The Effect of Peroxynitrite on Plasma Antioxidant Level and Tyrosine Nitration

PEROKSİNİTRİTİN PLAZMA ANTİOKSİDAN DÜZEYLERİNE VE TİROZİN NİTRASYONUNA ETKİSİ

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Summary

Aim: This study was designed to examine the effects of ONOO" mediated oxidative stress as in vivo and in vitro.

Materials and Methods: In vivo studies; 0.5-1-2-4 mmol/L concentration of exogenously prepared ONOO" was added to the rat plasma and the direct effects of ONOO" on plasma uric acid and ascorbic acid were investigated. In addition, plasma 3-NT levels were investigated.

In vivo studies; E.coli was injected intraperitoneally to the rats at a dosage of 12x10^{9} colony-forming units/ml/kg. Blood samples were collected 6 hour after administration of E.coli. The effects of endogen ONOO" on plasma uric acid and ascorbic acid were investigated. In addition, plasma 3-NT levels were investigated as a marker of ONOO" mediated oxidative damage.

Plasma uric acid level was determined by the uricase method and ascorbic acid level was measured by the modified method of Roe and Keuther based on the 2,4 dinitrophenylhydrazine. In order to demonstrate the effects of ONOO", plasma 3-NT levels were measured by HPLC method.

Results: In vitro studies; we have observed that addition of exogen ONOO' resulted in a dose-dependent increase in the formation of 3-NT in plasma. We have also observed a dose-dependent decrease in uric acid and ascorbic acid plasma levels.

In vivo studies; we have observed that lipopolysaccharide-induced stress resulted in a considerable increase in the 3-NT levels in comparison to the control group ($p< 0.001$). In addition, plasma uric acid levels in the E.coli injected group were nearly half of the control values ($p< 0.01$). Similarly, plasma ascorbic acid levels were decreased in the E.coli-injected group by 61% in comparison to the control group ($p< 0.01$).

In conclusion, as is the case in in vitro, in vivo formation of ONOO" also cause depletion of plasma ascorbate and uric acid level and increase of plasma 3-NT level.

Key Words: Peroxynitrite, 3-Nitrotyrosine, Uric acid, Ascorbate

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Ozet

Amaç: Bu çalışma, in vivo ve in vitro olarak ONOO" aracılıklı oksidatif stresin etkilerini görmek için yapılmıştır.

Materyal ve Metodlar: İn vitro çalışmalarda; 0.5-1-2-4 mmol/L konsantrasyonlarında hazırlanan ONOO" rat plazmalarına eklenerek, ONOO' in plazma ürik asit ve askorbik asit düzeylerine direkt etkisi araştırıldı. Ayrıca plazma 3-NT düzeylerine bakıldı.

In vivo çalışmalarda ise; 12x10^{9} koloni oluşturan ünite/ml/kg dozda E.coli intraperitonel olarak tatlara enjekte edildi. E.coli uygulamasından 6 saat soma rat kanları toplanarak, endojen olarak oluşan ONOO" in plazma ürik asit ile askorbik asit düzeylerine etkileri incelendi. Ayrıca ONOO" aracılıklı oksidatif stresin bir markın olarak plazma 3-NT düzeyleri de incelendi.

Plazma ürik asit düzeyleri ürikaz metodu ile, askorbik asit düzeyleri ise 2,4 dinitrofenilhidrazin üzerine kurulan modifiye bir metodla saptandı. Plazma 3-NT düzeyleri ise HPLC metodu ile yapıldı.

Bulgular: İn vitro çalışmalarda; eksojen olarak eklenen ONOO" in doza bağlı olarak plazma 3-NT düzeylerini artırırken, plazma ürik asit ve askorbik asit seviyelerini azalttığını gözledik.

İn vivo çalışmalarda ise; kontrol gruplarına göre lipopolisakkarit tarafından indüklenen stresin, plazma 3-NT düzeylerini önemli ölçüde artırdığını gözledik ($p< 0.001$). Ayrıca, E.coli enjekte edilen gruplarda plazma lirik asit değerleri hemen hemen kontrolün yarısına kadar inerken ($p< 0.01$), benzer şekilde plazma askorbik asit düzeyleri de kontrol grubu ile karşılaştırıldığında %61 oranında azaldı ($p< 0.01$).

Sonuç olarak, in vitro ve in vivo ONOO", plazma askorbat ve ürik asit azalmasına ve 3-NT seviyesinin artmasına sebep olmaktadır.

Anahtar Kelimeler: Peroksinitrit, 3-nitrotirozin, Ürik asit, Askorbat

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Nitric oxide (NO) and superoxide anion (0_2"), which are produced by several cell types, can react with each other in aqueous solution at an almost diffusion-controlled rate to yield peroxynitrite (ONOO") (1). ONOO" formation in the extracellular environment by generation of both NO and 0_2" by several cell types such as endothelial cells and phagocytes is a likely process. Many pathological states such as ischaemia-reperfusion injury (2), rheumatoid arthritis (3) and athero sclerosis (4) can lead to the formation of large amounts of ONOO" within the vasculature, blood cells and various organs. ONOO" induces oxidative damage to plasma, as has been demonstrated by its effects in vitro in decreasing both the total peroxyl-trapping capacity of plasma and the level of antioxidants such as ascorbate, uric acid, plasma thiol groups and ubiquinol-10 (5-7).

ONOO" nitrates tyrosine residues in proteins to form 3-nitrotyrosine (3-NT) which is a stable product that provides indirect evidence that ONOO" is involved in certain pathological conditions (8-11).

The aim of our study is to evaluate whether in vivo formation of ONOO" has an effect on plasma antioxidants e.g uric acid and ascorbic acid levels. In vivo ONOO" formation was induced by Escherichia coli (E coli) injection to rats and ONOO" involvement was checked by measuring 3-NT levels.

Materials and Methods

3-Nitro-l-tyrosine was obtained from Sigma (St. Louis, MO, USA). H_20_2, sodium acetate, citrate, NaOH, $Mn0_2$, H_3P0_4, KH_2P0_4, K_2HP0_4 were purchased from Merck (Deisenhofen, Germany). All organic solvents were at HPLC grade.

$ONOO^-$ was prepared using a quenched flow reaction as described previously (10) and was used fresh. We designed our in vitro experiments with control rat plasma. Control rat plasma samples were incubated with different concentrations of ONOO" (0-4 nmol/1) at 37 °C for 15 minutes.

The experiments described in this manuscript were performed with adherence to the National Institutes of Health Guidelines, the Care and Use of Laboratory Animals, and the approval of the ethics committee of GATA School of Medicine was obtained before beginning the study. Twenty male Wistar rats (250-300 g) were used in the present study. As a standard protocol, all rats were housed in a quiet non-stressful environment for three days before study. The rats were randomly divided into 2 groups (n=10). The first group which served as control, received intraperitoneal saline (1 ml/kg); the second group received intraperitoneal Escherichia coli (E. coli) at a dosage of $12x10^9$ colony-forming units/kg. Blood samples were collected 6 h after administration of E. coli and plasma samples were obtained. Control rat plasma samples were used for the control group.

Plasma uric acid level was determined by the uricase method. Ascorbic acid level was measured by the modified method of Roe and Keuther based on the 2,4 dinitro phenylhydrazine method (12).

Measurement of tyrosine nitration

Plasma was firstly hydrolysed in 6N HC1 at 100 °C for 18-24 h, then samples were analysed on an HP 1050 DAD HPLC apparatus (Hewlett Packard, Waldbron, Germany). The analytical column was a 5 urn pore size Spherisorb ODS-2 Ci_8 reverse-phase column (4.6 x 250 mm; Alltech, Deerfield, IL, USA). The guard column was a C|g cartridge (Alltech, Deerfield, IL, USA). The mobile phase was 50 mmol/1 sodium acetate/50 mmol/1 citrate/8% (v/v) methanol, pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml min^{-1} with a UV detector set at 274 nm. 3-NT peaks were determined according to its retention time and the peaks were confirmed by spiking with added exogenous 3-NT (10 umol/1) or by reduction to aminotyrosine by sodium dithionite. Concentrations of 3-NT were calculated from 3-NT standard curve and expressed as umol/1 (13).

All experiments were performed in duplicate. The differences between groups were analysed by Mann-Whitney U test using SPSS 9.0.0® (SPSS Inc, 1989-1999) package program and p values smaller than 0.05 were accepted as significant (14).

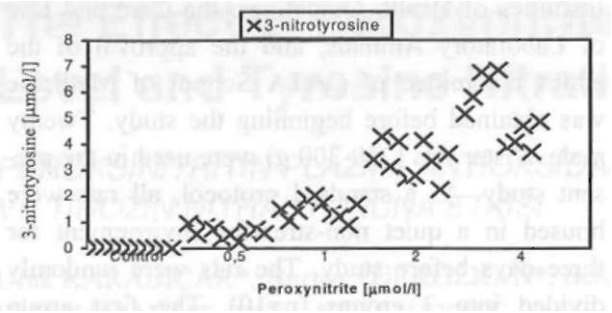

Figure 1. The effect of different dosage ONOO" on plasma 3-Nitrotyrosine levels. Increased dosage of ONOO' resulted in increased 3-nitrotyrosine formation. Ten plasma samples were treated with different doses of ONOO".

Results

Addition of exogenously prepared ONOO" resulted in a dose dependent increase in the formation of 3-NT in rat plasma (Figure 1). We have also observed a dose-dependent decrease in uric acid and ascorbic acid levels in response to added ONOO" in rat plasma (Figure 2).

None of the animals died or showed major signs of septic shock. In order to determine the presence of ONOO" formation in our in vivo experimental design, we checked plasma 3-NT levels. In the control groups plasma total 3-NT levels were hardly detectable. However the lipopolysaccharides-induced stress resulted in a considerable increase in the 3-NT levels (Figure 3). As can be seen from figure 3 plasma uric acid levels in the E.coli-injected group were nearly half of the control value ($p<0.01$). Similarly plasma ascorbate levels were decreased in the E.coli-injected group by 61% in comparison to the control group (Figure 3,$p<0.01$).

In Figure 4 and 5, the HPLC chromotograms of control and experiment animal were shown.

Discussion

Endothelial cells and activated phagocytes produce both NO and 0% which react to form ONOO" in the extracellular environment, a system rich in antioxidant defences. ONOO" causes depletion of important antioxidants and damage to proteins and lipids in plasma. It has been reported that ONOO" may oxidize plasma constituents by three possible mechanisms. First, the protonated form, peroxynitrous acid, decomposes rapidly to form a range of products, probably including OH and NO" $_2$, second, ONOO" itself has powerful oxidising capacity. Third, metal ions may catalyse heterolytic scission of the O - O bond in ONOO", resulting in the formation of nitrozium ion which is a powerful nitrating agent and readily nitrates tyrosines on proteins, resulting in the formation of 3-NT

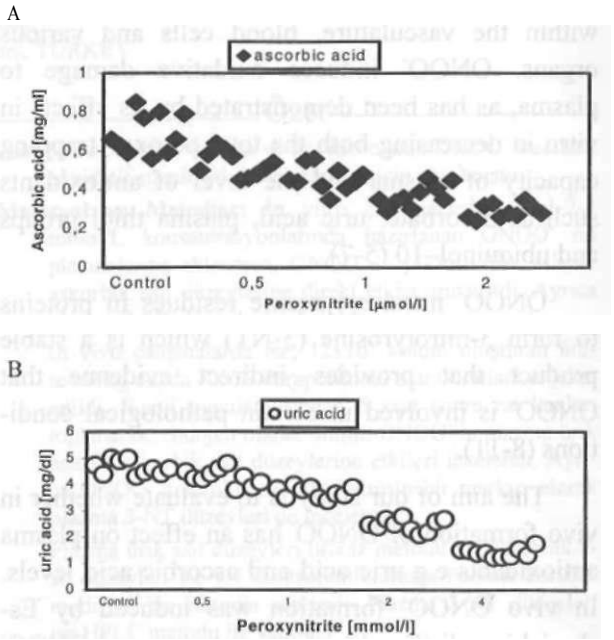

Figure 2. The effect of ONOO" on plasma ascorbic acid (A) and uric acid levels (B). 10 plasma samples were treated with different doses of ONOO".

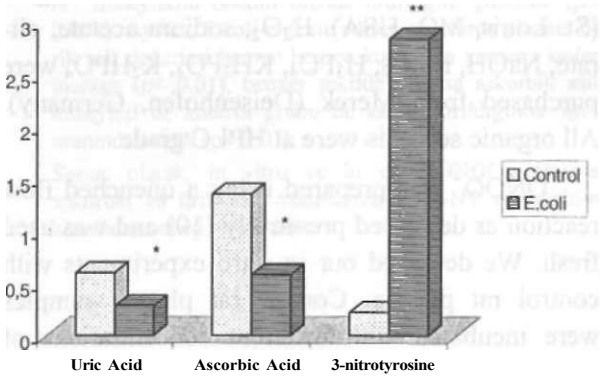

Figure 3. The levels of plasma uric acid, ascorbic acid and 3-nitrotyrosine levels in control and E.coli-injected groups. *$p<0.01$, **$p<0.001$ compared to control.

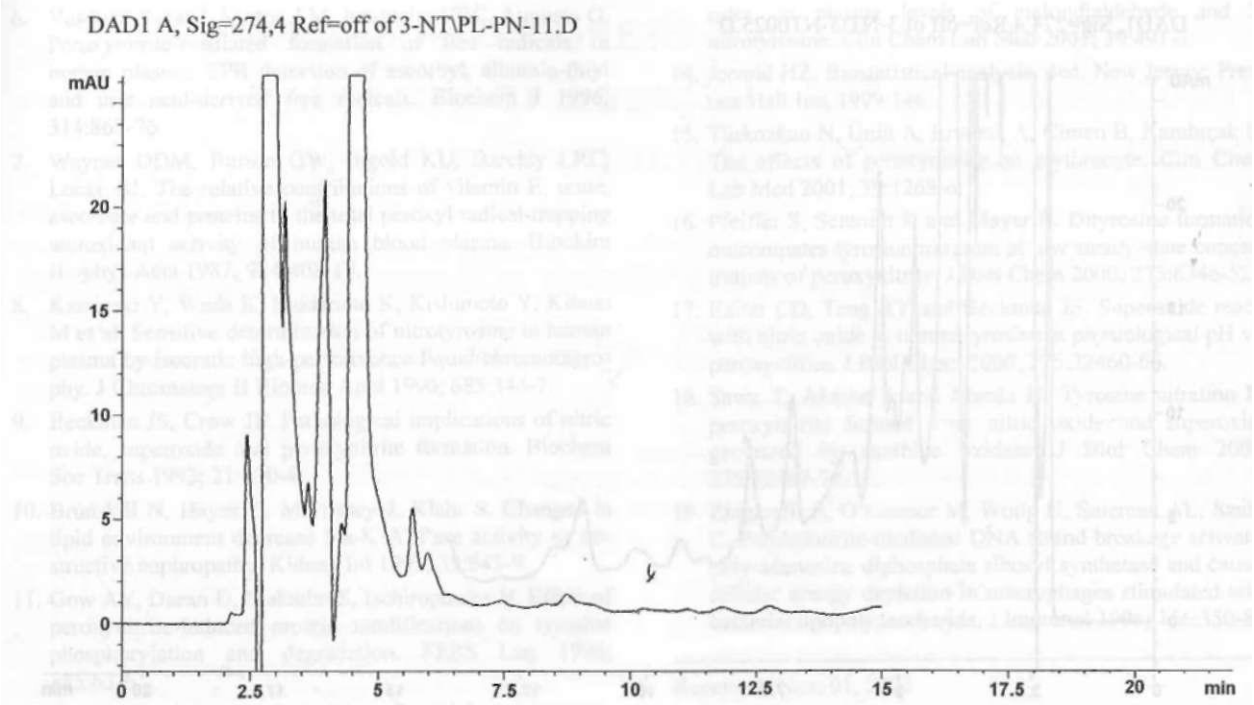

Figure 4. Kontrol rat plasma.

residues. 3-NT is a stable product that can also serve as indirect evidence that ONOO" is involved in certain pathological conditions. Endotoxins can stimulate production of free radicals from macrophages, endothelial cells and eosinophils. In this study LPS-induced ONOO" caused depletion of uric acid and ascorbic acid levels and formation of 3 NT. Wayner et al (7) reported the most important biological antioxidants would appear to be ascorbic acid, uric acid and vit E in plasma. In our study, six hours after administration of E.coli into rats, a significant increase in 3-NT level of plasma was observed. Administration of LPS in vivo is associated with over production of NO within vasculature, various organs and blood cells.

van der Vliet et al (5) reported that when ONOO" is added to plasma, rapid depletion of plasma ascorbic acid and uric acid and SH groups were observed, but they are not completely depleted and ascorbate is affected to the largest extent. These effects were observed 5 min after addition of ONOO" which was still able to oxidize lipids and nitrate protein tyrosine residues. Other workers suggest that uric acid may scavenge ONOO" and ONOO" derived reactive species. In plasma Vivor and co-workers reported (6) that ONOO" concentration of (0.1-1 mm) when added to plasma led to the formation of free radicals such as ascorbyl, uric acid-derived free radical, albumin thiol radical. Antioxidant depletion in plasma may be explained by the ONOO'-induced free radical formation.

In this study endogenously formed ONOO", in terms of 3-NT formation, caused an important depletion of uric acid and ascorbate levels in plasma. Direct comparisons of 3-NT formation in both in vitro and in vivo designs are diffucult since the concentration of ONOO" which may be achieved in vivo currently unknown. However, under oxidative stress, continuous production of ONOO" resulted in the formation of 3-NT levels which was slightly higher than single bolus of ONOO" (5,15). It has been shown that activated murine macrophages may generate up to 0.11 nmol. (10^6 cells)" min, which results in local concentrations of as much as 0.5 - 1.0 mm/min (5). In addition, myeloperoxidase-dependent pathways may also be responsible for 3-NT formation. How-

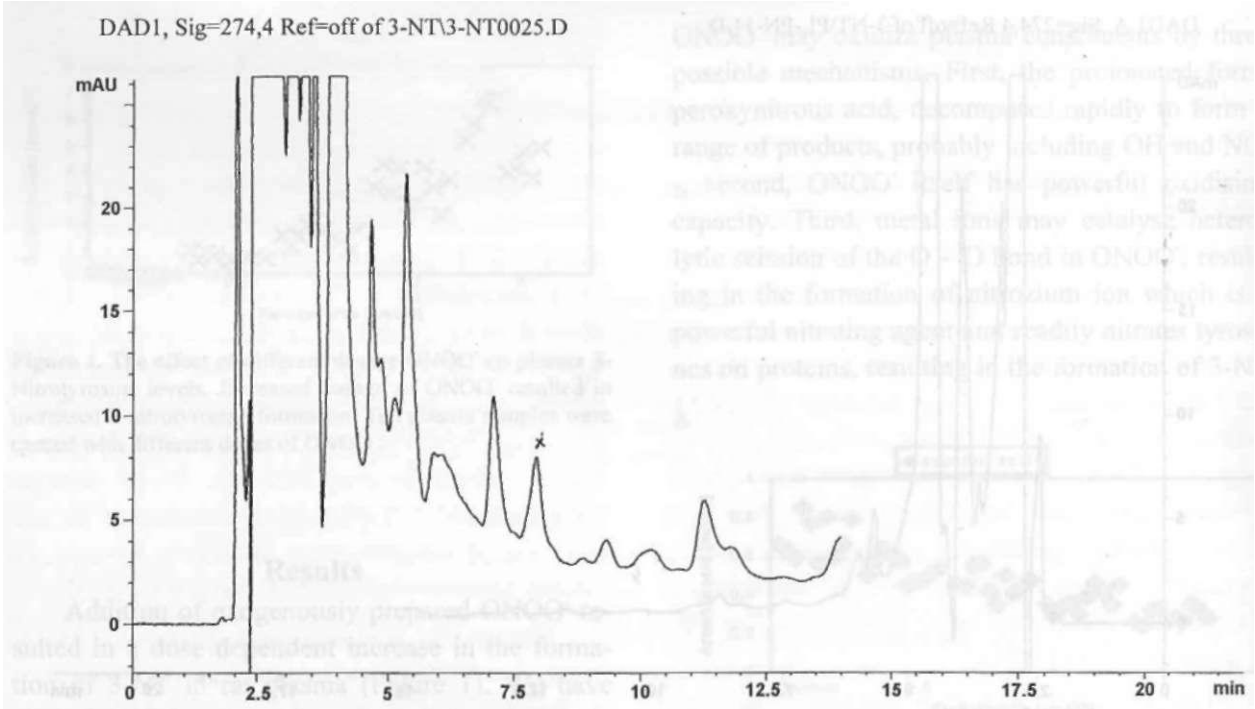

Figure 5. In vivo 3-NT formation.

ever, the real impact value of ONOO-dependent or myeloperoxidase-dependent 3-NT formation in both physiological and pathological conditions remains to be solved (16-18). In the light of the majority of the literatures, we also support the ONOO- dependent pathway. Although we did not particularly check neuthrophil myeloperoxidase activity, we did not observe any signs of systemic inflammation in response to endotoxin stimulation. The animals were normothermic and did not loose weight during the experimental procedure. Although there was an absence of major clinical signs, biochemical markers show the presence of oxidative and nitrosative stress in animals.

On the other hand it is not fair to claim that only ONOO' is responsible for the depletion of plasma antioxidants. In addition to the ONOO" effect, O_2" and OH" radicals may be also responsible for the depletion of antioxidants. However it is difficult to determine to what extent these radicals was involved and responsible for the depletion of plasma antioxidants. While the extent of ONOO-mediated damage is difficult to assess, ONOO" also exerts an important role in oxidative stress. However the formation of 3-nitrotyrosine supports ONOO- involvement. In another study (19), a PARS (Poly-Adenosine diphosphate Ribosyl Synthetase) inliibitor resulted in a 60% decrease in 3-NT formation.

In conclusion, as is the case in in vitro, in vivo formation of ONOO" also cause depletion of plasma ascorbate and uric acid level.

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