Serum S-glutathionylated proteins as a potential biomarker of carotid artery stenosis

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Abbreviations: CS, carotid artery stenosis; GSH, Glutathione; GSS-BSA, S-glutathionylated bovine serum albumin; DTT, dithiothreitol; GRX, glutaredoxins; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

Abstract

Objectives: As oxidative stress is known to be associated with the development of atherosclerosis, we investigated whether the serum S-glutathionylated proteins were increased in patients with carotid artery stenosis (CS).

Design and Methods: Fifty-four patients with CS and 20 age-matched non-CS patients were involved in this study. S-glutathionylated proteins in serum were examined by immunoblot analysis using an antibody against S-glutathionylated bovine serum albumin.

Results: The antibody against S-glutathionylated bovine serum albumin was confirmed to specifically recognize the serum S-glutathionylated proteins in patient samples. The S-glutathionylated proteins in serum were significantly increased in the patients with CS ($p<0.01$) compared to the non-CS patients, and the increase did not depend on the stage of CS. Logistic regression analysis revealed that the serum levels of S-glutathionylated proteins were associated with the development of CS ($p<0.01$).

Conclusions: Oxidative stress likely contributes to the development of CS, and serum S-glutathionylated proteins may be a potential biomarker of CS.

Keywords: carotid artery stenosis, *S*-glutathionylated protein, oxidative stress

Introduction

Atherosclerotic carotid artery stenosis (CS) is one of the common cerebrovascular diseases that can cause stroke. Carotid endarterectomy or carotid artery stenting are effective treatments to reduce the risk of stroke due to CS [1,2]. However, these treatments are associated with high rates of mortality and morbidity, and they are only acceptable for patients with high-risk symptomatic CS [1,2]. Therefore, the diagnosis of asymptomatic stenosis during the early stage is essential for preventing the progression of CS. Unfortunately, there is no specific and sensitive biomarker for the clinical diagnosis of CS, especially for asymptomatic patients at an early stage of the disease.

Oxidative stress can damage macromolecules such as DNA, lipids, and proteins. Various oxidants, such as those produced by lipid peroxidation and protein oxidation, induce the expression of vascular cell adhesion molecule-1 and monocyte chemotactic protein-1 in endothelial cells, which improve endothelial monocyte adhesion and monocyte infiltration and contribute to the initiation and/or progression of atherosclerosis [3-6]. Although oxidative stress is an important determinant in the development of inflammation, cancers, and cardiovascular disorders [7-9], its association with carotid stenosis has not been fully defined.

Glutathione (GSH) and GSH-related enzymes, such as GSH peroxidase and glutathione S-transferase, are thought to play an important role in protecting the vascular system against oxidative stress [7,10]. Under oxidative stress, S-glutathionylation of proteins occurs through thiol-disulfide exchange with oxidized glutathione or through the reaction of oxidant-induced protein thiyl radicals with reduced glutathione [10]. This modification helps to protect proteins against irreversible oxidation [11]. Recent studies have demonstrated that S-glutathionylation can produce discrete modulatory effects on protein function [12,13]. Therefore, S-glutathionylated proteins have been actively investigated with reference to problems of biological interest and as potential biomarkers of human diseases associated with oxidative stress [11,14-16].

In this study, we developed an antibody to specifically recognize the S-glutathionylated proteins, and then we investigated whether the serum S-glutathionylated proteins increased in patients with different stages of CS.

Materials and Methods

Patients

We enrolled 54 patients who visited the Department of Neurosurgery at Nagasaki University Hospital and underwent an examination of the carotid artery through carotid duplex ultrasound. The medical history was taken, and a physical examination and laboratory tests were performed for all subjects. Twenty age-matched patients without internal carotid artery stenosis were used as controls. This study was approved by the ethics committees of Nagasaki University Hospital, and all participants gave written informed consent prior to the commencement of study.

Development of an antibody to measure S-glutathionylated proteins

The development of an antibody that can specifically detect the serum S-glutathionylated proteins in patients was performed as described by Hjelle OP *et al*. [17] with slight modification. Briefly, S-glutathionylated bovine serum albumin (GSS-BSA) was used to immunize rabbits to generate the anti-GSS-BSA antibody. After 2 months of immunization, serum was collected for the following immunoblot analysis and used at a 2000-fold dilution.

Immunoblot analysis of S-glutathionylated proteins in serum

Blood samples were collected from patients 1-2 days before surgery. After centrifugation at 4°C, the serum was immediately collected and stored at -80°C until analysis. A total of 10 μl of serum was treated with 300 μl of Laemmli's denatured solution. The samples (10 μl) were electrophoresed on 7.5% SDS polyacrylamide gels in

the absence of dithiothreitol (DTT), and the proteins in the gels were transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and then incubated with the anti GSS-BSA antibody overnight at 4°C. After three washes, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG antibodies. Proteins in the membranes were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences). The intensity of the bands ranging from 35 to 250 kDa was measured as described previously [18], and we loaded different concentrations (5, 10, 20 ng) of S-glutathionylated bovine serum albumin as internal controls to establish a standard curve for each Western blot.

Immunohistochemical staining

The carotid artery tissues from endarterectomy were fixed, and tissues with obvious atherosclerotic lesions were selected to be embedded in paraffin. To detect the oxidative stress-associated damage in carotid artery tissues with atherosclerotic lesions, 5-μm-thick tissue slices were stained with an immunohistochemical technique, as described previously [19]. Primary monoclonal antibodies against human 8-OHdG (a marker for oxidative DNA damage), 4HNE (a marker for lipid oxidation), and dityrosine (a marker for protein oxidation) (JaICA, Shizuoka, Japan) were used to detect the oxidative stress-induced damage in the carotid artery tissues. Sections were washed three times and then incubated with a FITC-conjugated secondary antibody (Dako). Nuclei were stained with DAPI. Carotid artery tissues from patients who received surgical treatment for other diseases were used as control.

Statistical analysis

The statistical analysis was performed using Stat-View (version 4.5, Abacus Concepts Inc., Calabasus, CA, USA). One-way ANOVA was used to compare continuous

variables, and the Tukey-Kramer test was used for multiple comparisons. When appropriate, two-way cross-tabulation with the chi-square test was used for binary variables to compare differences between groups. Statistically significant differences among groups were analyzed by the Kruskal-Wallis test with Dunn's test. When S-glutathionylated proteins were undetectable by immunoblot analysis, a score of 0 was assigned. According to the North America Symptomatic Carotid Endarterectomy Trial (NASCET) criteria [20], we divided these CS patients into mild, moderate, and severe stages and assigned a score of 1, 2, or 3, respectively. The effect of *S*-glutathionylated proteins on the CS stage was analyzed using an ordinal logistic regression model with dyslipidemia, hypertension, smoking, diabetes, cardiovascular disease, and peripheral artery disease as covariates. Values of $p<0.05$ were considered statistically significant.

Results

Clinical characteristics of patients

According to the NASCET criteria [20], among the 54 patients with CS, 15 cases had mild stenosis (less than 50% stenosis), 10 cases had moderate stenosis (50-70% stenosis), and 29 cases had severe stenosis (more than 70% stenosis).

The characteristics of the patients, including their age, gender, and a collection of potential risk factors, are listed in the table (***Table 1***). Compared to control non-CS patients, male gender, current smoking, and hypertension were significantly more frequent among the CS patients (*p<0.05*), but all other parameters, including the complications of coronary artery disease and other peripheral artery diseases, were comparable between groups.

Serum levels of S-glutathionylated proteins increased in CS patients

To test the S-glutathionylation of proteins *in vitro*, 2 μl of serum from a healthy volunteer was incubated with 2 pmoles of ^{35}S-GSH, 20 nmoles of glutathione disulfide,

and 100 nmoles of H_2O_2 in 150 μl of phosphate-buffered saline at 4°C for 24 hrs. This incubation extensively induced the S-glutathionylation of serum proteins (lane 2, ***Figure 1A***), but the S-glutathionylation was reversed by the addition of DTT, a small-molecule redox reagent (lane 3, ***Figure 1A***), suggesting that the formation of S-glutathionylated proteins is redox dependent.

As shown in Figure 1B, the anti-GSS-BSA antibody that we produced reacted with GSS-BSA (positive control, lane 1, the left image in ***Figure 1B***). This antibody also reacted with multiple S-glutathionylated proteins in serum from a CS patient (lane 2) and a patient with arteriosclerosis obliterans who were found to have an increase of serum S-glutathionylated proteins in our previous study [21] (lane 3, the left image in ***Figure 1B***). Furthermore, compared to the patient with arteriosclerosis obliterans, the CS patient had thinner bands approximately 175 kDa but showed a distinct band approximately 35 kDa. As β-mercaptoethanol can recover the oxidized -SH residue, decomposition of S-glutathionylated proteins by stripping the membrane with β-mercaptoethanol resulted in a negative reaction with the anti-GSS-BSA antibody (the right image in ***Figure 1B***). Furthermore, we confirmed that the anti-GSS-BSA antibody also reacted with another glutathionylated protein that made from transferrin (GSS-transferrin, lane 2), but did not react with the intact form of transferrin (lane 1, the left image in ***Figure 1C***). The stripping of membrane with β-mercaptoethanol showed a negative reaction with the anti-GSS-BSA antibody (the right image in ***Figure 1C***). This confirmed that the anti-GSS-BSA antibody could specifically recognize various S-glutathionylated proteins in serum.

A typical immunoblot is shown in Figure 2 (***Figure 2A***). Although a distinct thick band was detected that was approximately 175 kDa, the anti-GSS-BSA antibody recognized different sizes of S-glutathionylated proteins in serum, indirectly indicated the pan-glutathione nature of the anti-GSS-BSA antibody. Considering that S-glutathionylation of proteins generally occurs under oxidative stress and we did not know which S-glutathionylated protein would contribute to the development of CS, we

measured all of the S-glutathionylated proteins ranging from 35 to 250 kDa, as marked (***Figure 2A***). The levels of S-glutathionylated proteins were significantly increased in patients with CS when compared to the control non-CS patients (***Figure 2B***).

We further investigated how the levels of S-glutathionylated proteins changed with the stages and characterizations of CS (***Figure 3***). The median level of S-glutathionylated proteins was 0.91 in the control subjects, 1.75 in the patients with mild CS, 2.16 in the patients with moderate CS, and 2.44 in the patients with severe CS, respectively. Compared with the control subjects, the levels of *S*-glutathionylated proteins were significantly increased at all stages of CS (***Figure 3A***, $p<0.05$). Although the levels of S-glutathionylated proteins were slightly increased in more severe stages of CS, there was no significant difference among the three stages of CS.

Among the 39 patients who received carotid endarterectomy with moderate to severe CS, 20 patients were symptomatic and 19 patients were asymptomatic. According to the previously published criteria [22], 31 of the 39 patients were pathologically diagnosed as having unstable CS and only 8 patients were diagnosed as having stable CS. The level of S-glutathionylated proteins was not significantly different between patients with stable and unstable CS (***Figure 3B***), although the patients with unstable CS showed higher levels of S-glutathionylated proteins than those with stable CS. The sample size in this study may have been too small to detect significance. Similarly, the level of *S*-glutathionylated proteins was also not significantly different between patients with symptomatic and asymptomatic CS (***Figure 3C***). Interesting, the level of S-glutathionylated proteins in these CS patients complicated with coronary artery disease (1.65±0.78) was measured relatively lower than in those CS patients without coronary artery disease (2.12±1.62, p=0.37). Although we have also measured the CRP, hsCRP, HbA1C, Protein C, total cholesterol, HDL-C, LDL-C in serum, all of these parameters in did not significantly differed between CS patients and control non-CS patients.

The relationship between S-glutathionylated proteins and CS was also analyzed

using an ordinal logistic regression model with covariates (***Table 2***). The coefficient, standard error, and *p* value for the S-glutathionylated proteins were 0.067, 0.020, and 0.0013, respectively. Similarly, the *p* values for hypertension and smoking were less than 0.05. The data suggest that the S-glutathionylation of proteins in serum is likely associated with the progress of CS**.** However, the S-glutathionylation of proteins in serum did not significantly related with the gender, age, and the levels of CRP protein, total cholesterol, and LDL in serum.

Oxidative stress-related damage in carotid artery tissue from patients with CS

Immunohistochemical staining revealed that many cells strongly expressed several oxidative stress markers, including 8-OHdG (upper), 4HNE (middle), and dityrosine (lower), suggesting oxidative damage to DNA, lipids, and proteins in the carotid artery tissue from patients with CS (left panel, ***Figure 4***). However, the expression levels of 8-OHdG, 4HNE, and dityrosine were negative or very low in the carotid artery from control subjects without CS (right panel, ***Figure 4***).

Discussion

The most important finding of this study is that the levels of S-glutathionylated proteins were elevated in serum from patients with CS, even at the early stage. This indicates that serum S-glutathionylated proteins may be able to serve as potential independent biomarkers for CS. Immunohistological staining clearly revealed oxidative damage to the DNA, lipids, and proteins in the carotid artery tissues from CS patients, indicating that oxidative stress is likely associated with the development and/or progression of CS.

In healthy individuals, plasma contains abundant anti-oxidants, such as vitamin C, vitamin D, vitamin E, albumin, and GSH, which provide powerful protection against oxidative stress [23]. GSH is one of the most abundant anti-oxidants and plays a critical

role in protection from oxidative damage. Levels of GSH and oxidized forms of GSH in human plasma are generally kept at 2.8 μM and 0.14 μM, respectively, but these concentrations are influenced by aging and many pathological conditions [24,25].

Protein S-glutathionylation, the reversible binding of glutathione to protein thiols, is involved in protein redox regulation, storage of GSH, and protection of protein thiols from irreversible oxidation [26]. Protein S-glutathionylation levels increase under conditions of oxidative stress and are controlled by glutaredoxins (GRX) that, under physiological conditions, preferentially deglutathionylate cysteines and restore sulfhydryls [27]. An impairment of redox regulation implies an increase in the susceptibility of compromised individuals to oxidative stress [28]. We have recently reported that estradiol protects cardiovascular function by upregulating the GSH/GRX redox system to increase anti-apoptotic activity [29,30].

Altered levels of S-glutathionylated proteins have been recently demonstrated to associate with various pathologies, such as diabetes, atherosclerosis, inflammatory, and cancer [11,14-16]. In the present study, serum S-glutathionylated proteins were significantly increased in patients with CS, suggesting an imbalance of the redox state and protein thiols in the serum. However, further investigations are needed to clarify the actual involvement of protein S-glutathionylation in the development and/or progression of CS. In another study, the S-glutathionylated proteins in serum were found to differ between CS patients and patients with arteriosclerosis obliterans [21]. It will be interesting to determine whether the pattern of S-glutathionylated proteins in serum has disease specificity.

Many risk factors, such as hypertension, diabetes, obesity, dyslipidemia, smoking, and inflammation, have previously been reported to be associated with the development of CS [31]. Although multiple risk factors are likely to be more predictive than a single risk factor for CS [32], it is still difficult to identify patients with asymptomatic CS at the early stage of the disease. The serum level of S-glutathionylated proteins was significantly

increased in CS patients at an early stage, and it was associated with the development of CS by ordinal logistic regression analysis. Our results suggest that the level of S-glutathionylated proteins in serum might be a potential independent indicator of the development of CS.

Cigarette smoking is well-known as a major risk factor for atherosclerotic vascular diseases, including CS and stroke. In this study, nearly 40% of the CS patients were currently smokers, and ordinal logistic regression analysis showed a significant relationship between CS and smoking. As cigarette smoking is well-known to induce oxidative stress, it will be interesting to determine the causal relationship between cigarette smoking and the S-glutathionylation of proteins in serum, which contribute to the development of CS.

Although the level of S-glutathionylated proteins may be a potential biomarker for CS, it is not likely to be sufficiently sensitive to distinguish the stages (early *vs.* severe) and types (unstable *vs.* stable; symptomatic *vs.* asymptomatic) of disease. We did not also show the advantages of S-glutathionylated proteins in serum as a biomarker of CS, by comparing with other biomarkers that have been reported previously by other groups [33-36]. Otherwise, the small numbers of patients in this study limited us to achieve significance of many other common risk factors, such as dyslipedemia and inflammatory, that has previously been demonstrated to contribute the development of CS. Further study involving in a large size of population will be needed to confirm the sensitivity and specificity of the S-glutathionylation of proteins in serum as a biomarker of CS.

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Table 1. Clinical characteristics of patients

	Control **(n=20)**	***Carotid stenosis*** **(n=54)**
Age (y)	**65±12**	**68±8**
Sex (Male/Female)	**11/9**	**44 /10**
Hypertension	**7 (35)**	**47(87) ***
Diabetes	**13 (65)**	**33(61)**
Dyslipidemia	**13 (65)**	**27 (50)**
Cardiovascular diseases	**2 (10)**	**13 (24)**
Peripheral artery diseases	**1 (5)**	**3 (6)**
Current smoking	**1 (5)**	**21 (39) ***

***p <0.05 *vs* Control**

Table 2. Ordinal logistic regression model for carotid stenosis

Variables	*Coefficient*	*Standard error*	*p-value*
S-Glutathionylated proteins	0.067	0.02	0.001
Hypertension*	0.24	0.08	0.004
Diabetes*	0.03	0.07	0.638
Dyslipedemia*	-0.09	0.07	0.204
Cardiovascular diseases*	0.11	0.09	0.253
Peripheral artery diseases*	0.02	0.16	0.900
Current smoking*	0.15	0.08	0.047

*** Positives are defined as 1, and negatives are defined as 0.**

Figure Legends

Figure 1. *S-glutathionylation of proteins.* ***A***) S-glutathionylation of proteins was induced *in vitro* by exposure to 100 nmoles H_2O_2 (lane 2), but it was reversed by the addition of DTT (lane 3). ***B***) An antibody against S-glutathionylated bovine serum albumin (GSS-BSA) reacted with the S-glutathionylated proteins in serum from both a patient with carotid artery stenosis (lane 2) and a patient with arteriosclerosis obliterans (lane 3). The specificity of the antibody was confirmed by stripping with β-mercaptoethanol (the right image). ***C***). The Anti-GSS-BSA also reacted with with S-glutathionylated transferrin (GSS-transferrin, lane 2), but did not react with the intact form of transferrin (lane 1, the left image). Again, the stripping of membrane with β-mercaptoethanol showed a negative reaction with the anti-GSS-BSA antibody (the right image).

Figure 2. *Immunoblot analysis of S-glutathionylated proteins in serum.* ***A***) The images show the increased level of S-glutathionylated proteins in serum from CS patients compared to control subjects. ***B***) Semi-quantitative measurement showed that the intensity of the S-glutathionylated proteins of 35-250 kDa (as marked) was significantly higher in CS patients than in control subjects.

Figure 3. *S-glutathionylated proteins in CS patients with different stages and types of disease*. ***A***) The serum levels of *S*-glutathionylated proteins did not differ significantly among patients with mild, moderate, and severe stages of CS, although the levels of *S*-glutathionylated proteins at all stages of CS were significantly higher than in control subjects without CS. The levels of S-glutathionylated proteins did not significantly differ between patients with stable and unstable CS (***B***) or between patients with symptomatic and asymptomatic CS (***C***).

Figure 4. *Detection of oxidative damage to DNA, lipids, and proteins in carotid artery*

tissue by immunohistochemical staining. The expression of oxidative stress markers for DNA (8-OHdG, upper), lipids (4HNE, middle), and proteins (dityrosine, lower) were clearly detected in the carotid artery tissue from patients with CS (left panel), but these markers were negative or low in carotid artery tissue from control subjects.

Figure 1.

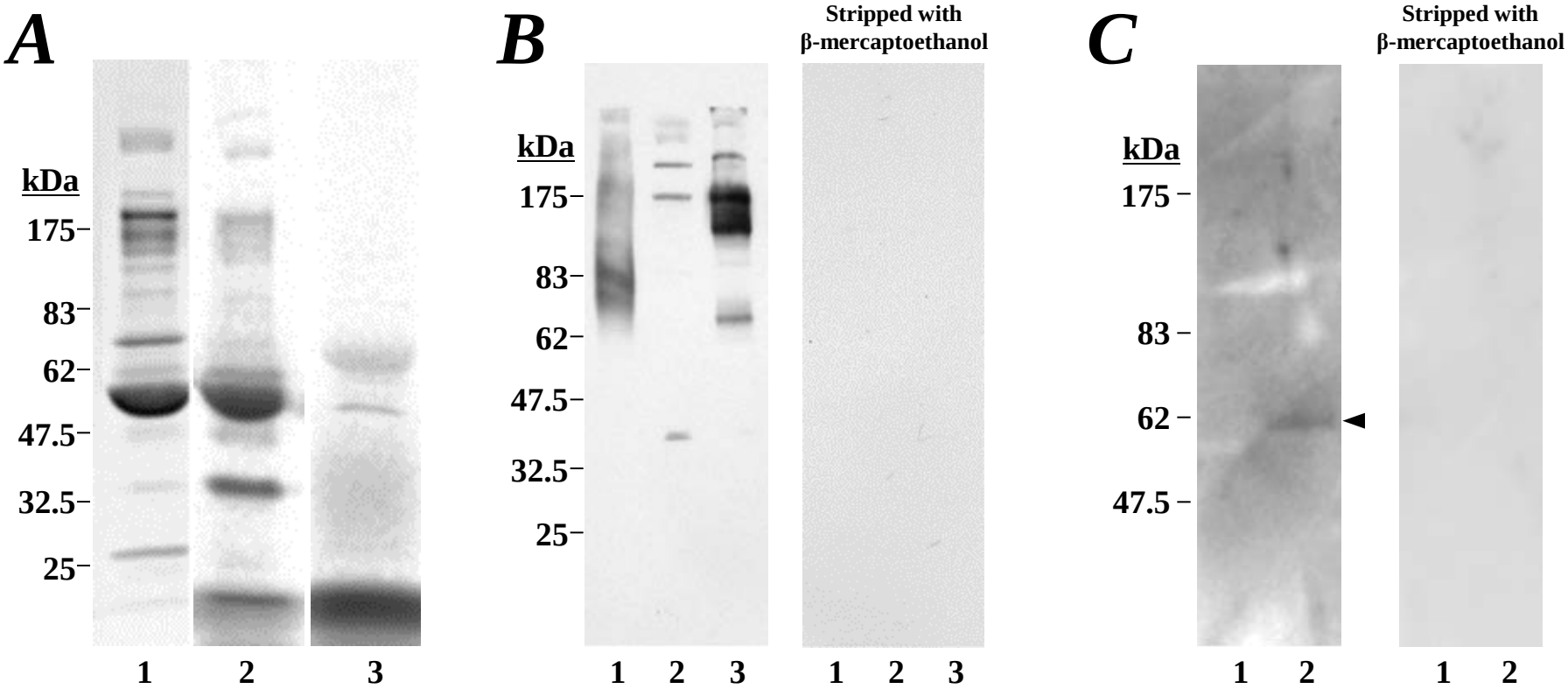
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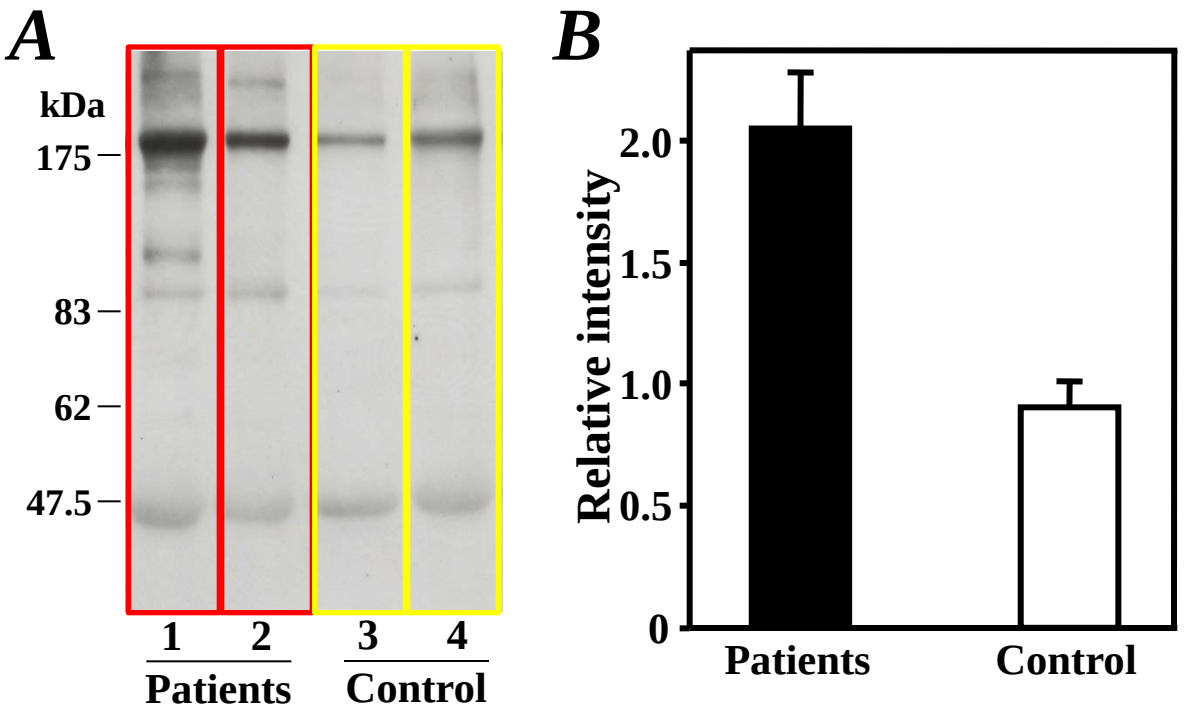
Figure 2.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Control

Figure 3.

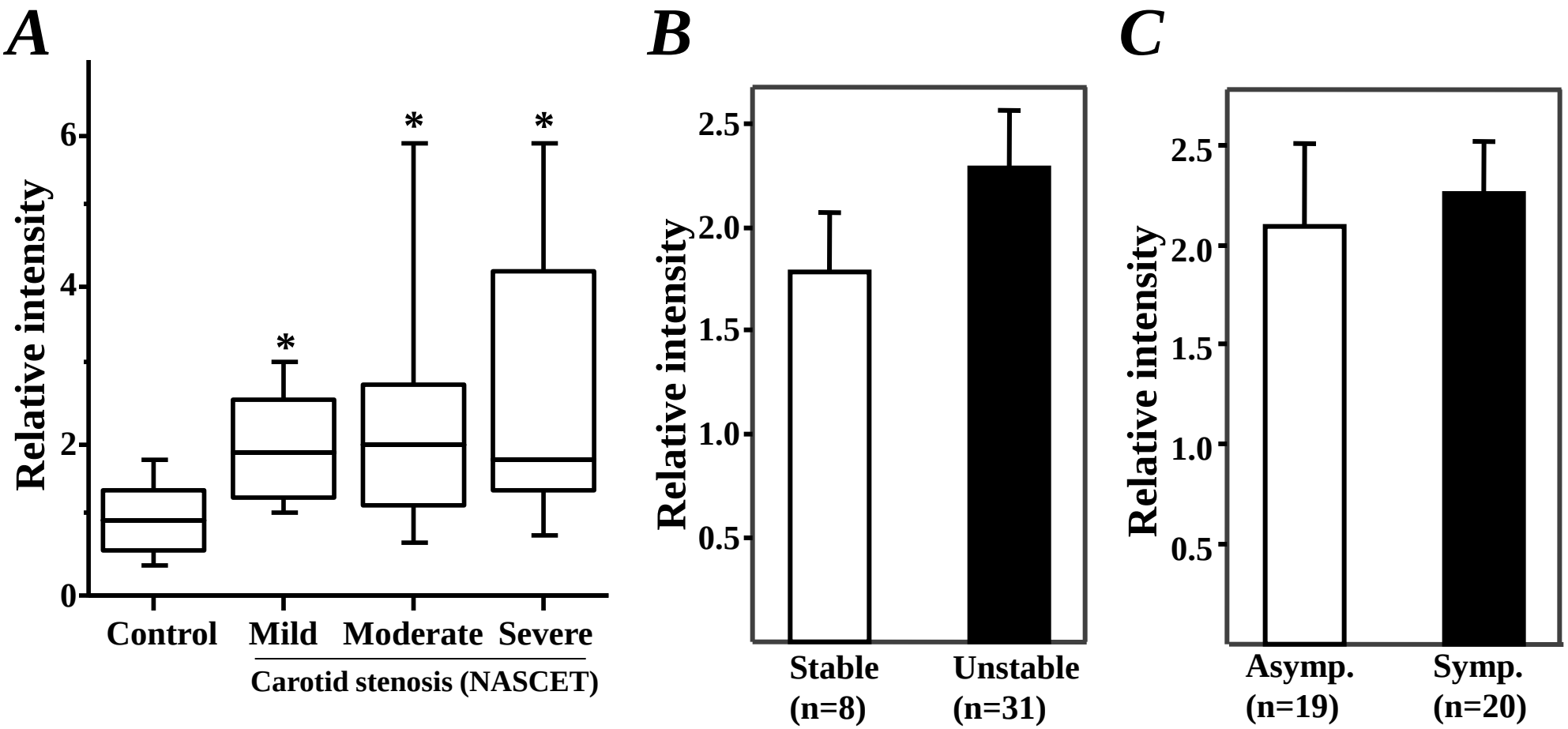
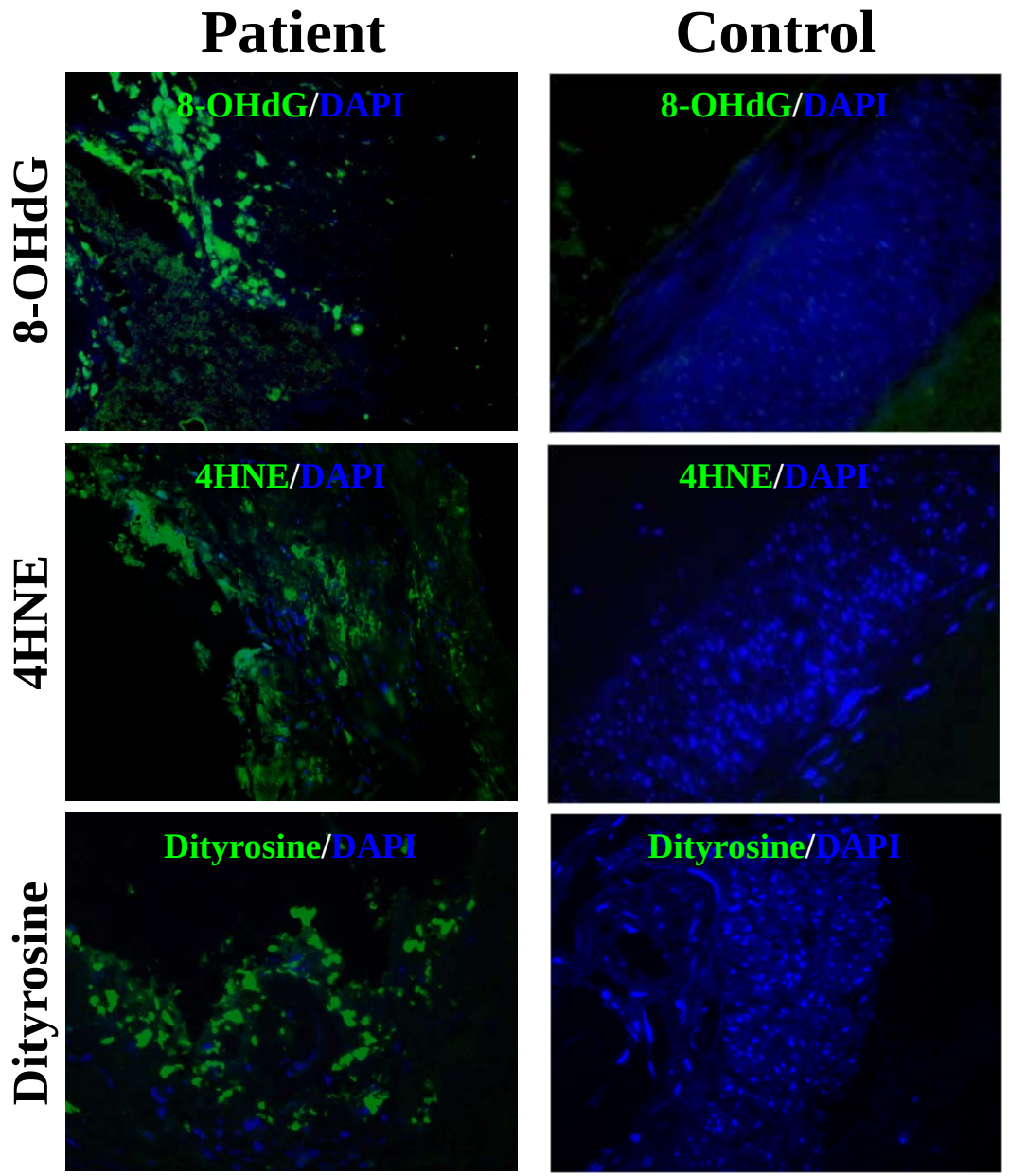

A
Relative intensity
0
2
4
6
*
*
*
Control
Mild
Moderate
Severe
Carotid stenosis (NASCET)
B
Relative intensity
0.5
1.0
1.5
2.0
2.5
Stable
(n=8)
Unstable
(n=31)
C
Relative intensity
0.5
1.0
1.5
2.0
2.5
Asymp.
(n=19)
Symp.
(n=20)

Figure 4.


Patient
Control
8-OHdG
4HNE
Dityrosine
8-OHdG/DAPI
8-OHdG/DAPI
4HNE/DAPI
4HNE/DAPI
Dityrosine/DAPI
Dityrosine/DAPI