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Proton pump inhibitors suppress iNOS-dependent DNA damage in Barrett's esophagus by increasing Mn-SOD expression

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ABSTRACT

Barrett's esophagus (BE), an inflammatory disease, is a risk factor for Barrett's esophageal adenocarcinoma (BEA). Treatment of BE patients with proton pump inhibitors (PPIs) is expected to reduce the risk of BEA. We performed an immunohistochemical study to examine the formation of nitrative and oxidative DNA lesions, 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxygaunosine (8-oxodG), in normal esophageal, BE with pre- and post-treatment by PPIs and BEA tissues. We also observed the expression of an oxidant-generating enzyme (iNOS) and its transcription factor NF-κB, an antioxidant enzyme (Mn-SOD), its transcription factor (Nrf2) and an Nrf2 inhibitor (Keap1). The immunoreactivity of DNA lesions was significantly higher in the order of BEA > BE > normal tissues. iNOS expression was significantly higher in the order of BEA > BE > normal tissues, while Mn-SOD expression was significantly lower in the order of BEA < BE < normal tissues. Interestingly, Mn-SOD expression and the nuclear localization of Nrf2 were significantly increased, and the formation of DNA lesions was significantly decreased in BE tissues after PPIs treatment for 3–6 months. Keap1 and iNOS expression was not significantly changed by the PPIs treatment in BE tissues. These results indicate that 8-nitroguanine and 8-oxodG play a role in BE-derived BEA. Additionally, PPIs treatment may trigger the activation and nuclear translocation of Nrf2 resulting in the expression of antioxidant genes, leading to DNA damage suppression. These DNA lesions can be useful biomarkers to predict both the risk of BEA and the efficacy of PPIs treatment to prevent BEA in BE patients.

1. Introduction

Barrett's esophagus (BE), a metaplastic columnar-lined esophagus, is caused by gastro-esophageal acid reflux disease (GERD). BE patients are 30- to 40-fold more likely to develop Barrett's esophageal adenocarcinoma (BEA) [1,2]. BE progresses to an intestinal-like structure during chronic GERD. It is suggested that BE is differentiated from adult stem cell lining at the basal layer of esophageal epithelium and bone marrow stem cells [3–5]. Molecular mechanisms of inflammation-mediated carcinogenesis followed by GERD still remain unclear. Proton pump inhibitors (PPIs), which also have anti-inflammatory activity [6,7], are usually used to treat BE. PPIs target H^+, K^+-ATPase through covalent binding to a sulfhydryl (–SH) group of the protein to impair its function [8]. Several studies have found that PPIs are associated with a reduced incidence of dysplasia [9–11] and neoplasia [12] in BE patients. Moreover, a case control study in a cohort of patients with BE indicated that taking PPIs, NSAID/aspirin or statin reduced the risk of BEA [13]. These studies suggested that PPIs could be used as chemopreventive agents for protection from BEA in patients with BE.

Chronic inflammation during GERD is an important factor causing esophageal carcinogenesis [14]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated during inflammation are considered to contribute to inflammation-mediated carcinogenesis [15]. ROS and RNS can generate 8-oxo-7,8-dihydro-2′-deoxygaunosine (8-oxodG) and 8-nitroguanine, markers of oxidative and nitrative DNA damage, respectively. Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) reacts with superoxide anions ($O_2^{\cdot-}$) from NAD(P)H oxidase to form peroxynitrite ($ONOO^-$), producing 8-nitroguanine [16]. Therefore, 8-nitroguanine is a more specific biomarker for inflammation than

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8-oxodG. Moreover, 8-nitroguanine and 8-oxodG were reported to be biomarkers for predicting the risk of and to play significant roles in inflammation-related carcinogenesis which may involve genetic instability and epigenetic change [17]. Although oxidative DNA damage has been reported [18], 8-nitroguanine has not yet been investigated in Barrett's esophagus.

We have hypothesized that the formation and accumulation of 8-nitroguanine and 8-oxodG may play an important role in BE-derived primary BEA through inflammation-mediated nitrative/oxidative stress via an imbalance of the oxidant and antioxidant systems. To understand the carcinogenic mechanisms induced by nitrative/oxidative stress, we examined inflammation-related DNA damage (8-nitroguanine and 8-oxodG) and protein expression involved in oxidant (iNOS and NF-κB) and antioxidant (Mn-SOD and Nrf2) systems in biopsy sections of BE compared with normal esophagus and BEA tissues. To clarify the effect of PPIs in relation to oxidative/nitrative stress, we also determined the formation of DNA lesions and the expression of iNOS, Mn-SOD, Nrf2 and Keap1 in BE tissues after PPIs treatment.

2. Material and methods

2.1. Human subjects

All tissues were obtained from endoscopic biopsies or endoscopic mucosal resections at the Tohoku University Hospital. Formalin-fixed and paraffin-embedded biopsy sections from 19 BE patients (14 males, mean age (S.D.): 64 (13)) and 12 BEA patients (11 males, mean age (S.D.): 65 (13)). Seven sections from normal esophagus (4 men, mean age (S.D.): 59 (6)) were used. Among the BE patients, only those with histologic confirmation of specialized intestinal metaplasia and three or more centimeters of macroscopic Barrett's epithelium were included. BEA was defined as an adenocarcinoma predominantly involving the tubular distal esophagus and histological evidence of adjacent Barrett's epithelium. In addition, patients with endoscopically and histologically normal esophagus undergoing an endoscopy for a routine diagnostic checkup were recruited as normal controls.

The PPIs used were Pariet (R) (Sodium rabeprazole, 10 mg) or Takepron (R) (Lansoprazole, 30 mg). After 3–6 months, BE tissues were collected as post-treatment samples. The study was approved by the Tohoku University Hospital Ethics Committee (No. 2003–149) and written informed consent was obtained from all subjects.

2.2. Immunohistochemical study

The rabbit polyclonal anti-8-nitroguanine antibody was produced as described previously [19]. Double immnunofluorescence was performed to examine the colocalization of 8-nitroguanine and 8-oxodG [19]. Paraffin-embedded sections were incubated with the primary antibodies (rabbit polyclonal anti-8-nitroguanine (1 μg/mL) and mouse monoclonal anti-8-oxodG (1:200, Japan Institute for the Control of Aging, Fukuroi, Japan) overnight at room temperature. The sections were next incubated with fluorescent secondary antibodies (Alexa 488-labeled goat anti-mouse IgG and Alexa 594-labeled goat anti-rabbit IgG antibodies, 1:400 each, Molecular Probes Inc., Eugene, Oregon, USA) for 3 h at room temperature. Finally, the nuclei were stained by 4'-6-diamidino-2-phenylindole (DAPI) and the sections were examined with a fluorescence microscope (LX70, Olympus, Tokyo, Japan) and a laser scanning confocal microscope (Fluoview FV1000-D, Olympus).

To examine the localization of NF-κB, iNOS, Mn-SOD, Keap1 and Nrf2, an immunofluorescence technique was performed using a mouse monoclonal anti-NF-κB p65 (1:50, Santa Cruz Biotechnology, CA, USA), mouse monoclonal anti-iNOS (1:200, Sigma, MO, USA), rabbit polyclonal anti-Mn-SOD (1:200,Millipore, CA, USA), rabbit anti-Keap1 (1:100, ProteinTech Group, Chicago, USA) or rabbit polyclonal anti-Nrf2 (1:100, AnaSpec, CA, USA) antibody as the primary antibody.

2.3. Immunohistochemical grading

We defined immunohistochemical grading (IHC grading) based on the intensity and frequency derived from the staining results in normal mucosal, columnar and cancer cells of normal esophageal, BE and BEA tissues, respectively. The staining intensity was scored as negative (0), weak (+1), moderate (+2), or strong (+3). The frequency of positive cells in a section was scored as negative (0), less than 25% (+1), 25–50% (+2), 51–75% (+3), or more than 75% (+4). An IHC score was assigned by multiplying the intensity score by the frequency score. IHC grading was assigned by an IHC score as follows: −, negative expression (0); +, weak expression (1–3), ++, moderate expression (4–6); +++, high expression (7–9) or ++++, very high expression (10–12).

2.4. Statistic analysis

The significance of differences among normal, BE and BEA groups was analyzed by Chi-square test. The difference between pre- and post-treatment with PPIs in BE patients was analyzed using the Wilcoxon signed rank test. $P < 0.05$ was considered to be statistically significant. The statistical analyses were performed using SPSS 19.0 for Windows software.

3. Results

3.1. Formation of 8-nitroguanine and 8-oxodG in esophageal samples

A double immunofluorescence study shows the formation of 8-nitroguanine and 8-oxodG in normal esophageal, BE, and BEA tissues (Supplementary Fig. 1). Little or no immunoreactivity for 8-nitroguanine and 8-oxodG was observed in epithelial and mucosa cells of normal esophageal tissues. Strong immunoreactivity was found in BE and BEA tissues. 8-Nitroguanine and 8-oxodG were colocalized in the nucleus of mucosa, columnar, inflammatory and stroma cells of BE tissues and in cancer cells of BEA tissues. Table 1 shows that the formation of nitrative and oxidative DNA lesions increased significantly in the order of BEA > BE > normal tissues.

3.2. Expression of iNOS, NF-κB, Mn-SOD and Nrf2 in esophageal samples

To clarify the mechanism by which gastric reflux agents generate 8-nitroguanine in BE and BEA, we examined the expression of iNOS, NF-κB, an antioxidant enzyme Mn-SOD and Nrf2. Supplementary Fig. 2 shows the distribution of iNOS, NF-κB, Mn-SOD and Nrf2 in normal esophageal, BE, and BEA tissues. iNOS and NF-κB were overexpressed in BE and BEA tissues compared with normal esophageal tissues as shown in Table 1. iNOS was found in the cytoplasm of columnar cells and some mucosa cells in BE tissues and also in the cytoplasm of cancer cells in BEA tissues, whereas not in normal esophageal tissues. NF-κB was found in the cytoplasm and translocated into the nucleus in inflammatory, columnar, and stroma cells of BE tissues and cancer cells in BEA tissues. Table 1 shows that the expression of iNOS was significantly increased in the order of BEA > BE > normal tissues. NF-κB expression tended to increase in the same order.

Mn-SOD was expressed in the cytoplasm of normal epithelium, columnar and cancer cells in normal, BE and BEA tissues, respectively. Moreover, it was expressed in fibroblasts and inflammatory cells in BEA tissues as shown in Supplementary

Table 1
Formation of DNA lesions and expression of iNOS, NF-κB, Mn-SOD and Nrf2 in esophageal tissues.

Biomarker	[$]Group	[#]IHC grade					[*]*P*-value	
		–	+	++	+++	++++	vs. Normal	vs. BE
8-Nitroguanine	Normal	6	1	0	0	0		
	BE	0	4	7	7	1	$P < 0.001$	
	BEA	0	0	3	4	5	$P = 0.001$	$P = 0.048$
8-OxodG	Normal	5	2	0	0	0		
	BE	0	4	7	7	1	$P = 0.001$	
	BEA	0	0	3	4	5	$P = 0.001$	$P = 0.048$
iNOS	Normal	6	1	0	0	0		
	BE	1	9	3	6	0	$P = 0.001$	
	BEA	0	0	6	3	3	$P = 0.001$	$P = 0.007$
NF-κB	Normal	6	1	0	0	0		
	BE	3	2	7	5	2	$P = 0.014$	
	BEA	0	0	2	5	5	$P = 0.001$	$P = 0.096$
Mn-SOD	Normal	0	1	6	0	0		
	BE	3	15	1	0	0	$P < 0.001$	
	BEA	6	3	1	1	1	$P = 0.017$	$P = 0.042$
Nuclear localization of Nrf2	Normal	1	1	2	2	0		
	BE	2	8	6	1	0	$P = 0.296$	
	BEA	1	2	2	2	4	$P = 0.536$	$P = 0.052$

[#] An IHC grade was assigned to each specimen according to the degree of staining as described in the Materials and methods. Normal = normal esophagus, BE = Barrett's esophagus, BEA = Barrett's esophageal adenocarcinoma.
[*] *P*-values were calculated by Chi-square test (versus (vs.) Normal and vs. BE).
[$] IHC grade was analyzed in normal mucosal, columnar and cancer cells in normal esophageal, BE and BEA tissues, respectively.

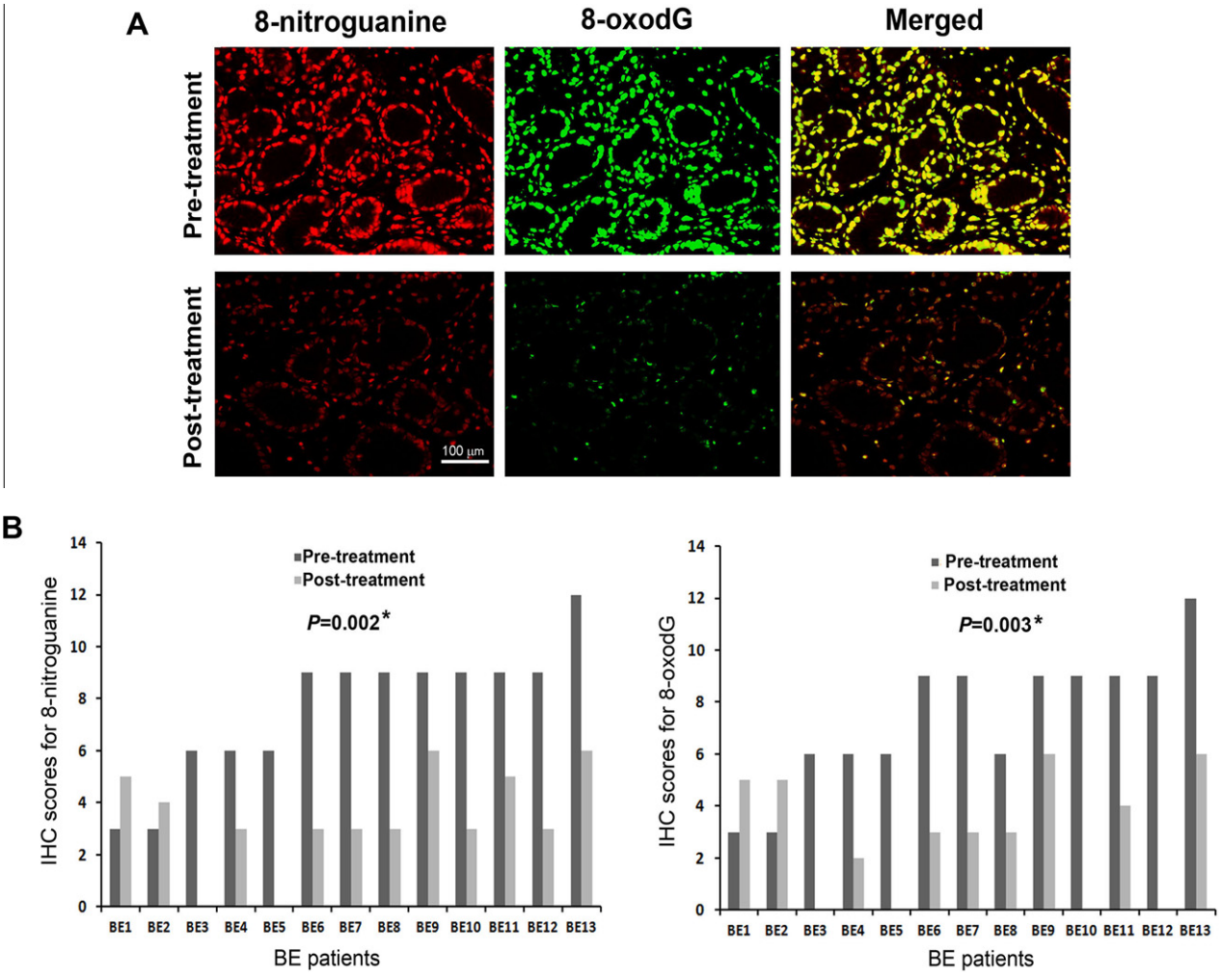

Fig. 1. Inhibitory effect of PPIs on DNA damage in Barrett's esophagus patients. (A) immunofluorescence analysis of 8-nitroguanine (red) and 8-oxodG (green). The original magnification is 200×. (B) IHC scores for 8-nitroguanine and 8-oxodG. [*]*P*-values (pre- and post-treatment) were analyzed with the Wilcoxon signed rank test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Mn-SOD expression was significantly higher in the order of normal mucosa > columnar cells of BE > cancer cells of BEA (Table 1). Nrf2 was expressed in the nucleus of mucosal cells in normal esophagus and located in the cytoplasm and nucleus of columnar cells in BE tissues. In BEA tissues, Nrf2 was highly expressed in both the cytoplasm and nucleus of cancer cells. However, the immunoreactivity for nuclear Nrf2 was not significantly different among normal mucosa, columnar, and cancer cells in normal, BE and BEA tissues.

3.3. Effect of PPIs on the decrease in DNA damage in Barrett's esophagus patients

Fig. 1A shows the effect of PPIs on DNA damage in BE patients. Immunoreactivity for 8-nitroguanine and 8-oxodG was decreased in the columnar cells of BE tissues after the treatment. Fig. 1B shows the change in the immunoreactivity of DNA lesions in individual BE tissues of 8-nitroguanine and 8-oxodG, respectively. IHC scores of DNA lesions were significantly decreased after the treatment ($n = 13$).

3.4. Effect of PPIs on the antioxidant system in Barrett's esophagus patients

To clarify the mechanism of the PPIs-induced reduction in 8-nitroguanine and 8-oxodG in BE tissues, we examined the expression of oxidant (iNOS) and antioxidant (Mn-SOD, Keap1 and Nrf2) systems by immunohistochemical analysis (Fig. 2A). Mn-SOD highly expressed and the nuclear localization of Nrf2 was also increased in the columnar cells in BE tissues after PPIs treatment. The expression levels of iNOS and Keap1 were not significantly different between pre- and post-treatment with PPIs in BE patients. Fig. 2B shows the change in immunoreactivity for nuclear Nrf2 and Mn-SOD in individual BE tissues. IHC scores for Mn-SOD and nuclear Nrf2 were significantly increased after the treatment.

4. Discussion

BE and BEA are inflammation-related diseases induced by gastric reflux [14]. Here, we demonstrated that nitrative (8-nitroguanine) and oxidative (8-oxodG) DNA lesions were formed in the order of BEA > BE > normal tissues. The results suggest that these lesions were formed in genomic DNA and accumulated during esophageal carcinogenesis. 8-Nitroguanine was previously observed in inflammation-related cancers [15,20]. 8-Nitroguanine is a mutagenic lesion because it is chemically unstable and can be spontaneously released, resulting in the formation of apurinic site, which can cause point mutations during DNA synthesis, such as G to T transversions [21,22]. Relevantly, Wu et al. reported critical roles for DNA polymerase ζ in cellular tolerance to nitric oxide-induced DNA damage [23]. 8-OxodG is also a potential mutagenic

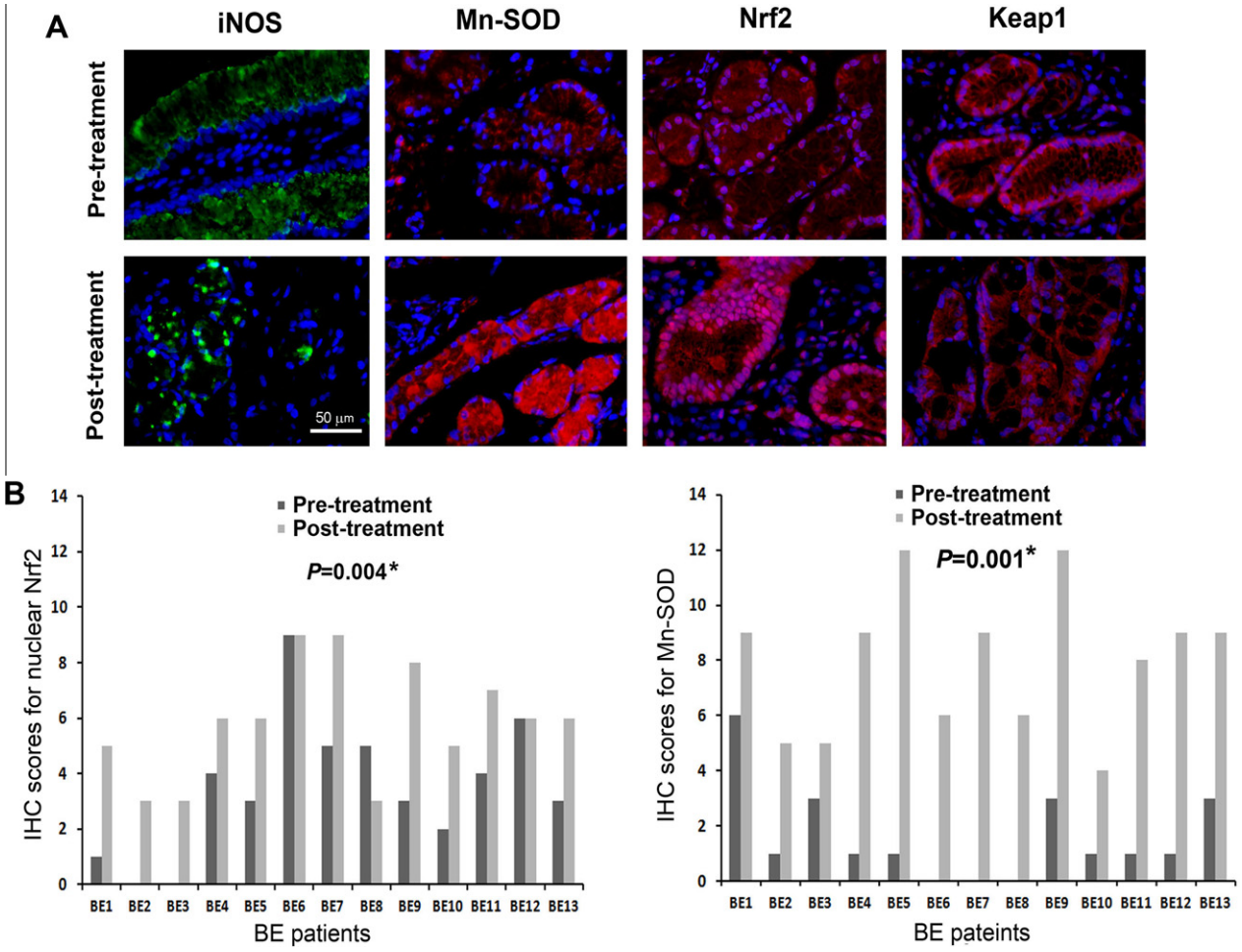

Fig. 2. Effect of PPIs on the expression of oxidants and antioxidants in Barrett's esophagus patients. (A) immunofluorescence analysis of iNOS (green), Mn-SOD (red), Nrf2 (red) and Keap1 (red). The nucleus was stained by DAPI (blue). The original magnification is 200×. (B) IHC scores for the nuclear localization of Nrf2 and the expression of Mn-SOD. *P-values (pre- and post-treatment) were analyzed using the Wilcoxon signed rank test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DNA lesion resulting in point mutations such as G to T transversions by the misincorporation of adenine during DNA synthesis [24,25]. Interestingly, these DNA lesions were significantly decreased in BE tissues after PPIs treatment. Our findings are important to clarify the mechanism behind the carcinogenesis of BEA initiated by 8-nitroguanine and 8-oxodG in BE tissues. 8-Nitroguanine and 8-oxodG could be used as biomarkers to predict the risk of BEA in BE patients.

To clarify the mechanism of gastric acid reflux-induced DNA damage in BE and BEA, we examined the expression of iNOS, NF-κB, Mn-SOD and Nrf2. Our results indicated that the expression of an oxidant-generating enzyme (iNOS) and NF-κB increased in the order of BEA > BE > normal tissues while the antioxidant enzyme (Mn-SOD) expression was significantly decreased in the order of BEA < BE < normal tissues. NF-κB mediates the expression of pro-inflammatory proteins, including iNOS, and plays an important role in the promotion of inflammation-associated carcinogenesis [26–28]. Accumulation of NF-κB in the nucleus leads to the up-regulation of iNOS expression, resulting in the overproduction of NO. NO reacts with $O_2^{\cdot-}$, produced by NAD(P)H oxidase, to form $ONOO^-$. $ONOO^-$ can directly form DNA lesions, including not only 8-oxodG but also 8-nitroguanine [15,16]. Nrf2 seemed to be highly expressed in cancer cells of BEA ($P = 0.052$ compared to BE), however the downstream protein Mn-SOD showed significantly weaker expression in the cancer cells (Table 1, compared to normal and BE tissues). Mn-SOD is an antioxidant involved in defense against $O_2^{\cdot-}$ [29]. Mn-SOD expression was found to be reduced in columnar cells in BE tissues and esophageal adenocarcinoma cells [30,31]. Reduced expression of Mn-SOD in the columnar cells of BE and cancer cells of BEA tissues may result in increased production of $O_2^{\cdot-}$, which reacts with NO generated by iNOS resulting in the formation of large amount of $ONOO^-$. Our results suggest that the 8-nitroguanine and 8-oxodG in BE and BEA can be explained by both iNOS over-expression and Mn-SOD under-expression.

The formation of nitrative and oxidative DNA lesions was significantly decreased in BE tissues after PPIs treatment. To clarify the mechanism by which PPIs reduce DNA damage, we examined the expression of oxidative/nitrative stress-related proteins including iNOS, Mn-SOD, Keap1 and Nrf2 in BE tissues pre- and post-treatment. Interestingly, the expression of iNOS did not change significantly after the treatment, although the expression of Mn-SOD was increased. The nuclear localization of Nrf2 was also significantly increased in BE tissues after the treatment with PPIs. The expression of the Nrf2 inhibitor (Keap1) was unchanged after treatment. Nrf2 is a transcription factor of several antioxidants and cytoprotective proteins including Mn-SOD, which acts by binding to the antioxidant response element (AER) [32–34]. The transcription factor and its target genes have been reported to be involved in not only protection against oxidative stress but also the suppression of inflammatory responses [35,36]. Keap1 is a sulfhydryl-rich protein, which binds to Nrf2 in the cytoplasm leading to its ubiquitination and degradation [37–39]. Lansoprazole, a PPI, was reported to be involved in the oxidation of Keap1 and activation of Nrf2 leading to the up-regulation of the antioxidant heme oxygenase-1 [40]. Morri et al. reported that Omeprazole, a PPI, was transformed into an SH-reactive strong fluorescent molecule in an acidic medium and the addition of glutathione- or protein-containing sulfhydryl groups, such as pepsin, to the medium decreased the fluorescence [41], suggesting that PPI can react with sulfhydryl-rich proteins in certain conditions. Therefore, our results suggest that PPIs treatment mediated the activation and nuclear translocation of Nrf2 via the reaction with Keap1 leading to the up-regulation of several antioxidants such as Mn-SOD resulting in DNA damage suppression, which may reduce the risk of esophageal cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.152.

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