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Effect of MPO/H_2O_2/NO^- system on nitric oxide-mediated modification of TTR amyloid and serum TTR in FAP ATTR Val30Met patients

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ABSTRACT. Amyloid deposits consist of protein fibrils and amorphous material, and this deposition is related to oxidative stress. Previously, we demonstrated the presence of high-density lipoproteins and/or lipids in amyloid deposits of familial amyloid polyneuropathy patients. In this study, the presence of myeloperoxidase (MPO) in amyloid deposits was demonstrated using immunohistochemical staining. In contrast, normal surrounding tissues were consistently negative for MPO. Nitrotyrosine was present in amyloid deposits after being exposed to the MPO/H_2O_2/NO^- system by immunohistochemical staining, and the oxide mediated modification of serum transthyretin (TTR) was observed upon exposure to the MPO/H_2O_2 system using two-dimensional gel electrophoresis and TTR Western blotting. This observation revealed that the TTR amyloid deposits and serum TTR were oxidized by the MPO/H_2O_2/NO^- system. Nitric oxide-mediated modification of TTR may play a role in

amyloidogenesis *in vivo*.

Key words: Familial amyloid polyneuropathy; Nitrotyrosine; High-density lipoprotein; Amyloid; Myeloperoxidase

INTRODUCTION

In familial amyloid polyneuropathy (FAP) and senile systemic amyloidosis (SSA), extracellular protein fibrils are combined with deposits of amorphous material (Inoue et al., 1998). Protein fibrils are composed of a surface layer and a core, which is the amyloid P component in a microfibril-like structure. In FAP and SSA, the surface layer is nearly completely composed of transthyretin (TTR) (Westermark et al., 1990; Serpell et al., 1995; Inoue et al., 1998).

Recently, it was reported that lipoprotein and/or lipids are present in amyloid deposits in systemic amyloidosis (Ikeda et al., 2011). Additionally, the products of lipid peroxidation, such as 4-hydroxy-trans-2-nonenal (HNE), are components of amyloid formation. Furthermore, the level of thiobarbituric acid reactive substances (TBARS), another marker of lipid peroxidation, is increased in amyloid-rich tissues of FAP patients compared with those in control subjects (Ando et al., 1997; Sun et al., 2006).

It is well known that amino acid substitutions in TTR destabilize the tetramer into monomeric subunits that undergo conformational changes, which self-associate into amyloid fibrils (Lai et al., 1996; Lashuel et al., 1998). *S*-Nitrosylation of TTR induces structural changes, as well as instability of the tetramer conformation. This phenomenon leads to a greater fibril formation in a low-pH environment (Saito et al., 2005).

Myeloperoxidase (MPO) is a heme enzyme present in high concentrations in the granules of leukocytes and monocytes (Koelsch et al., 2010). MPO is released during phagocytosis and it catalyzes the oxidation of halide ions in the presence of hydrogen peroxide (H_2O_2) to oxidize and halogenate microbial components. Additionally, MPO can produce nitrogen dioxide radical (NO_2^-), a reactive species that converts tyrosine to nitrotyrosine (Gaut et al., 2002; Ford, 2010; Swirski et al., 2010). Recently, it was reported that HDL contains MPO as well as nitrotyrosine as a marker of MPO oxidization (Pennathur et al., 2004; Malle et al., 2006). We previously reported that HDL colocalizes with amyloid deposits in FAP and dialysis-related amyloidosis (DRA) (Sun et al., 2006).

To determine whether nitric oxide-mediated modification of TTR amyloid and serum TTR are induced by the MPO/H_2O_2/NO^- system, we investigated of association between MPO and amyloid deposits in FAP patients. We also examined the anti-nitrotyrosine immunoreaction on serum TTR and TTR amyloid deposits exposed to the MPO/H_2O_2/NO^- system.

MATERIAL AND METHODS

Research subjects

Autopsied frozen cardiac and kidney samples from 3 FAP ATTR Val30Met patients (1 42-year-old male, 1 45-year-old female, and 1 52-year-old female), were prepared by Congo red staining. The amyloid-positive sections were exposed to the MPO/H_2O_2/NO^- system. Eight healthy volunteers (5 males and 3 females) were selected as controls. Blood samples from the sub-

jects were obtained after identification as wild-type TTR using matrix-assisted laser desorption/ionization (MALDI)/time-of-flight/mass-mass spectrometry (TOF-MS) (Terazaki et al., 1999).

Congo red staining

Frozen tissues prepared for light microscopic study were stained with alkaline Congo red. Amyloid deposits were confirmed by apple green birefringence under polarized light.

Immunohistochemical staining

Immunohistochemistry using a polyclonal rabbit anti human myeloperoxidase (MPO) antibody (AssayPro, Winfield, MO, USA) was performed on the same tissues examined by Congo red staining. Samples were incubated in blocking buffer [0.5% bovine serum albumin in 10 mM phosphate-buffered saline (PBS) at pH 7.4]. An anti-MPO antibody diluted 1:80 in blocking buffer and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) diluted 1:100 in blocking buffer were used as the primary and the secondary antibodies, respectively. Visualization of reactivity was performed using a DAB Liquid System (DAKO, Glostrup, Denmark) as described by manufacturer. On parallel control sections, the primary antibody was replaced with blocking buffer (Hazell et al., 2001).

Identification of nitrotyrosine in amyloid deposits using immunohistochemical staining

In vitro oxidative reactions of frozen tissue sections were carried out in phosphate buffer (20 mM sodium phosphate, pH 7.0, 100 µM DTPA) supplemented with 150 mIU myeloperoxidase, 100 µM H_2O_2, 10 µM sodium nitrite, and 10 µM N-acetyl-tyrosine (Wako Pure Chemical Industries, Ltd., Oska, Japan) at 27°C for 12 h. The reactions were initiated by adding oxidant and terminated by flushing with water for 5 min. Reactions were probed at room temperature with the monoclonal anti-3-nitro-L-tyrosine antibody (1:50 dilution; Dako Cytomation, Carpinteria, CA, USA) for 30 min, and goat anti-rabbit globulin antibody conjugated HRP was incubated at room temperature for 40 min (Pennathur et al., 2004).

Oxide-mediated modification of serum TTR with myeloperoxidase/H_2O_2 system

After removing albumin and Ig from the serum samples of healthy volunteers using the ProteoSeekTM Albumin/Ig G Removal Kit (Pierce, Rockford, IL, USA), oxidation reactions were carried out at 27°C in phosphate buffer [10 mM sodium phosphate, 100 µM diethylenetriaminepentaacetic acid (DTPA), pH 7.0] containing 10 µL serum. For the myeloperoxidase/H_2O_2 system, the reaction mixture was supplemented with the indicated concentration of myeloperoxidase (Cambridge Isotope Laboratories Inc., MA, USA) and H_2O_2 (Beckman et al., 1994).

pI of serum TTR in the presence and absence of the MPO/H_2O_2 system

After serum exposure to the MPO/H_2O_2 system, a second step involving isoelectric focusing (IEF) was performed: 10 µL each sample containing 0.5 µL serum and 8 M urea was added

to the IEF gels (pH 4-6; Bio Co., Tokyo, Japan). Next, the IEF gels were run at a limit of 750 V for 3.5 h, after an initial phase at constant power of about 26 mA, the gels were stained with 0.8% sliver in water and were spotted by 6% citric acid monohydrate for 5 min (Altland et al., 1999).

TTR Western blotting

Identification of TTR protein was performed by Western blotting. Briefly, according to two-dimensional gel electrophoresis, the protein was probed at 4°C overnight with primary rabbit anti-human TTR antibody (1:1000; Dako Japan Co., Ltd., Tokyo, Japan). An HRP-conjugated goat anti-rabbit antibody was incubated at room temperature for 1 h. This approach was used to quantify modified TTR to identify the radical-based isoform serum TTR for isoelectric point (pI) analysis.

RESULTS

Association of MPO with amyloid deposition

Amyloid deposition was confirmed in the heart, kidney, and aorta of FAP patients by Congo red staining under polarized light. The positive of anti-human TTR antibody immunoreaction showed colocalization with the amyloid deposition. Additionally, the positive reaction of the anti-MPO antibody colocalized with both the anti-human TTR antibody used in the immunoreaction and the Congo red staining used in the tissue specimen (Figure 1).

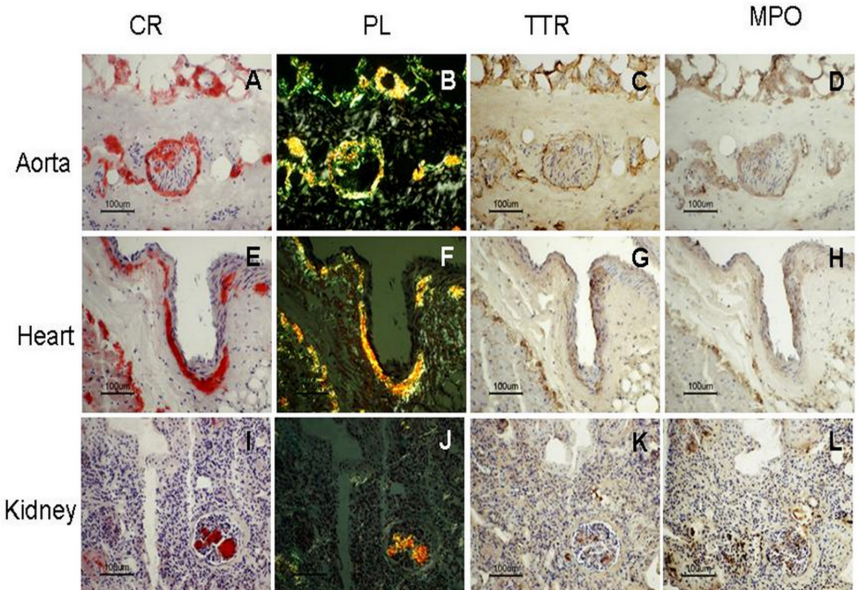

Figure 1. Association of myeloperoxidase in amyloid deposition in FAP patients. **A. E. I.** Congo red staining; **B. F. J.** its polarized light examination; **C. G. K.** immunohistochemical staining using the anti-TTR antibody; **D. H. L.** immunohistochemical staining using the anti-MPO antibody. **A.-D.** pars thoracic aorta; **E.-H.** heart; **I.-L.** kidney.

Identification of nitrotyrosine associated with amyloid deposition

Amyloid-rich sections were exposed to the MPO/H_2O_2/NO^- system as described previously. The anti-3-nitro-L-tyrosine antibody immunoreaction was positive for amyloid deposits in FAP ATTR Val30Met patients (Figure 2E). In contrast, the immunoreaction on the exposed sections was negative for the control samples (Figure 2C, D). Negative results were also observed when only one element (MPO, H_2O_2, or NO^-) was used (data not shown).

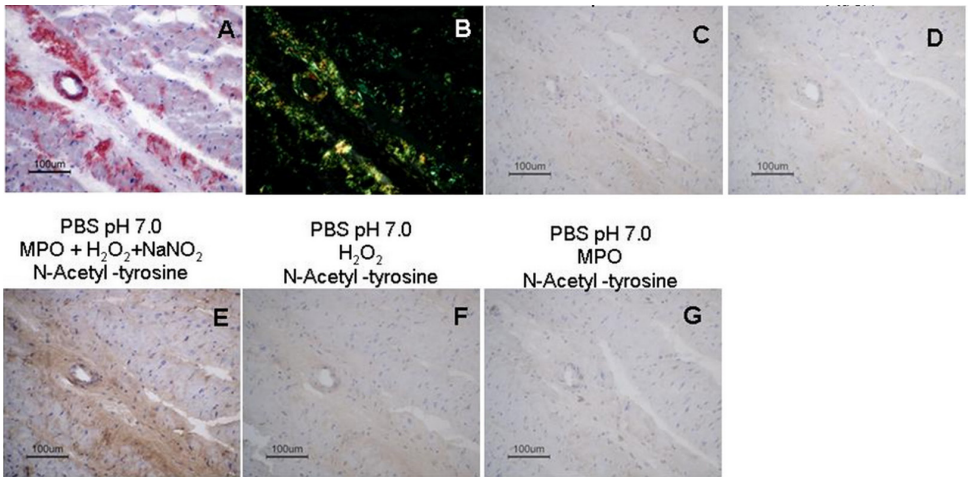

Figure 2. Analysis of MPO due to N-acetyl-tyrosine conjugate to amyloid deposit in heart tissue from FAP patients. **A.** Congo red staining; **B.** Congo red staining under polarized light; **C. D.** immunohistochemical staining on exposed sections of control system; **E.** immunohistochemical staining using polyclonal anti-N-acetyl-tyrosine antibody; **F.** incubating solution containing PBS, H_2O_2, and N-acetyl-tyrosine reagent; **G.** incubating solution containing PBS buffer, MPO, and N-acetyl-tyrosine reagent.

Density of anti-nitrotyrosine antibody immunoreaction is dependent on MPO reaction time

The anti-nitrotyrosine antibody immunoreaction was increased in the amyloid deposits of FAP ATTR Val30Met patients as reaction time increased (Figure 3D-H). In contrast, there was nearly no positive immunoreaction in amyloid deposits exposed to the control system (Figure 3C).

Effect of MPO/H_2O_2 system on oxide-mediated modification of serum TTR

The pI of wild-type serum TTR was analyzed by two-dimensional gel electrophoresis. Sliver staining showed 1 point (as arrow indicated) at which the protein reacted with the polyclonal anti-TTR antibody according to western blotting (Figure 4B-b). Three points were silver-stained following exposure to the MPO/H_2O_2 system. The pattern of the anti-human TTR antibody immunoreaction was the same as that of sliver staining (Figure 4A-a and 4B-b).

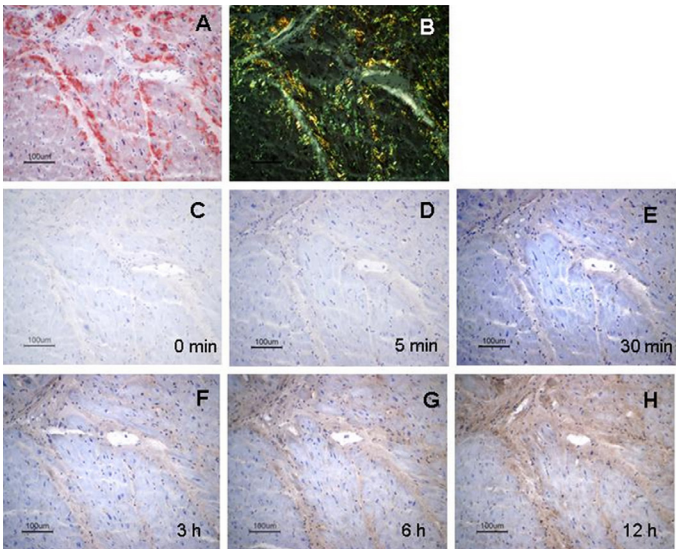

Figure 3. Analysis of anti-N-acetyl-tyrosine antibody immunoreaction in the hearts of FAP patients. Immunohistochemical analysis of anti-N-acetyl-tyrosine reaction was dependent on the MPO-H_2O_2 system incubation time in Congo red-positive tissues. **A.** Congo red staining; **B.** Congo red staining under polarized light; (C-H): immunohistochemical staining for different incubation times (**C.** 0 min; **D.** 5 min; **E.** 30 min; **F.** 3 h; **G.** 6 h and **H.** 12 h) using the MPO-H_2O_2 system.

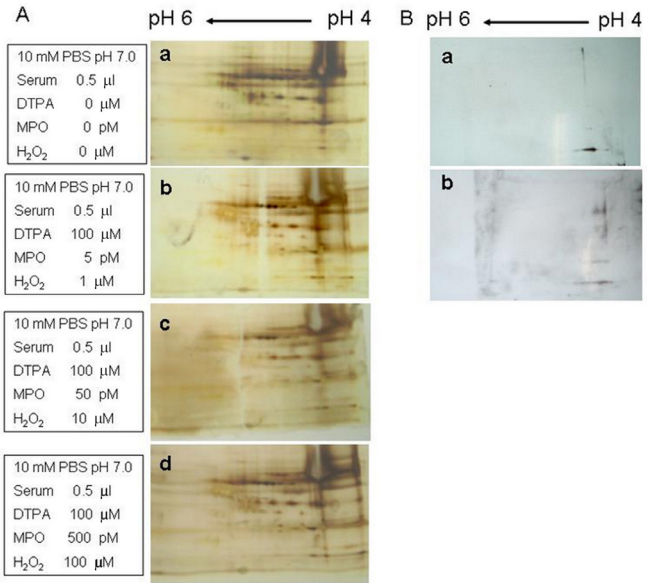

Figure 4. Isoelectric focusing and immunoblotting of serum TTR. **A.** Silver staining after two-dimensional electrophoresis. Healthy volunteer serum (0.5 µL) was exposed to the myeloperoxidase-H_2O_2 system at the indicated molar ratio of activity of MPO IU to serum protein for 2 h at 27°C in phosphate buffer (**a-d**). After the reaction, TTR modified by the indicated condition was confirmed using two-dimensional electrophoresis. **B.** Western blotting with polyclonal anti-human TTR antibody. TTR was modified when exposed to the myeloperoxidase system.

DISCUSSION

In this study, we demonstrated that the anti-MPO antibody immunoreaction appears in amyloid deposits, but is negative in normal tissue (amyloid-negative sites) (Figure 1). We also demonstrated that amyloid deposits consist of protein fibrils as well as amorphous material (Inoue et al., 1998) and that HDL and/or lipid droplets are present in amyloid deposits (Sun et al., 2006; Ikeda et al., 2011). TTR is known to have affinity for serum HDL (Sousa et al., 2000; Sun et al., 2003; Zheng et al., 2004; Pennathur et al., 2004). According to our results, the MPO present in amyloids may be localized to 3 possible sites within the amyloid deposits. The first is associated with the protein fibril; the second is coupled with the amorphous material such as HDL and lipid drops; and the third is associated with both protein fibrils and amorphous material.

We analyzed the affinity between MPO and TTR amyloid fibrils isolated from tissues of FAP ATTR Val30Met patients. Our results revealed no affinity between MPO and TTR protein fibrils according to BIAcore analysis (data not shown). Our findings agree with the results of other studies showing that the HDL contains MPO (Suhr et al., 2001; Sousa et al., 2001; Zheng et al., 2004; Pennathur et al., 2004), and HDL has been found in amyloid deposits in FAP and in dialysis-related amyloidosis (DRA) (Sun et al., 2006). Our study showed that HDL has an affinity for TTR (Beckman et al., 1994), which supports our hypothesis that MPO is associated with HDL and/or lipids contained in amyloid deposits.

Several reports have found that biomarkers of oxidative stress are colocalized with in amyloids, such as HNE and nitrotyrosine (Ando et al., 1997; Suhr et al., 2001). Additionally, levels of oxidations were found to be elevated in amyloid-rich tissues, as evidenced by the presence of thiobarbituric acid reactive substances (TBARS) and advanced glycation end products (RAGE) (Ando et al., 1997; Sousa et al., 2001).

To investigate whether nitric-mediated modification of TTR amyloid was induced by the MPO/H_2O_2/NO^- system, we examined the presence of anti-3-nitro-L-tyrosine in amyloid deposits of FAP ATTR Val30Met patients when exposed to the MPO/H_2O_2/NO^- system. There was no reaction in the control system (Figure 2). The density of the anti-3-nitro-L-tyrosine immunoreaction also increased with reaction time (Figure 3). These results indicate that TTR amyloid deposits were oxidized when exposed to the MPO/H_2O_2/NO system *in vivo*. Furthermore, it was found that nitrotyrosine is related to TTR deposition in amyloid deposits, even before the appearance of detectable amyloid fibrils in transgenic mice for mutant TTR (Sousa et al., 2002). However, whether nitric conjugated TTR enhances TTR aggregation and TTR amyloid formation is unclear. It was difficult to demonstrate if nitric oxide-mediated modification of TTR enhances fibril formation *in vivo*. However, the presence of nitric-oxide nitric-mediated modified TTR in circulation may reveal a role in amyloidogenesis *in vivo*.

Approximately 80% of total TTR is conjugated and posttranslationally modified in serum as shown by electrospray ionization (ESI)-mass spectrometry (MS)/MS (Ueda et al., 2009). In this study, we used isoelectric focusing and TTR western blotting and found that nitric oxide-mediated modification of serum TTR occurs when reacted with the MPO/H_2O_2 system (Figure 4). Additionally, the amount of nitric oxide-modification of serum TTR increased relative reaction time in the MPO/H_2O_2 system (Figure 3C-H). Our results indicated that the nitric-oxide-mediated modification of TTR was affected by the MPO/H_2O_2 system *in vivo*. The ratio of conjugated serum ATTR Val30Met increases in FAP patients, including cysteine, cysteine-glycine, or glutathione-conjugated TTR (Suhr et al., 1998). Interestingly, nitric oxide-mediated

modification of TTR leads to structural modification and destabilizes the tetramer conformation, enhancing amyloid fibril formation in low-pH environments (Saito et al., 2005).

In conclusion, MPO was colocalized in amyloid deposits. Additionally, nitric oxide-mediated modification of TTR amyloid was enhanced by the MPO/H_2O_2/NO^- reactive system. Nitric oxide-mediated modification of TTR may play a role in amyloidogenesis *in vivo*.

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