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Note

Sialic acid attenuates the cytotoxicity of the lipid hydroperoxides HpODE and HpETE

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ABSTRACT

Reduction of peroxide molecular species is an essential function in living organisms. In previous studies, we proposed a new function for the sialic acid *N*-acetylneuraminic acid (Neu5Ac)—that of antioxidant/hydrogen peroxide scavenging agent. On the basis of the reaction scheme, Neu5Ac is thought to act as a general antioxidant of all hydroperoxide-type species (R–OOHs). The concentration of *tert*-butyl hydroperoxide (*t*-BuOOH) decreased after co-incubation with *N*-acetylneuraminic acid. Neu5Ac also decreased the R–OOH concentration in solutions of peroxylinolenic acid (13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid, HpODE) and peroxyarachidonic acid (15(*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid, HpETE)—two lipid hydroperoxides that participate in many physiological events. Moreover, the cytotoxicity of both these lipid hydroperoxides was attenuated by reaction with Neu5Ac acid. Our results suggest that *N*-acetylneuraminic acid is a potential antioxidant of most hydroperoxides that accumulate in organisms.

N-Acetylneuraminic acid (Neu5Ac) is a major sialic acid species in humans, and its various functions such as water retention in mucosal tissues and cellular communication are well known.[1,2] In previous studies, we demonstrated the potential of Neu5Ac with regard to its hydrogen peroxide (H_2O_2)-scavenging activity.[3,4] This antioxidant activity of Neu5Ac is ascribed to its pyruvic acid moiety. Pyruvic acid is the smallest α-ketocarbonic acid, and its carbonyl group is highly reactive toward H_2O_2.[5,6] The pyruvic acid moiety of Neu5Ac converts H_2O_2 to H_2O and CO_2 in a reaction involving the α-ketocarboxylic group of pyruvic acid, and Neu5Ac itself is converted to the decarboxylated product 4-(acetylamino)-2,4-dideoxy-D-*glycero*-D-*galacto*-octonic acid (ADOA).[3]

H_2O_2 is a hydroperoxide (ROOH)-type molecular species with a very simple structure. In living organisms, other types of hydroperoxide species—lipid hydroperoxides—exist.[7–9] The fatty acid moiety of lipid hydroperoxides is often oxidized as a result of autoxidation or a lipoxygenase-mediated reaction.[10] The products, that are lipid hydroperoxides, are unstable and damage tissues by acting as either oxidizing agents[7,8] or sources of various radical species. Moreover, lipid hydroperoxides induce inflammation by stimulation of prostaglandin synthesis and induction of cytokine production.[11,12] Consequently, accumulation of lipid hydroperoxides can cause disease and tissue/organ damage. The peroxylinolenic acid (HpODE) moiety of oxidized cholesterol, which is a major trigger of arteriosclerosis, and peroxyarachidonic acid (HpETE) are intermediates in the arachidonic acid cascade. Both these lipid hydroperoxides produce direct cytotoxic effects in vitro. In this study, we examined the attenuation effect of Neu5Ac on these lipid hydroperoxides.

First, we attempted to determine whether Neu5Ac reacts with organic hydroperoxides. *tert*-Butyl hydroperoxide (*t*-BuOOH), a small organic hydroperoxide, was selected as the test compound. As expected, Neu5Ac reduced the hydroperoxide group (–OOH) concentration in *t*-BuOOH solution in a dose-dependent manner (Fig. 1A). In a previous experiment, we observed that Neu5Ac decreased the same concentration of H_2O_2 within 4 h.[3] Thus, the ability of Neu5Ac to decrease the concentration of *t*-BuOOH is corresponds to approximately 10% of its ability to decrease the concentration of H_2O_2. We used HpODE and HpETE as test compounds because these two lipid hydroperoxides are known to be involved in certain biological disorders and chronic diseases. Neu5Ac also acts as an antioxidant of these lipid hydroperoxides (Fig. 1B). The H_2O_2-quenching action of Neu5Ac, which is based on its α-ketocarboxylic acid structure, was demonstrated in a previous report.[3] We found that 3 typical α-ketocarboxylic acids, namely, pyruvic acid, oxalacetic acid, and α-ketoglutaric acid, exhibited a R–OOH-quenching activity (Fig. 1C). The results of these experiments strongly indicate the broad reactivity of Neu5Ac toward R–OOH-type peroxides; this reactivity can be ascribed to the α-ketoglutaric acid structure of Neu5Ac.

In the next experiment, we attempted to demonstrate the attenuating effect of Neu5Ac on HpODE and HpETE. The LC_{50} va-

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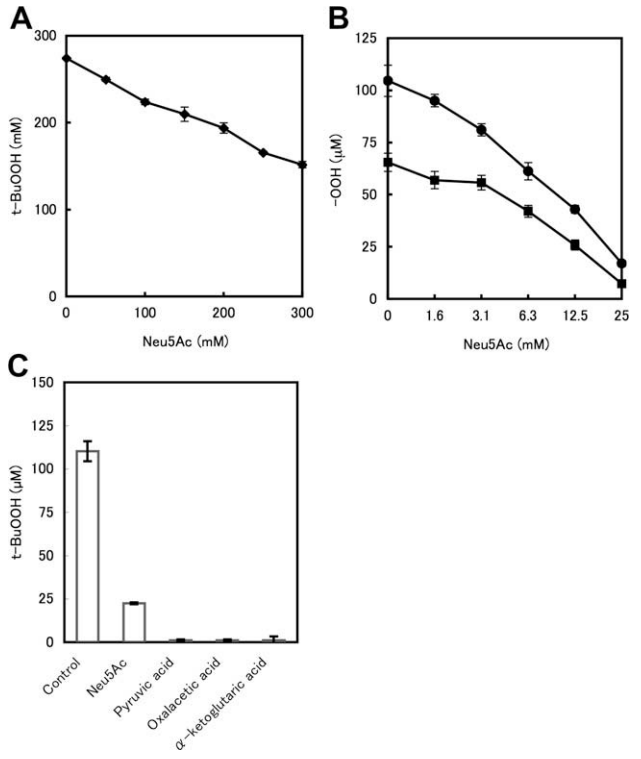

Figure 1. Neu5Ac reduces organic peroxides. (A) *t*-BuOOH (300 mM) in 50% MeOH–50% water was incubated with Neu5Ac (300 mM) at 37 °C. After 22 h, the *t*-BuOOH concentration was measured, as described in Section 1.1. (B) HpODE (100 μM, ■) or HpETE (100 μM, ●) in 50% MeOH–50% water was incubated with 0–25 mM Neu5Ac at 37 °C. After 20 h, the –OOH concentration in both reaction mixtures was measured. Approximately 40 μM HpODE was spontaneously destroyed because of its inherent instability. (C) HpODE (100 μM) in 20% MeOH–80% water was incubated with 40 mM Neu5Ac, pyruvic acid, oxalacetic acid, or α-ketoglutaric acid at 37 °C. After 40 h of incubation, the –OOH concentration was measured.

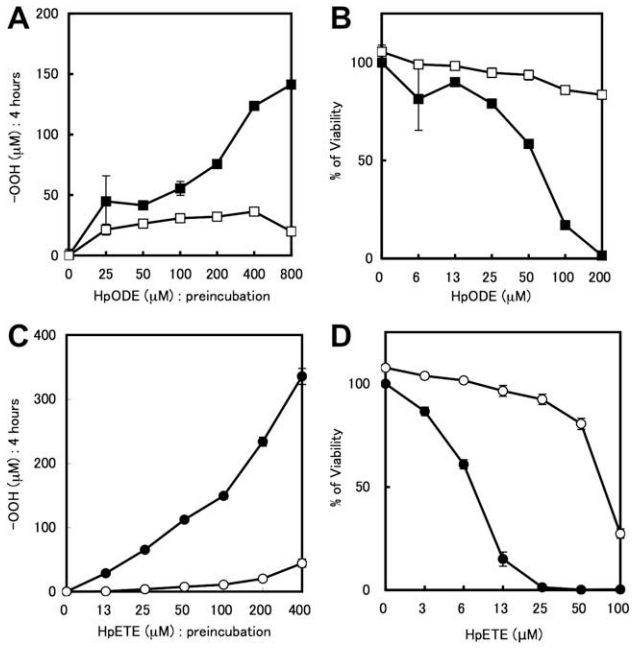

Figure 2. Neu5Ac attenuates the cytotoxicity of HpODE and HpETE. (A) Before cell culture, 0–800 μM HpODE was incubated (preincubation) with (□) or without (■) 25 mM Neu5Ac for 4 h, and the R–OOH concentration was measured. (B) After preincubation with (□) or without (■) 25 mM Neu5Ac, EL-4 murine leukemia cells were cultured in the preincubated media for 20 h. Cell viability was determined by the MTT reduction assay. (C and D) The 2 above experiments were performed with (○) or without (●) 25 mM HpETE. Because of differences in the stability and toxicity of the two lipid hydroperoxides, 0–400 μM HpETE was used.

lue (50% Lethal Concentration) of these two lipid hydroperoxides for HL-60 human leukemia cells is approximately 60 and 10 mM, respectively (Fig. 2B and D). On preincubation for 4 h at 37 °C, Neu5Ac reduced the –OOH concentrations of culture media containing HpODE and HpETE (Fig. 2A and C). As expected, on assessing the cell viability in these media, it was found that the LC_{50} values were remarkably diminished (Fig. 2B and D, >200 and 70 mM, respectively). In conclusion, the attenuating action of Neu5Ac on lipid hydroperoxides was revealed.

Lipid hydroperoxides are generated in vivo by the reaction of fatty acids with reactive oxygen species or by the action of lipoxygenase on fatty acids. These substances are highly detrimental to organic molecules such as other lipids, proteins, and nucleic acids. Therefore, the prompt removal of lipid hydroperoxides is an extremely important physiological process. Neu5Ac, which is a major sialic acid species in human tissues, detoxifies H_2O_2 by oxidizing it to H_2O and CO_2.[3]

Lipid hydroperoxides exist in human blood and organs as toxic substances. In this study, we used HpETE and HpODE as representative lipid hydroperoxides; these two lipid hydroperoxides exhibited severe cytotoxicity in vivo. Furthermore, it has been previously demonstrated that esterified HpODE is a major product of oxidization of human LDL and that oxidized LDL induces apoptosis.[13–15] We tested the antioxidant effect of Neu5Ac toward HpODE and HpETE. We confirmed that Neu5Ac decreased –OOH concentrations in both lipid hydroperoxide solutions and attenuated the toxicity of these hydroperoxides toward cultured cells. The fact that lipoproteins in cholesterol complexes are sialoglycoproteins is thought to indicate the physiologic importance of the anti-hydroperoxide function of sialic acid. In the experiments shown in Figure 2, Neu5Ac produced an attenuation effect at concentrations greater than its physiological concentrations. However, it is known that Neu5Ac is released from sialyl Lewis X located on the cell surface by treatment with reactive oxygen species.[16] Furthermore, mucin, which is present in large amounts in the trachea and digestive organs, can serve as a source of free Neu5Ac.[3] Therefore, it is expected that the local Neu5Ac concentration increases in response to an oxidative stress such as that due to inflammation.

In this study, it was confirmed that Neu5Ac reduced –OOH by oxidizing lipids in the same way as it oxidizes H_2O_2. This reaction lowered the toxicity of lipid hydroperoxides toward cultured cells. These results may indicate that Neu5Ac reacts with not only H_2O_2 but also most R–OOH-type species, for example, lipid hydroperoxides. This finding strongly supports the utility of sialic acid as an endogenous antioxidant factor.

1. Experimental

1.1. Materials

N-Acetylneuraminic acid (code no. 00648-24, Nacalai Tesque, Japan) was dissolved in water, and the pH of the resultant solution was adjusted to 7.0–7.5 by using sodium hydroxide. *t*-BuOOH was obtained from Sigma–Aldrich Co., USA (code no. B-1633). The lipid hydroperoxides HpODE (13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid) and HpETE (15(*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid) were obtained from Wako Pure Chemical Ind., Japan (code nos. 082-08003 and 087-08031, respectively).

1.2. Determination of organic or lipid hydroperoxides concentration in solution

The concentration of –OOH in the reaction mixture was measured with a PeroxiDetect™ Kit (code no. PD1; Sigma–Aldrich Co., USA). Peroxides convert Fe^{2+} to Fe^{3+} ions under acidic conditions, and Fe^{3+} ions form a blue adduct of xylenol orange.[17] The reaction mixture or a standard *t*-BuOOH soln (1 vol) in a 96-well microtiter plate was incubated with the aq peroxide color reagent (9 vol) for 30 min at room temperature, and subsequently, the optical density at 550 nm was measured. *t*-BuOOH was used as a control for determining the –OOH concentrations.

1.3. Determination of cell death

For the cytotoxicity assay, EL-4 murine lymphoma cells were seeded at 2×10^5 cells/well in a 96-well microplate and cultured in 5% CO_2 at 37 °C for 16 h. Before the assay, lipid hydroperoxides and Neu5Ac in a RPMI 1640 cell culture medium (code no. R8758; Sigma–Aldrich, USA) containing 10% fetal bovine serum (cat. no. 15-010-0500V; Thermo Trace Ltd, Australia) were preincubated for 3–6 h, and subsequently, the cells were added. Cellular viability was assessed using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. After preincubation, the MTT solution was added to the culture (final concentration, 500 μg/mL MTT), and the incubation was continued for 4 h. The absorbance of MTT formazane was measured at an optical density of 550–650 nm. Note that 100% indicates the viability of untreated controls.

References

1. Schauer, R. *Zoology (Jena)* **2004**, *107*, 49–64.
2. Crocker, P. R.; Redelinghuys, P. *Biochem. Soc. Trans.* **2008**, *36*, 1467–1471.
3. Iijima, R.; Takahashi, H.; Namme, R.; Ikegami, S.; Yamazaki, M. *FEBS Lett* **2004**, *561*, 163–166.
4. Iijima, R.; Takahashi, H.; Ikegami, S.; Yamazaki, M. *Biol. Pharm. Bull.* **2007**, *30*, 580–582.
5. Jefford, C. W.; Boschung, A. F.; Bolsman, T. A.; Moriatry, R. M.; Melnick, B. *J. Am. Chem. Soc.* **1976**, *98*, 1017–1018.
6. Mazzio, E.; Soliman, K. F. *Neurosci. Lett.* **2003**, *337*, 77–80.
7. Halliwell, B. *FASEB J.* **1987**, *1*, 358–364.
8. Esterbauer, H.; Wäg, G.; Puhl, H. *Br. Med. Bull.* **1993**, *49*, 566–576.
9. Parthasarathy, S.; Litvinov, D.; Selvarajan, K.; Garelnabi, M. *Biochim. Biophys. Acta* **2008**, *1781*, 221–231.
10. Folcik, V. A.; Nivar-Aristy, R. A.; Krajewski, L. P.; Cathcart, M. K. *J. Clin. Invest.* **1995**, *96*, 504–510.
11. Higgs, G. A.; Salmon, J. A.; Spayne, J. A. *Br. J. Pharmacol.* **1981**, *74*, 429–433.
12. Khan, B. V.; Parthasarathy, S. S.; Alexander, R. W.; Medford, R. M. *J. Clin. Invest.* **1995**, *95*, 1262–1270.
13. Folcik, V. A.; Cathcart, M. K. *J. Lipid Res.* **1994**, *35*, 1570–1582.
14. Kato, Y.; Osawa, T. *Arch. Biochem. Biophys.* **1998**, *351*, 106–114.
15. Salvayre, R.; Auge, N.; Benoist, H.; Negre-Salvayre, A. *Biochim. Biophys. Acta* **2002**, *1585*, 213–221.
16. Eguchi, H.; Ikeda, Y.; Ookawara, T.; Koyota, S.; Fujiwara, N.; Honke, K.; Wang, P. G.; Taniguchi, N.; Suzuki, K. *Glycobiology* **2005**, *15*, 1094–1101.
17. Jiang, Z. Y.; Woollard, A. C.; Wolff, S. P. *FEBS Lett.* **1990**, *268*, 69–71.