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Nitrite-Mediated Inactivation of Human Plasma Paraoxonase-1: Possible Beneficial Effect of Aromatic Amino Acids

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Abstract Paraoxonase-1 (PON-1) is a high-density-lipoprotein-bound enzyme, and its major function is to prevent oxidation of low-density lipoprotein. Atherogenesis could be related to decreased activity of this enzyme. Nitrites (NO_2^-), either present as a contaminant and/or the main metabolic end product of nitric oxide (NO) degradation, may trigger nitrative damage to PON-1 enzyme. Minimal information is available concerning the effect of nitrite on the enzyme activity and the mechanism which it exerts its effect. The aim of this study was to analyze whether nitrites could play a role in modifying human PON-1 activity. Our results revealed that PON-1 activity was inhibited by nitrite in dose- and time-dependent manner. Site-specific nitration focused on phenolic residues, particularly tyrosine residues of the enzyme, may result in modification of its biological functions. Nitration of phenolic residues occurs via peroxynitrite ($ONOO^-$) formation, which requires peroxides and nitrite. Thus, we tested the presence of peroxides, which are found in all plasma samples regardless of nitrite concentration. The inhibition of PON-1 activity by nitrite was significantly reduced by tryptophan, reduced glutathione (GSH), and catalase additions. Therefore, we concluded that nitrites may have a role in the inactivation of PON-1, probably through nitration of enzyme phenyl residues, and additions of individual aromatic amino acids, with highlighting on tryptophan, could be of important value in minimizing the nitrite-induced inhibition of PON-1 enzyme.

Keywords Nitrites · Paraoxonase · Peroxynitrite

Abbreviations

GSH	reduced glutathione
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
LDL	low-density lipoprotein

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NO	nitric oxide
NO_2^-	nitrite
$O_2^{\bullet -}$	superoxide radical
$ONOO^-$	peroxynitrite anion
PON	paraoxonase

Introduction

The paraoxonase (PON) gene family contains at least three members, including PON-1, PON-2, and PON-3. All gene family members have been reported to possess antioxidant properties and antiatherogenic capacities. PON-2 and PON-3 are unlike PON-1, as they lack the ability to hydrolyze paraoxon [1, 2].

PON-1 is a calcium-dependent organophosphatase that hydrolyzes organophosphates, which are widely used as insecticides and nerve gases. PON-1 is responsible for determining the selective toxicity of these compounds in mammals [3, 4].

Serum levels of PON-1 activity fluctuate extensively among individuals, according to the differences in susceptibility to organophosphate poisoning [5]. Polymorphism in the PON-1 gene explains the molecular basis for these differences. Single amino acid substitution results in an observable difference in the enzyme's ability to hydrolyze paraoxon. PON-1 A, an isoform that has a glutamine at position 192, is characterized by low activity toward paraoxon, whereas PON-1 B isoform, which contain arginine instead, is distinguished with high activity toward paraoxon [6].

Many epidemiological studies have shown a strong negative association between the serum concentration of high-density lipoprotein (HDL) and the incidence of atherosclerosis [7]. The mechanism by which HDL renders its protective effect against atherosclerosis continues to be the subject of considerable debate. The useful effects attributed to HDL are probably linked to its ability to promote cholesterol transfer from cell wall and to transport cholesterol to the liver for catabolism. In addition, it appears that HDL can also protect low-density lipoprotein (LDL) against oxidation [8–10].

PON-1 activity is correlated directly with the total antioxidant capacity of plasma [11]. PON-1 is carried in plasma by HDL and protects against vascular disease through inhibition of LDL oxidation [12–14].

The increasing use of nitrogen-based fertilizers in modern farming, particularly with corn, vegetables, other row crops, and forages have influenced accumulation of nitrates/nitrites in the environment. Poultry production and urban sewage treatment also contribute nitrogenous wastes to the soil and water [15, 16]. Nitric oxide, either synthesized in vivo from L-arginine or as a component of cigarette smoke and automobile exhaust, can be oxidized to nitrite or nitrate [16, 17]. Nitrogen-containing drugs/chemicals (e.g., nitroglycerin, and sodium nitroprusside) also represent a class of NO-donating agents [18]. Thus, man and animals are subjected to significant nitrate and nitrite levels in foods, feed and water, and those formed in vivo [16].

Exposure of proteins to reactive nitrogen species results in modification of amino acid residues, altering the protein structure and function [19, 20]. The stable product of tyrosine (Tyr) residues nitration (nitrotyrosine) has been measured as a biomarker of protein damage induced by peroxynitrite and other reactive nitrogen species [20]. Increased levels of free and protein-bound nitrotyrosine during aging, oxidative stress, and some pathological conditions have been reported [21–23].

Therefore, we studied the effect of nitrites on the activity of human plasma PON-1 in an attempt to correlate the proposed nitration of enzyme phenolic residues to its activity. Furthermore, we examined the effects of individual aromatic amino acids on the PON-1 activity. We, thus, aimed to evaluate the possible protective role of phenolic amino acids against enzyme inactivation.

Materials and Methods

Chemicals

The following chemicals were used (Suppliers in parenthesis): phenylalanine (Phe), Tyr, and tryptophan (Trp) were a product from (Fluka Buchs, Switzerland). Sodium nitrite was purchased from (Winlab, UK). Catalase, L-ascorbic acid, α tocopherol, Tris HCl, reduced glutathione (GSH), and paraoxon were purchased from (Sigma Chemical Co., St. Louis, MO, USA). All the remaining chemicals were of the highest analytical grade. All the solutions used for enzyme assays were prepared using deionized water to eliminate trace metal contaminations.

Experimental Procedures

Blood samples were collected from ten fasting, nonsmoking, normolipidemic, and apparent healthy donors (aged 20–40 years). After removal of cells by centrifugation at 3,000 rpm for 10 min, 100 μl of plasma was added to 1 ml Tris–HCl buffer (pH 8.0, 50 mM), together with 100 μl from increasing concentrations of sodium nitrite solutions (1–6 mM). Sodium nitrite was dissolved in phosphate buffer (pH 7.4, 100 mM). The reaction mixtures were then incubated at 37°C for 1 h. PON-1 activity and lipid peroxides were determined by the assay described below. The same reaction mixture was prepared again using single sodium nitrite concentration (1 mM), incubated at 37°C for various time periods (1–6 h), and PON-1 activity was thereafter estimated. One milliliter of buffer solutions (Tris–HCl buffer; pH 8.0, 50 mM) of phenyl alanine (Phe), Trp, Tyr, ascorbic acid, alpha tocopherol, and GSH 1 mM each, and catalase (100 μg/ml) were added separately to human plasma (100 μl). One hundred microliters of sodium nitrite (1 mM) was added to each sample, and all reaction mixtures were then incubated at 37°C for 1 h. All incubations were carried out in duplicate parallel tubes and three independent experiments. A plasma control sample was prepared and treated similarly, except that nitrites, aromatic amino acids, or antioxidants were not added.

PON-1 Activity Assay

The PON-1 activity was determined using paraoxon (1 mM) as substrate. The rate of hydrolysis of paraoxon was measured after 90 s by monitoring the increase in absorbance at 412 nm and 25°C due to the formation of *p*-nitrophenol using JENWAY spectrophotometer model 6105 UV/VIS. Enzyme activity was measured in 50 mM Tris–HCl buffer (pH 8.0) containing calcium chloride (1 mM). The sample to be tested was added to start the reaction, and the increase in absorbance of each sample was recorded against reagent blank contains reaction mixture only. The amount of *p*-nitrophenol generated was calculated from the molar extinction coefficient at pH 8.0 of 17,000 M^{-1} cm^{-1}. One unit of PON-1 activity is defined as 1 nmol *p*-nitrophenol generated per minute under the above conditions [24].

Determination of Lipid Peroxides

Serum lipid peroxides were assayed spectrophotometrically according to El-Saadani et al. [25]. The analysis was based on the ability of lipid peroxides to convert iodide to iodine.

Statistical Analysis

Data are expressed as mean±S.D of at least three independent experiments in duplicate. Data analysis was evaluated by one-way analysis of variance followed by Bonferroni corrected Student's *t* test for individual points using Graph Pad Software (San Diego, CA). A 0.05 level of probability was used as the criterion for significance.

Results

Incubation of human plasma with different concentration of sodium nitrite revealed marked inhibition of plasma PON-1 activity. As can be seen, the lowest concentration of sodium nitrite (1 mM) inhibited 23% of PON-1 activity, while the highest concentration used (6 mM) repressed 85% of the enzyme activity (Fig. 1).

Figure 2 displays the relationship between incubation time and PON-1 activity. A 22% decrease in PON-1 activity was observed after 1 h. Further exposures resulted in up to 81% loss of activity, after 6 h, when compared to control sample. On the other hand, 93% of the activity remained after 6 h of incubation at 37°C in the absence of sodium nitrite.

Peroxide levels were evaluated in plasma samples used. Table 1 indicates the presence of peroxides regardless the nitrite concentration.

The possible protective effect of Phe, Tyr, Trp, ascorbic acid, alpha tocopherol, GSH, and catalase on PON-1 activity that was suppressed with nitrite (1 mM) was examined. Trp was the only amino acid able to conserve significantly almost completely the inhibition caused by nitrite. Besides, GSH and catalase were able to exert significant protective effect against nitrite-induced inhibition of PON-1 (Fig. 3).

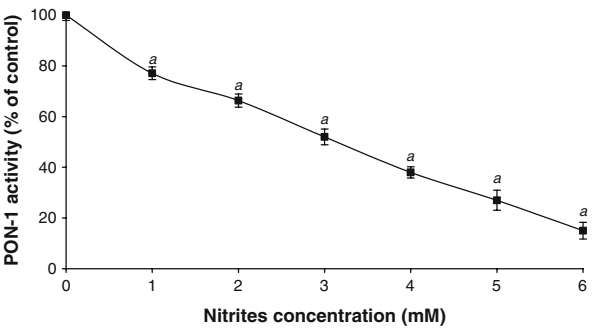

Fig. 1 Inhibition of plasma PON-1 activity at various concentrations of sodium nitrite. Human plasma (100 μl) was incubated for 1 h at 37°C with 100 μl of various concentration of sodium nitrite (1–6 mM). The results were expressed as a percentage of control PON-1 activity in plasma before treatment. Data analysis was achieved using one-way analysis of variance (ANOVA) followed by Bonferroni as post-ANOVA test. *a* indicate significant inhibition from nitrites-free plasma (control) at $p<0.05$, which was observed at the concentration of the nitrite as low as 1 mM

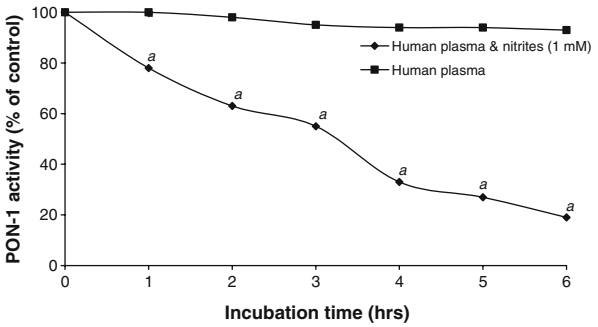

Fig. 2 Inhibition of plasma PON-1 activity by sodium nitrite at various time periods. Human plasma (100 μl) was incubated at 37°C with 100 μl of sodium nitrite (1 mM). PON-1 activity was assayed at different time periods (1–6 h). The results were expressed as a percentage of control PON-1 activity in plasma at zero time. Data analysis was achieved using ANOVA followed by Bonferroni as post-ANOVA test. *a* indicate significant inhibition ($p<0.05$) from nitrites-free plasma (control), which was incubated without sodium nitrite for the same time periods

Discussion

In the present study, PON-1 activity was measured in the presence of nitrite, and it has been demonstrated that inhibition of PON-1 activity was directly proportional to the nitrite concentration and time of exposure (Figs. 1 and 2). This has been attributed to the presence of phenolic amino acid residues in PON-1 enzyme [26, 27]. Nitrites could react with phenolic residues in PON-1 leading to confirmation changes and consequently enzyme inhibition [19, 20, 28, 29].

Nitrations of phenolic compounds especially aromatic amino acids have attracted much attention because they are active in modifying various proteins. Many proteins and enzymes change their conformation and lose their function after modification of amino acid residues by reactive oxygen and nitrogen species. Nitration of protein aromatic amino acids is a covalent protein modification, and evidence that nitrated proteins could be removed by different cellular proteolytic systems has been reported [30–33]. In addition, the elevated level of nitrated proteins in many pathological conditions has been reported [21–23, 28, 34].

The reaction mixtures of plasma samples with different concentrations of nitrite were investigated for the peroxide levels (Table 1), as it has been reported that nitration of phenolic residues mediated via peroxynitrite formation. The latter is generated by the fast reaction of nitric oxide with hydrogen peroxide [35–38].

Table 1 Plasma peroxide levels at different concentrations of sodium nitrite.

	Nitrites concentration (mM)						
	Control	1 mM	2 mM	3 mM	4 mM	5 mM	6 mM
Peroxides (nmol/ml)	1.5±0.13	1.41±0.19	1.26±0.16	1.31±0.19	1.42±0.23	1.32±0.18	1.24±0.21

Data are presented as mean±SD. Data analysis was achieved using one-way analysis of variance (ANOVA) followed by Bonferroni as post-ANOVA test. Non-significant changes of peroxide levels were observed in plasma samples with different concentration of nitrites when compared to nitrites free plasma (control).

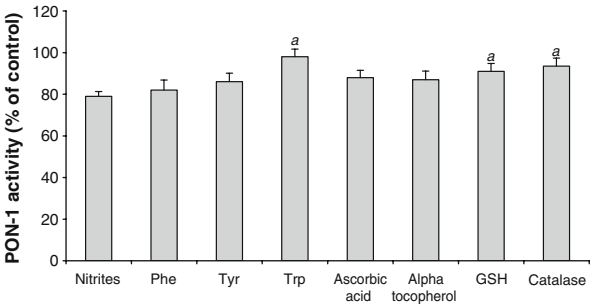

Fig. 3 Effect of aromatic amino acids, non-enzymatic and enzymatic antioxidants on nitrite-induced inhibition of plasma PON-1 activity. Phenyl alanine (*Phe*), tryptophan (*Trp*), tyrosine (*Tyr*), ascorbic acid, alpha tocopherol, and reduced glutathione (*GSH*) 1 mM each, and catalase (100 μg/ml) were added separately to human plasma (100 μl). One hundred microliter of sodium nitrite (1 mM) was added to each sample, and all reaction mixtures were then incubated at 37°C for 1 h. The results were expressed as a percentage of control PON-1 activity in plasma without treatment. Data analysis was achieved using ANOVA followed by Bonferroni as post-ANOVA test. *a* indicate significant inhibition from nitrites treated plasma at $p<0.05$

Supplementation of plasma with individual aromatic amino acids differentially protects PON-1 activity against nitrite-induced inhibition (Fig. 3). Each amino acid produces a different level of protection. This variation of protection level could be related either to the recurrences of each residue in the enzyme sequence or to kinetic characteristics of the reaction of nitrite radicals with each individual amino acid. Tryptophan (Trp) gives the highest level of protection, which is probably due to the kinetics of peroxynitrite reaction with free amino acids. Alvarez et al. [39] proved that Trp is the most reactive amino acid with peroxynitrite and rapidly affect its decay rate [39]. In addition, the hypothesis of the presence of at least one Trp residue in the PON-1 active site was previously reported [26, 40]. The present study also investigated the protective ability of particular non-enzymatic and enzymatic antioxidants. However, GSH and catalase were only able to protect significantly against the inhibitory effect of nitrite (Fig. 3). As these antioxidants have the ability to quench most peroxides, these results suggest that peroxides have a potential role in the observed loss of the enzyme activity.

In conclusion, the current study indicates that nitrite could be responsible for the inhibition of PON-1 activity, which is possibly mediated through the formation of peroxynitrite, the potent nitrating agent. Nevertheless, additional study is required to sharply declare the nitrite-induced inhibition of plasma PON-1 activity through calculation of reaction characteristics such as Michaelis constant (K_m) and maximum velocity (V_{max}). Aromatic amino acids and different antioxidants are able to exert a protective effect through inhibition of peroxynitrite formation and thus preventing its damage to the enzyme. Furthermore, our results suggested that particular treatment with Trp may have beneficial effects on PON-1 activity and thus could play an important role in the protection of LDL oxidation and avoidance of cardiovascular risk. However, further study is required to clarify the detailed mechanism and to add extra information on the possibility to make use of Trp as a part of protective strategies against cardiovascular disease.

References

1. Li, H. L., Liu, D. P., & Liang, C. C. (2003). *Journal of Molecular Medecine*, *81*(12), 766–779.
2. Ng, C. J., Shih, D. M., Hama, S. Y., Villa, N., Navab, M., & Reddy, S. T. (2005). *Free Radical Biology & Medicine*, *38*(2), 153–163.
3. Mackness, B., Durrington, P. N., & Mackness, M. I. (1998). *General Pharmacology*, *31*(3), 329–336.
4. Jarvik, G. P., Tsai, N. T., McKinstry, L. A., Wani, R., Brophy, V. H., Richter, R. J., et al. (2002). *Arteriosclerosis, Thrombosis, and Vascular Biology*, *22*, 1329–1333.
5. Blatter-Garin, M. C., Abbott, C. A., Messmer, S., Mackness, M. I., Durrington, P. N., Pometta, D., et al. (1994). *Biochemical Journal*, *304*, 549–554.
6. Davies, H. G., Richter, R. J., Keifer, M., Broomfield, C. A., Sowalla, J., & Furlong, C. E. (1996). *Nature Genetics*, *14*, 334–336.
7. Gordon, G. B., Bush, D. E., & Weisman, H. F. J. (1988). *Journal of Clinical Investigation*, *82*(2), 712–720.
8. Steinberg, D., Carew, T. E., Fielding, C., Fogelman, A. M., Mahley, R. W., Sniderman, A. D., et al. (1989). *Circulation*, *80*(3), 719–723.
9. Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., et al. (1995). *Circulation*, *91*(9), 2488–2496.
10. Ayub, A., Mackness, M. I., Arrol, S., Mackness, B., Patel, J., & Durrington, P. N. (1999). *Arteriosclerosis, Thrombosis, and Vascular Biology*, *19*, 330–335.
11. Delgado Alves, J., Ames, P. R., Donohue, S., Stanyer, L., Nourooz-Zadeh, J., Ravirajan, C., et al. (2002). *Arthritis and Rheumatism*, *46*(10), 2686–2694.
12. Mackness, M. I., & Durrington, P. N. (1995). *Lancet*, *346*(8979), 869–872.
13. Aviram, M., Hardak, E., Vaya, J., Mahmood, S., Millo, S., Hoffman, A., et al. (2000). *Circulation*, *101*(21), 2510–2517.
14. Sutherland, W. H., de Jong, S. A., & Walker, R. J. (2004). *Nephrology Dialysis Transplantation*, *19*(1), 75–82.
15. Mirvish, S. S. (1995). *Cancer Letter*, *93*(1), 17–48.
16. Chow, C. K., & Hong, C. B. (2002). *Toxicology*, *180*, 195–207.
17. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., & Byrns, R. E. (1993). *Proceedings of the National Academy of Sciences of the United States of America*, *90*(17), 8103–8107.
18. Bauer, J. A., Booth, B. P., & Fung, H. L. (1995). *Advances in Pharmacology*, *34*, 361–381.
19. Berlett, B. S., & Stadtman, E. R. (1997). *Journal of Biological Chemistry*, *272*, 20313–20316.
20. Ischiropoulos, H. (1998). *Archives of Biochemistry and Biophysics*, *356*, 1–11.
21. Petruzzelli, S., Puntoni, R., Mimotti, P., Pulera, N., Baliva, F., Fornai, E., et al. (1997). *American Journal of Respiratory and Critical Care Medicine*, *156*, 1902–1907.
22. Lamb, N. J., Gutteridge, J. M., Baker, C., Evans, T. W., & Quinlan, G. J. (1999). *Critical Care Medicine*, *27*, 1738–1744.
23. Gole, M. D., Souza, J. M., Choi, I., Hertkorn, C., Malcolm, S., Foust, R. F., et al. (2000). *American Journal of Physiology Lung Cellular and Molecular Physiology*, *278*, L961–L967.
24. Gan, K. N., Smolen, A., Eckerson, H. W., & La Du, B. N. (1991). *Drug Metabolism and Disposition*, *19*(1), 100–106.
25. El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A. Y., & Jürgens, G. (1989). *Journal of Lipid Research*, *30*, 627–630.
26. Josse, D., Xie, W., Masson, P., Schopfer, L. M., & Lockridge, O. (1999). *Chemistry & Biology Interact*, *119-120*, 79–84.
27. Yeung, D. T., Lenz, D. E., & Cerasoli, D. M. Y. (2005). *FEBS Journal*, *272*(9), 2225–2230.
28. Pignatelli, B., Chun-Qi, L., Paolo, B., Qiping, C., Wolfgang, A., Fredrik, N., et al. (2001). *Cancer Research*, *61*, 778–784.
29. Ahmed, Z., Ravandi, A., Maguire, G. F., Emili, A., Draganov, D., La Du, B. N., et al. (2002). *Biochemical and Biophysical Research Communications*, *290*, 391–396.
30. Naseem, K. M., Low, S. Y., Sabetkar, M., Bradley, N. J., Khan, J., Jacobs, M., et al. (2000). *FEBS Letters*, *473*(1), 119–122.
31. Gow, A. J., Farkouh, C. R., Munson, D. A., Posencheg, M. A., & Ischiropoulos, H. (2004). *American Journal of Physiology Lung Cellular and Molecular Physiology*, *287*, L262–L268.
32. Zou, M. H. (2007). *Prostaglandins & Other Lipid Mediators*, *82*, 119–127.
33. Takemoto, K., Ogino, K., Wang, D., Takigawa, T., Kurosawa, C. M., Kambayashi, Y., et al. (2007). *Acta Medica Okayama*, *61*(1), 17–30.
34. Peluffo, G., & Radi, R. (2007). *Cardiovascular Research*, *75*, 291–302.

35. Sampson, J. B., Ye, Y., Rosen, H., & Bechman, J. S. (1998). *Archives of Biochemistry and Biophysics*, *356*, 207–213.
36. Radi, R. (2004). *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 4003–4008.
37. Bartesaghi, S., Valez, V., Trujillo, M., Peluffo, G., Romero, N., Zhang, H., et al. (2006). *Biochemistry*, *45* (22), 6813–6825.
38. Kambayashi, Y., Hitomi, Y., Kodama, N., Kubo, M., Okuda, J., Takemoto, K., et al. (2006). *Acta Biochimica Polonica*, *53*(3), 577–584.
39. Alvarez, B., Ferrer-Sueta, G., Freeman, B. A., & Radi, R. (1999). *Journal of Biological Chemistry*, *274* (2), 842–848.
40. Josse, D., Lockridge, O., Xie, W., Bartels, C. F., Schopfer, L. M., & Masson, P. (2001). *Journal of Applied Toxicology*, *21*(1), S7–S11.