2\text{O}_2 \)-stressed HUVECs was significantly increased by treatment with 3% \( \beta \)-o-glucan. The addition of exogenous growth factors in the culture medium did not increase the magnitude of ROS-driven angiogenesis (Fig. 4C, right panel) nor enhances the pro-angiogenic effect of \( \beta \)-o-glucan. In addition, the treatment with a similar dose of \( \beta \)-o-glucan significantly increased the angiogenic ability of HMVEC-C in vitro (Fig. S1).

\( \beta \)-o-glucan treatment rescues the vasculogenic activity under oxidative stress in vivo

The chronic exposure to PMA alone significantly halted the formation of the caudal artery at 24 hpf, measured as an increase in the distance...
between the end of the dorsal longitudinal anastomotic vessel (DLAV) and the caudal vein plexus (CVP) of Tg (kdrl: EGFP)s843Tg embryos (Fig. 5). In an additional group, 3% β-D-glucan counteracted the ROS-induced vascular toxicity and preserved the vasculogenic activity essential to lead the normal formation of the caudal artery (Fig. 5A, quantified in Fig. 5B). No impairment of vasculogenesis was detected in samples from β-D-glucan -treated embryos under normal conditions.

β-β-glucan modulation of cell viability and MnSOD expression does not rely on histone acetylation

In both microenvironmental conditions, 24 hrs treatment with 50 and 500 μM of NaBu had a slight but significant adverse effect on cell survival (Fig. 6A); although, 5 μM of NaBu did not affect the cell viability. As shown in Figure 6B and quantified in Figure 6C, NaBu increased the level of H4 acetylation in a dose-dependent manner without changing the endothelial MnSOD expression level in all the experimental conditions.

The simultaneous 24 hrs treatment of HUVECs with 3% w/v β-β-glucan and NaBu 5 μM did not interfere with β-β-glucan effects on cell viability (data not shown) and MnSOD expression (Fig. 7A, quantified in Fig. 7B, upper panel) even in the presence of high level of H4 acetylation (Fig. 7A, quantified in Fig. 7B, lower panel). Finally, the treatment of cells with 3% w/v β-β-glucan, alone or in combination with NaBu 5 μM, prevented the rise of O2/CO level (Fig. 8A for quantification of dEDT staining; Fig. 8B for representative images).

β-β-glucan modulation of human capillary formation does not rely on histone acetylation

Even if the 24 hrs treatment with NaBu 5 μM did not attenuate the angiogenic ability of HUVECs, it slightly increased capillary formation
β-D-glucan. B) Measurement of the level of MnSOD and H4 expression normalized over loading control (GAPDH). Acetylation level of H4 was quantified normalizing the amount of Ace tally-H4 protein over total protein. Cells were exposed to H2O2 for 24 hrs, alone or in combination with 3% β-D-glucan, NaBu (5 μM), or both. Unstressed and untreated cells were used as control (mean ± SD; n = 4). *P < 0.05 versus control; §P < 0.05 versus H2O2.

Discussion

The enhancement of endogenous angiogenic activity may hold promises for restoring adequate myocardial perfusion in ischemic heart. It is emerging that the optimization of antioxidant gene expression may promote mature capillary formation [33]. MnSOD is the most effective antioxidant enzyme [34] that protects myocardium via scavenging mitochondrial O2− [35]. Cardiac overexpression of MnSOD limits cardiac cell loss and renders the heart more resistant to the oxidative burst [36]. In addition, MnSOD gene transfer restores endothelial function through increasing activity of eNOS [37], which exerts direct pro-angiogenic effect [38]. Consistent with these observations, it has been suggested that increased MnSOD expression might enhance ROS-driven angiogenesis.

A recent study suggested that the use of natural molecules could be sufficient to induce endothelial MnSOD expression [39]. Hence, the identification of novel dietary compounds able either to increase MnSOD level or to enhance angiogenesis may prove rewarding in sight of therapeutic myocardial angiogenesis, therefore, avoiding invasive approaches.

We provided evidence that barley β-0-glucan is an active natural enhancer of the angiogenic potential of ROS-exposed endothelial cells related to increased MnSOD expression.

The treatment of monolayered H2O2-stressed HUVECs with 3% w/v β-0-glucan increased MnSOD level without reducing the expression of HSP70, a stress protein sufficient to active MnSOD [40]. In agreement with previous studies, the increase in the expression of MnSOD was useful to improve its enzymatic activity [41], which prevails over that of SOD1 in attenuating superoxide anion level [42]. The rise of β-0-glucan-induced MnSOD expression, which reduced the intracellular load of O2− in stressed endothelial cells, was related to the normal level of HIF-1α, thus rendering the cells more resistant to the oxidative burst. In fact, the reduction in O2− level suppressed the expression of HIF-1α [43], which plays a role in promoting cell death by autophagy [44]. On the other hand, the eNOS activation via phosphorylation of serine 1177 was enhanced by β-0-glucan without involving the activation of AKT/PKB, which is an established eNOS activator [27, 45]. Increased nitric oxide generation contributes to neutralize O2− in stressed cells [46], and the endothelial eNOS activation is also regulated in an AKT-independent manner [47]. In our study, the nitric oxide synthesis was significantly increased in stressed β-0-glucan-treated adult and newborn endothelial cells. Therefore, we have shown that β-0-glucan-induced MnSOD up-regulation encompasses both endothelial pro-survival features, by preventing the increase in HIF-1α expression, and the potential to reduce superoxide anion level, by activating eNOS in an AKT-independent manner.

Taking into account that the β-0-glucan effects were considerably more accentuated under oxidative stress than in normal conditions, we tested whether the increased MnSOD expression affects the angiogenic response to ROS. Notably, β-0-glucan-treated endothelial cells generated more vessels either with or without exogenous growth factors in the medium. In addition, we found that the extent of increasing tube formation from cultured HMVEC-C treated by similar dose of β-0-glucan was smaller than HUVECs (Fig. S1). Our data were
Fig. 8 NaBu does not affect β-D-glucan-induced superoxide anion down-regulation under chronic oxidative stress. (A) Quantification of the relative intensity of fluorescence in DHE positive cells in each experimental condition at rest or during oxidative stress (H2O2). (B) Representative images of DHE staining of HUVECs at rest or with H2O2, in the presence of either β-D-glucan, NaBu (5 μM), or both (mean ± SD; n = 4). *P < 0.05 versus control; ***P < 0.001 versus control; §P < 0.05 versus H2O2.

Fig. 9 NaBu does not affect β-D-glucan-induced capillary formation under chronic oxidative stress. (A) In vitro angiogenesis assay without exogenous growth factors. Intrinsic tube formation ability was tested, alone (untreated) or with 3% β-D-glucan, NaBu (5 μM), or both; at rest (left side) or during oxidative stress (right side). (C) In vitro angiogenesis assay with exogenous growth factors. Tube formation ability was tested, alone (untreated) or in combination with 3% β-D-glucan, NaBu (5 μM), or both; at rest (left side) or during oxidative stress (right side; mean ± SD; n = 4). *P < 0.05 versus control; §P < 0.05 versus β-D-glucan.

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in accordance with a previous study showing lower angiogenic response to exogenous factors of adult endothelial cells compared to
HUVECs [48]. The finding that β-o-glucan-treated cells exhibited a
significant yield of angiogenesis in the absence of either exogenous
growth factors or oxidative stress prompted the hypothesis that β-o-
-glucan exerts a direct pro-angiogenic activity. It is known that en-
dothelial cells form a dense capillary network regardless the con-
ventional paracrine growth factors [19, 49, 50]. Since MnSOD
overexpression promotes endothelial cell sprouting [41], it is conceiv-
able that β-o-glucan-induced angiogenesis depends upon MnSOD
level. In addition, we cannot exclude the direct pro-angiogenic effect
also exerted by NO generated from eNOS [38], which is activated by
MnSOD [37].

To confirm in vitro data, we performed in vivo experiments using
transgenic zebrafish embryos. In an additional experiment, we treated
PMA-exposed embryos with 3% w/v β-o-glucan. Prolonged PMA
treatment inhibits VEGF expression [51] and VEGF-induced angiogen-
esis by down-regulation of PKC [52]. In our model, long-term PMA
exposure remarkably hampered the development of the dorsal longi-
tudinal anastomotic vessel and the simultaneous treatment of PMA-
exposed embryos with β-o-glucan rescued the physiological vascular
development. β-o-glucan did not affect vessel density of healthy
embryos.

Unraveling mechanisms would offer options to better modulate
MnSOD-related angiogenesis by β-o-glucan. Endothelial cells express
dectin-1, a C-type lectin-like receptor, [53] and β-o-glucan effects are
suppressed with Dectin-1-specific blocking monoclonal antibody [54]. It suggests that the engagement of Dectin-1 might represent a
major initial step in endothelial gene expression modulation. It is
worth noticing that β-o-glucan-treated HUVECs showed a consistent
histone acetylation level, yet it was higher in H2O2-exposed cells. Our
results prompted the hypothesis that the binding of Dectin-1 to β-o-
-glucan induces MnSOD expression via histone acetylation. Shimazu
et al. [55], in fact, have shown that histone acetylation as a result of
inhibition of class I HDAC increases MnSOD expression. In our
model, with rising of histone acetylation level, the MnSOD expression
was unaffected by increasing doses of NaBu, a class I HDAC inhibitor.
At higher doses of NaBu (50 or 500 μM), we detected cell death in
the presence of higher level of histone acetylation and O2− (data not
shown). Conversely, the exposure of HUVECs to a lower dose of NaBu
(5 μM) added to complete medium enhanced angiogenesis. Nonethe-
less, the β-o-glucan effects were unaffected by co-treatment with
5 μM NaBu.

Our study verified that the β-o-glucan-induced rise of MnSOD
level and tube formation was not because of inhibition of class I
HDAC. Even if the pro-angiogenic effect of water-soluble β-o-glucan
was independent of HDAC activity, we cannot exclude that higher
doses of β-o-glucan may promote cell death similarly to NaBu. In fact,
a previous study demonstrated that treatment of HUVECs with a cock-
tail containing a higher dose of fungal β-o-glucan exhibited an anti-
angiogenic effect [18].

Studies are in progress to further dissect the panel of signaling
pathways modulating gene profile recruited by barley β-o-glucan.

In conclusion, we identified barley β-o-glucan as natural activator
of MnSOD expression and the angiogenic ability of ROS-exposed
endothelial cells regardless histone acetylation. Since mature endo-
thelial cells exhibit intrinsically lower expression of MnSOD compared
to endothelial progenitor cells [56], our results assume significance
to develop β-o-glucan-based approaches of therapeutic angiogenesis
for the prevention of heart failure.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online
version of this article:

Figure S1 Effects of β-o-glucan treatment on cardiac HMVECs.

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