Short Communication

In Vivo Disposition of 3-Nitro-L-Tyrosine in Rats: Implications on Tracking Systemic Peroxynitrite Exposure

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ABSTRACT:

In many pathological conditions such as inflammatory and neurodegenerative diseases, the in vivo toxicity of nitric oxide has been attributed to the toxic oxidant peroxynitrite. Interaction of peroxynitrite with biological molecules can modify tyrosine residues on the proteins at the ortho position resulting in the formation of the stable end-product, 3-nitro-L-tyrosine (3-NT). Recent investigations indicate that changes in the circulating concentrations of 3-NT in pathological conditions may reflect the extent of nitric oxide-dependent oxidative damage and peroxynitrite toxicity. In the present study, we examined the in vivo disposition characteristics of 3-NT in rats after either a single i.v. bolus dose (10 mg/kg) or a loading and maintenance infusion at 10 or 30 mg/kg. Plasma concentrations of 3-NT were analyzed by a reversed-phase HPLC method. After a single bolus dose of 3-NT at 10 mg/kg, the average half-life of the elimination phase for the drug was 68.5 ± 18.4 min (n = 5). Infusions of 3-NT at two different doses (10 and 30 mg/kg) indicated that the pharmacokinetic properties of 3-NT below plasma concentrations of 100 μM were both linear and stationary. Urinary excretion of unchanged 3-NT was minimal, but two distinct metabolites of 3-NT were identified in the urine collected throughout the study. These findings may be useful in the interpretation of the plasma and urine 3-NT concentrations as possible indices of systemic peroxynitrite exposure.

Peroxynitrite is an important toxic product of the reaction between nitric oxide and superoxide anion (O₂⁻). In many pathological conditions such as atherosclerosis, septic shock, cerebral ischemia, and Alzheimer’s disease, peroxynitrite has been implicated as a major contributing factor to the underlying pathology (Beckmann et al., 1994; Haddad et al., 1994; Smith et al., 1997; Tanaka et al., 1997). Peroxynitrite is a potent oxidant that reacts with major biological molecules. Nitration of tyrosine residues by peroxynitrite on the ortho position has been shown to result in formation of a stable product, 3-nitro-L-tyrosine (3-NT)³ (Crow and Beckman, 1995; Beckman, 1996). Nitratated tyrosine residues have been demonstrated to exist in proteins of damaged tissues (Beckmann et al., 1994; Haddad et al., 1994; Smith et al., 1997) and have been shown to be involved in regulating the functional activity of various proteins (Ischiropoulos et al., 1992). Recently, steady-state concentrations of free 3-NT in human plasma were determined and estimated to be about 0.05% of that observed for tyrosine (Kamisaki et al., 1996). In pathological conditions such as rheumatoid arthritis, septic shock, and amyotrophic lateral sclerosis, free 3-NT levels have been shown to be increased, possibly reflecting the degree of oxidative damage induced by peroxynitrite (Kaur and Halliwell, 1994; Bruijn et al., 1997; Fukuyama et al., 1997). Measurements of 3-NT levels in blood and synovial fluid samples obtained from patients with rheumatoid arthritis indicated an 80- to 100-fold increase in the free 3-NT concentrations in comparison with those observed in normal volunteers (Kaur and Halliwell, 1994). These data indicate that the changes in the circulating 3-NT plasma concentrations in pathological conditions may reflect the extent of nitric oxide-dependent oxidative damage and peroxynitrite toxicity.

Because a basic understanding of the disposition properties of 3-NT can provide useful information in assessing the degree of peroxynitrite exposure, we have examined the in vivo disposition properties of 3-NT in rats after i.v. bolus administration. Furthermore, to simulate conditions under which a rapid surge in 3-NT concentrations is followed by a new steady-state and then a washout phase, we devised an infusion regimen that allowed us to examine both the effect of changes in dose and time on the in vivo disposition properties of 3-NT in rats.

Experimental Procedures

Materials. 3-NT, N-acetyl-tryptophan, heptane sulfonic acid (sodium salt), 3-amino-L-tyrosine, and p-hydroxyphenylacetic acid were purchased from Sigma Chemical Company (St. Louis, MO). 3-Nitro-4-hydroxyphenylacetic acid (NHPA) and tetrani-tromethane (TNM) were obtained from Aldrich Chemical Company (Milwaukee, WI). All solvents used were of HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA).

HPLC Assay. Plasma concentrations of 3-NT were analyzed by a reversed-phase HPLC method. Briefly, 10 μl of the internal standard (N-acetyl-tryptophan, 0.5 mM) was added to 90 μl of plasma. Plasma proteins were then precipitated with 70 to 72% perchloric acid and samples were vortexed and centrifuged. The resultant supernatant was removed and a 50-μl aliquot was injected into a C18 reversed-phase Ultrasphere column (250 × 4.6 mm i.d., 5 μm) that was preceded by a guard column (45 × 4.6 mm i.d.), both from

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Beckman Instruments (San Ramon, CA). The mobile phase used was 18.5 mM heptane sulfonic acid: 30% methanol (pH 2.7) at a flow rate of 1.5 ml/min. UV detection was accomplished at 275 nm. The calibration curves in plasma were linear between 5 and 100 μM. The identity of the 3-NT peak after elution from the column in plasma samples was verified by comparing the spectrum of the eluted peak to that of the authentic 3-NT using a photodiode array detector (Waters 991, Millipore Corp., MA).

**Determination of Urinary Concentrations of NHPA and 3-Nitro-4-Hydroxyphenylacetic Acid (NHPA).** Concentrations of NHPA, a urinary metabolite of 3-NT, were analyzed using the HPLC method as described above. Briefly, 45 μl of the internal standard (3-amino-L-tyrosine, 10 mM) was added to 55-μl urine samples; samples wereacidified by addition of 10 μl of perchloric acid (70–72%). After vortexing and centrifugation, a 50-μl aliquot of the resulting sample was injected into the HPLC column. The calibration curves for NHPA in urine were obtained by spiking blank urine (45 μl) with 35 μl of internal standard and 10 μl of each working standard of NHPA to achieve the final concentrations of 1 to 10 mM. The identity of the NHPA peak in urine after elution was verified by photodiode array detection. NHPA was made from the reaction of TNM with p-hydroxyphenylacetic acid and overnight incubation at room temperature as described previously (Sokolovsky et al., 1966). The identity of the NHPA peak was verified by photodiode array detection.

**Animal Studies.** Male Sprague-Dawley rats (318 ± 30 g) were anesthetized with ketamine-xylazine (90–10 mg/kg) 2 days before the pharmacokinetic study and indwelling cannulas were implanted in the femoral vein and artery for administration of drug and blood sampling, respectively. In single bolus dosing studies, animals (n = 5) received an i.v. bolus dose of 10 mg/kg 3-NT in saline through the femoral vein cannula followed by flushing with normal saline. Blood samples (300 μl) from the artery were collected in heparinized tubes, immediately centrifuged (2 min at 13,000 g), and the separated plasma was stored at −20°C until analysis. In infusion studies, male rats received 3-NT at either 10 or 30 mg/kg (n = 3 at each dose) as a loading i.v. infusion (200 μl/min for 20 min, at a rate of either 1.27 or 3.81 μmol/min/kg), which was followed by a constant maintenance infusion (42.5 μl/min for 60 min, at a rate of 0.27 or 0.81 μmol/min/kg). In all experiments, the total volume of saline administered was held constant (6.5 ml) by varying the drug concentration in the infusion medium. During the infusion, blood samples (200 μl) were collected from the femoral artery at various time points and stored until analysis. Urine samples were also collected in these rats and stored until analysis.

**Data Analysis.** The Spline polynomial method (Yeh and Kwan, 1978) was used to calculate the area under the plasma concentration-time curve (AUC) and the first moment curve after i.v. bolus administration of 3-NT at the 10 mg/kg dose. The mean residence time (MRT) was determined as the ratio of area under the first moment curve/AUC. Systemic clearance (Clp) was calculated as dose/AUC. The volume of distribution at steady-state (Vss) was obtained from Clp × MRT. For the infusion studies, loading and maintenance infusion rates were calculated based on the targeted steady-state plasma 3-NT concentrations (30 and 90 μM) using the pharmacokinetic parameters obtained from the noncompartmental analysis of the data in the single bolus dosing studies and the following equations (Wagner, 1974):

\[
\begin{align*}
    k_{01} &= k_{02}(1 - e^{-\frac{A}{T}}) \\
    k_{02} &= \frac{V_{ss}}{Clp}
\end{align*}
\]

where k01 and k02 are the loading and maintenance infusion rates, A is the slope of the terminal phase, Vss is the targeted steady-state concentration (30 or 90 μM), T is the time during which the loading dose was infused (20 min) and Clp is the systemic clearance obtained from single bolus studies after administration of a 10 mg/kg dose. The systemic clearance at steady-state (Clpss) was calculated using the following equation and the steady-state plasma concentrations obtained from the samples collected at t = 60 min (C80):

\[
    Clp_{ss} = k_{02}C_{ss}^{0.60}
\]

Computer simulations were performed to generate the predicted concentration-time profile for 3-NT during the course of infusion using the ADAPT computer program (Biomedical Simulations Resource, Los Angeles, CA) and differential equations describing a two-compartment model with a zero-order input rate.

**Results**

After administration of a bolus dose of 10 mg/kg of 3-NT, plasma concentrations of 3-NT declined rapidly, indicating a fast distribution phase which was followed by a more prolonged elimination phase (Fig. 1A). The average half-life of the elimination phase for the drug was 68.5 ± 18.4 min (n = 5). The values of Vss, Clp, and MRT obtained through noncompartmental analysis of the data were 0.78 ± 0.22 l/kg, 8.12 ± 0.99 ml/min/kg, and 95.5 ± 26.2 min, respectively. Using these pharmacokinetic parameters, computer simulations were performed to predict the plasma concentration-time profile for 3-NT after a loading and maintenance infusion, which was followed by a washout phase (Fig. 1B, solid lines). Experiments were carried out to verify the integrity of the predictions obtained through the use of computer simulations. Figure 1B shows plasma concentration-time profiles for 3-NT after a loading and maintenance infusion at 10 and 30 mg/kg doses (symbols). As shown, the simulated lines gave an excellent description of the experimental data during the infusion and washout phases. The average value for Clpss obtained in these studies was 10.2 ± 1.38 ml/min/kg (n = 6) which was similar to the Clp obtained after bolus administration (P > 0.05, Student’s t test).

Throughout the infusion studies, urine samples were collected and samples were examined for 3-NT. However, there was no detectable drug (<3 μM or <1% of the administered dose based on the volume of urine collected) in urine after infusion of either the 10 or 30 mg/kg dose. The volume of urine excreted over the length of the study was 6.2 ± 3.5 ml and 4.8 ± 0.76 ml, for 10 and 30 mg/kg dose,
respectively (n = 3 at each dose, P > .05). Consistent with previous observations in rats and humans (Ohshima et al., 1991), we observed two urinary metabolites of 3-NT in these samples. These metabolites were previously identified and reported to be NHPA and NHPL (Ohshima et al., 1991). The identity of the NHPA peak was verified by comparing its absorbance spectrum as well as its retention time to those of the authentic NHPA that was obtained commercially (data not shown). Concentrations of NHPA in urine appeared to increase proportionally with increasing the 3-NT dose (0.14 ± 0.11 mM and 0.34 ± 0.11 mM, at 10 and 30 mg/kg dose, respectively). Total amount of NHPA recovered in the urine samples was 0.67 ± 0.3 μmol and 1.86 ± 0.67 μmol, at 10 and 30 mg/kg, which accounted for 6.1 ± 2.7% and 4.7 ± 1.6% of the total administered dose of 3-NT, respectively. The spectrum of the two metabolite peaks observed in the urine appeared to be similar to that obtained for 3-NT and different from 3-amino-tyrosine, indicating that the nitro functional group of 3-NT was not changed in any of the two metabolites detected. We were unable to obtain authentic samples of NHPL so we could not quantify the extent of its urinary excretion. However, the spectrum and the retention time of the NHPL peak in urine was similar to that of the NHPA peak obtained from reaction of TNM with p-hydroxyphenylactic acid. The magnitudes of the spectrophotometric signals for NHPL in urine were similar to those observed for NHPA. Neither metabolite was detectable in plasma under our experimental conditions.

Discussion

In many pathological conditions, the extent of peroxynitrite production has been determined indirectly by measuring the tissue or plasma 3-NT concentrations, the stable nitrated product of tyrosine reaction with peroxynitrite. Through the use of immunohistochemical staining with polyclonal antibodies, the widespread occurrence of 3-NT in damaged tissues has been demonstrated. For example, in the ischemic gerbil cerebral cortex (Tanaka et al., 1997), brain and lung tissues from human patients (Haddad et al., 1994; Smith et al., 1997), and atherosclerotic lesion of human coronary arteries (Beckmann et al., 1994), 3-NT was detected. Recently, Fukuyama et al. (1997) determined plasma concentrations of 3-NT in septic shock patients with renal failure using an HPLC assay and reported plasma 3-NT concentrations above 100 μM in these patients.

In the present study, we determined the fundamental disposition characteristics of 3-NT. We showed that the in vivo half-life of 3-NT was short and the apparent volume of distribution of 3-NT was about 20-fold larger than the plasma volume of the rat (40 ml/kg), indicating extensive distribution of the drug into extravascular tissues. The apparently large distribution of 3-NT into tissues may be due to the cellular uptake of 3-NT by various amino acid transporters. The neutral amino acid transporter, the L system, has been reported to be responsible for the cellular transport of tyrosine. However, further investigations are required to establish the role of this or other amino acid transporter(s) in the cellular uptake of 3-NT.

To simulate conditions (e.g., possibly in septic shock) under which a rapid surge in 3-NT concentrations is followed by a new steady state, we devised a dosing regimen consisting of a loading and maintenance infusion that resulted in establishing different steady-state plasma concentrations, i.e., 30 and 90 μM over an 80-min infusion interval. As predicted through the use of computer simulations, experimental data indicated that pharmacokinetic properties of 3-NT were linear below plasma concentrations of 100 μM. The linearity in the pharmacokinetics of 3-NT was evident from the calculated plasma clearance over a wide range of concentrations of 3-NT that were examined. Furthermore, no apparent time dependence was observed in the disposition properties of 3-NT over the infusion interval that was used in this study. The slope of elimination phase during the washout phase was similar to that obtained after i.v. bolus administration of the drug. The mean residence time of 3-NT after bolus administration was short, suggesting that 50% of the drug was eliminated from the body in about 100 min. These pharmacokinetic properties should be considered when monitoring rapid surges in the free plasma 3-NT concentrations.

Ohshima et al. (1991) have previously examined the urinary elimination of 3-NT metabolites in rats after administration of a 100 μg oral dose of 3-NT, using gas chromatography with a thermal energy indicator. In agreement with these results, we also observed the two metabolites of 3-NT in the urine of the rats infused with the drug. Using the HPLC assay, we were not able to detect any intact 3-NT in urine samples (<1% of the administered dose). The on-column sensitivity of our assay was 30 ng/injection, which may be a limiting factor in determining 3-NT in the urine samples that we collected. However, a more sensitive assay using gas chromatography with a thermal energy indicator (0.5 ng/injection) that was used in previous studies (Ohshima et al., 1991) also was unable to detect 3-NT traces in rat and human urine. These results suggest that urinary elimination of the intact 3-NT may be minimal but 3-NT metabolites may serve as useful noninvasive urinary markers of peroxynitrite exposure.

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