STABILITY AND INTERACTIONS OF RECOMBINANT HUMAN NERVE GROWTH FACTOR IN DIFFERENT BIOLOGICAL MATRICES: IN VITRO AND IN VIVO STUDIES

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ABSTRACT:

The purpose of this investigation was to characterize the stability, activity, and interactions of recombinant human nerve growth factor (rhNGF) in various biological matrices in vitro and in vivo. rhNGF (10 µg/ml) remained stable in human plasma for up to 4 days at 37°C. There was a decrease in the recovery of rhNGF after incubation at lower concentrations (20 ng/ml) and for longer time periods (3 and 5 days at 37°C). Size exclusion HPLC analysis indicated that rhNGF forms high molecular weight (HMW) complexes after long incubation periods. We confirmed that α2-macroglobulin (α2M) is the major plasma component that binds to rhNGF. Furthermore, this interaction was considerably increased by treatment of plasma with primary amines such as CH₃NH₂. Changes in the pH environment did not affect the interaction of rhNGF with α2M. We also determined that the binding of rhNGF to CH₃NH₂-treated pure α2M or α2M present in human plasma substantially diminished its immunoreactivity and bioactivity detection. The interaction of rhNGF with activated α2M was reversed and inhibited by coinubcation with dimethyl sulfoxide. Released rhNGF under these conditions was fully bioactive. 125I-rhNGF also binds to α2M by forming similar 125I-rhNGF/HMW complexes in plasma after i.v. administration in rats and mice. Sixty minutes after dosing in rats, most of the labeled material was in the form of a 125I-rhNGF/HMW complex. These studies have provided a better understanding of the nature of the interactions of rhNGF with plasma components as well as methods to enhance, reverse, and inhibit these interactions.

Nerve growth factor (NGF) is required by sensory and neural crest-derived sympathetic neurons for survival and differentiation during embryonic and early postnatal life (Thoenen and Barde, 1980). NGF is also critical for the normal function of these neuronal types in adult animals. Based on the action of NGF on these cells, a potential therapeutic role has been proposed for recombinant human nerve growth factor (rhNGF) in peripheral neuropathies, which are characterized by dysfunction of the small unmyelinated fibers and sympathetic neurons. In a phase II clinical trial study, rhNGF was demonstrated to be active, and it improved some of the sensory impairments in diabetic neuropathy (Apfel et al., 1998).

Although the structure, biosynthesis, receptors, biological effects, and physiological roles of NGF have been well characterized, there is controversial evidence about its presence in the bloodstream (Levi-Montalcini and Booker, 1960; Henry, 1972; Banks et al., 1973). Analysis of rhNGF by Korsching and Thoenen (1983) and Furukawa et al. (1983) failed to detect any significant levels in plasma. More recently, different laboratories have reported detectable NGF levels in human plasma in the low pM range (Faradji and Sotelo, 1990; Bracci-Laudiero et al., 1993). Hogue-Angeletti (1969) and Suda et al. (1978) have shown that mouse NGF in serum interacts with high molecular proteins that interfere with the measurements of NGF. Subsequently, several investigators have shown that NGF binds to α2-macroglobulin (α2M) present in serum (Ronne et al., 1979; Koo and Stach, 1989) with Kd values in the low nanomolar range (Koo and Stach, 1989). Most of the studies to date characterizing the interactions of NGF in serum have used mouse NGF purified from the submaxillary gland, known as β-NGF.

Human α2M (M₉ ~718,000) is a major plasma component present at high (2-4 mg/ml) concentrations (Sotrup-Jensen, 1987). This tetrameric glycoprotein is composed of four identical polypeptide chains held together in pairs by disulfide bridges and by noncovalent bonds (Swenson and Howard, 1979; Sotrup-Jensen et al., 1984). Each subunit contains a protease-sensitive sequence called the bait region and a thiol ester. After proteolytic degradation and thiol ester cleavage, α₂M undergoes a conformational change or activation, which traps and blocks the protease activity (Gonias et al., 1982). A similar conformational activation change has been postulated in the specific binding of nonprotease growth factors, including NGF to α2M. Although the mechanism of these interactions is not well understood, the activation of α₂M is dramatically enhanced by treatment with primary amines such as serotonin and CH₃NH₂ (Gonias, 1992; Hall et al., 1992), and reagents such as thiocyanate and iodine have been shown...
to inhibit or partially reverse the activation of α2M (Cunningham et al., 1990).

In this article, we report studies performed to assess the stability and potential interactions that may occur between rhNGF and plasma components in a series of in vitro and in vivo studies. In these experiments, we have further characterized the associations between rhNGF and plasma components, mainly α2M, and the effects of these interactions on the bioactivity and metabolism of rhNGF. Analysis of rhNGF stability and bioactivity in plasma would provide information to further understand potential pharmacological effects of rhNGF in the clinic.

Materials and Methods

Preparation of rhNGF. rhNGF (Genentech, Inc., South San Francisco, CA) was purified after production from a Chinese hamster ovary cell line as described previously (Burton et al., 1992) and stored at 4°C in an excipient buffer of 10 mM sodium acetate/140 mM NaCl, pH 5.5.

Human Plasma Collection. Fresh blood was drawn from healthy humans in citrate (1000 U/ml). Plasma was obtained after centrifugation at 2000 rpm for 20 min at 4°C. Equivalent volumes were pooled together and stored frozen at −70°C until assay.

Iodination of rhNGF. rhNGF was iodinated with sodium I-125 (NEN Life Science Products, Boston, MA) using the lactoperoxidase method. Twenty micrograms of rhNGF was labeled in 10 mM sodium acetate/140 mM NaCl, pH 5.5 with lactoperoxidase (1 U/ml) and 2 μCi sodium I-125. The reactions were initiated by the addition of 15 μl of H2O2 diluted 1:174,000. After a 5-min incubation at room temperature, 15 μl of H2O2 was added and the reaction was stopped 5 min later by adding 15 μl of N-acyetyl-D,L-tyrosine (20 mM). The iodinated proteins were separated from unincorporated I-125 using PD-10 size exclusion (SEC) columns (Pharmacia, Upsalla, Sweden). I25I-rhNGF was stored at 4°C in 10 mM sodium acetate/140 mM NaCl, pH 5.5 containing 1 mg/ml human serum albumin and 0.5 mg/ml protamine sulfate. The final material was >98% trichloroacetic acid-precipitable, and SDS-polyacrylamide gel electrophoresis of the radiolabeled material indicated one single radioactive band with a molecular mass of approximately 13 kDa.

NGF Bioassays. The biological activity of NGF was determined by two methods. Plasma samples containing rhNGF were tested for the capacity to support the survival of NGF-conditioned pheochromocytoma-12 (PC12) cell line using a cell viability stain as described previously (Shih et al., 1994). Data are presented as PC12-derived bioactive NGF concentration. The limit of detection was 5 ng/ml. The novel kinase receptor activation assay (KIRA) was used to determine the bioactivity of rhNGF by measuring NGF-induced activation of the NGF tyrosine kinase receptor (TrkA) in terms of receptor phosphorylation as described previously (Sadick et al., 1997). Data are presented as bioactive NGF concentration with an assay range of 1 to 20 ng/ml.

NGF Enzyme-Linked Immunosorbent Assay (ELISA). Human plasma samples were analyzed for immunoreactive rhNGF concentration by an ELISA. The two-site ELISA used polyclonal antibodies raised to rhNGF in rabbits as described previously (Bennett et al., 1990). The assay range was 0.4 to 6.0 ng/ml.

Nondenaturing SEC of NGF. Samples were analyzed on a TSK-4 SWXL (7.8 × 30 cm) G2000, G3000, or G4000 column (TosoHaas, Montgomeryville, PA) with a precolumn filter (no. A315; Upchurch Scientific, Oak Bay, WA) on a model 1090 Series II HPLC apparatus (Hewlett Packard Co., Palo Alto, CA). The mobile phase was 0.4 M potassium phosphate, pH 6.5 at a flow rate of 1.0 ml/min with a 20-min assay time. The effluent was monitored at 280 nm. Gel filtration protein standards were used to calibrate the assay bath. The samples (26 ng of I25I-rhNGF) were analyzed on a TSK-4 SWXL SEC column.

Effect of pH on Activation of α2M in Human Plasma. The effect of pH on the interactions of rhNGF with human plasma components was assessed under the following conditions:

1. Pooled citrated human plasma was mixed with various buffers in a 1:1 volume ratio, and the final pH of the solution was measured. I25I-rhNGF (1.3 μg/ml) was added to the following plasma buffer solutions: sodium acetate, pH 4.1; sodium acetate, pH 5.5; KH2PO4, pH 6.5; PBS, pH 7.4; Tris-HCl, pH 8.0; and Tris-HCl, pH 9.2 and incubated for 4 h at 37°C. Twenty six nanograms of I25I-rhNGF in each mixture was analyzed on a TSK-4 SWXL SEC column.

2. I25I-rhNGF (1.3 μg/ml) was incubated in pooled citrated human plasma for 24 h at 37°C in the presence or absence of 5% CO2. The samples (26 ng of I25I-rhNGF) were analyzed on a TSK-4 G2000 SWXL SEC column.

3. I25I-rhNGF (1.3 μg/ml) was subjected to repeated cycles (10 times) of quick freezing over dry ice and thawing to room temperature in a water bath. The samples (26 ng of I25I-rhNGF) were analyzed on a TSK-4 G2000 SWXL SEC column.

Modification of α2M Activation in Human Plasma. CH3NH2 treatment of human plasma was prepared by incubating 200 mM CH3NH2 in equivalent volumes of pooled citrated human plasma or pure α2M and 0.4 M Tris-HCl, pH 8.0. I25I-rhNGF (1.3 μg/ml) incubated in the CH3NH2-treated plasma or CH3NH2-treated pure α2M (2.5 mg/ml, M-7151; Sigma, St. Louis, MO) for 3 h at 37°C. To assess the specificity of the modifications of I25I-rhNGF with α2M, an excess of cold rhNGF (300 ng) was incubated with I25I-rhNGF in CH3NH2-activated plasma or I25I-rhNGF in CH3NH2-treated pure α2M for 3 h at 37°C.

The recovery of immunoreactive and bioactive rhNGF in human plasma was measured by ELISAs and KIRAs, respectively. rhNGF (25 μg/ml) was incubated in citrated human plasma with and without 200 mM CH3NH2 and pure α2M for increasing time intervals at 37°C. Aliquots were taken at 0, 2, 4, 6, 8, 10, 12, 16, 20, 48, and 72 h. Levels of rhNGF were quantitated by ELISAs and KIRAs.

Reversal of α2M Activation in Human Plasma. To determine the effects of dimethyl sulfoxide (DMSO) on rhNGF interactions with α2M, citrated human plasma was activated with CH3NH2 for 3 h at 37°C, followed by incubation with 1, 2.5, 5, 10, and 25% DMSO (v/v) for 3 h at 37°C. In another careful experiment test the reversal of α2M activation, rhNGF (50 μg/ml) was incubated in untreated citrated human plasma or CH3NH2-treated human plasma for 3 h at 37°C. Dimethyl sulfoxide (5, 10, or 25%; v/v) was then added to the samples. The concentration of rhNGF in the samples was measured by ELISA.

Interactions of NGF In Vivo. Studies in animals were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care. Three male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 250 to 274 g were used. Sodium iodide (5 mg) was administered i.p. at 48, 24, and 0.5 h before administering the radiolabeled material. Two days before the start of the study, animals were anesthetized with ketamine (35–50 mg/kg) and xylazine (0.75 mg/kg), and cannulae were implanted into the jugular and femoral veins of the rats. The cannulae were flushed with heparin/saline (200 U/ml), coiled, and inserted into a s.c. pouch on the back of each animal. Animals were allowed to recover for 48 h after cannulation. Food and water were supplied ad libitum. Rats received a single i.v. bolus dose of 274 μCi/rat (average dose = 1.06 mCi/kg) of I25I-rhNGF via the femoral venous catheter. Blood was collected in EDTA (final 3% concentration) via the jugular venous catheter at 5, 10, 30, and 60 min postdose. Animals were sacrificed at 60 min postdose by exsanguination under deep anesthesia (using ketamine/xylazine). Plasma was obtained
Stability of rhNGF in Human Plasma. In a preliminary study, the stability of rhNGF was assessed by several methods. rhNGF (10 and 25 µg/ml) was incubated in fresh citrated human plasma for increasing time periods, and aliquots were subjected to KIRA and PC12 assays, respectively. There was no decline in immunoreactivity and bioactivity for up to 4 days incubation at 37°C (Fig. 1, A and B, respectively). The levels of rhNGF remained constant, indicating minimal degradation at these concentrations. Additional studies at lower and more pharmacologically relevant concentrations were performed. Decreasing concentrations rhNGF were incubated (20 µg/ml, 2 µg/ml, 200 ng/ml, and 20 ng/ml) of rhNGF in human plasma for 1, 3, and 5 days at 37°C. Levels of rhNGF were quantified by ELISA. Assay sensitivity was the limiting factor in determining the lowest concentration of rhNGF used in this assessment. Figure 1C shows that the recovery of rhNGF was dependent on incubation time and concentration. At a high concentration of rhNGF (20 µg/ml), the percent recovery was high (88.3% ± 5.1 after 1 day of incubation at 37°C) and decreased slightly with time (68.0% ± 3.9 after 5 days of incubation at 37°C). However, at the lowest concentration tested (20 ng/ml), the recovery of rhNGF was significantly decreased (57.7% ± 9.8 at day 1) and there was additional decrease in recovery with a longer incubation time (20.7% ± 3.9 at day 5). These results indicated that at least 50% of the original material was recovered after 24-h incubation at all measured concentrations, but the decrease in rhNGF recovery at the lowest concentration tested (20 ng/ml) became significant after 5 days of incubation in plasma.

To further characterize the fate of rhNGF, labeled rhNGF was incubated in fresh citrated human plasma for increasing periods of time and subjected to SEC HPLC. Chromatographs of 125I-rhNGF incubated in citrated human plasma for 1, 3, and 5 days at 37°C. Levels of rhNGF were quantified by ELISA. The radioactivity from 125I-rhNGF was shown to be present in one main radioactive peak, corresponding to the position of 125I-rhNGF dimer and a minor peak corresponding to free I-125 (Fig. 2). At later incubation timepoints, an 125I-rhNGF/high molecular weight (HMW) peak appeared at or near the void volume with an apparent molecular mass of at least 600 kDa. The predominant peak of radioactivity for up to 6 h of incubation corresponded to the 125I-rhNGF dimer. After 1 day of incubation and continuing on to 5 days, there was a significant decrease in the 125I-rhNGF/HMW component concomitant with a decrease in the 125I-rhNGF dimer peak. By 7 days of incubation, the 125I-rhNGF/HMW complex was the dominant peak. The shift in the radioactive profile from 125I-rhNGF dimer to the 125I-rhNGF/HMW complex suggested an interaction or aggregation of 125I-rhNGF in human plasma. The recovery of total radioactivity over these increasing incubation periods remained approximately constant (70–80%, data not shown).

**Effects of pH on the Interactions of rhNGF in Human Plasma.**

The effect of pH on the formation of 125I-rhNGF/HMW complexes in human plasma was determined. The pH of fresh human plasma was adjusted by mixing equal volumes of plasma with various buffers, resulting in the following final pH values: 4.1, 6.0, 6.8, 7.0, 7.5, 8.0, and 8.9. 125I-rhNGF was incubated under these conditions for 4 h at 37°C, and aliquots of the incubations were subjected to SEC HPLC as above. No changes in the ratios of the 125I-rhNGF/HMW complex and 125I-rhNGF dimer peaks were observed, with the exception of plasma at pH 4.1 (Fig. 3A). At pH 4.1, the percentages of integrated peaks of 125I-rhNGF/HMW complex and 125I-rhNGF were 36.3 and 44.5%, respectively. In contrast, the average percentage of integrated peaks of 125I-rhNGF/HMW complex and 125I-rhNGF for the remaining plasma/buffers were 26.9 ± 3.5 and 54.3 ± 3.6%, respectively. It has
been previously reported that incubation of rhNGF under a 1% CO₂ environment prevented the formation of 125I-rhNGF/HMW complexes (Keck et al., 1994). In our study, 125I-rhNGF was incubated in fresh human plasma in the presence or absence of a 5% CO₂ environment and also subjected to repeated freezing and thawing. We found no noticeable differences in the SEC profiles under these conditions (only SEC profile of CO₂ is shown in Fig. 3B). The main peak consisted of 125I-rhNGF dimer. The presence of the 125I-rhNGF/HMW complex only became apparent after longer incubation periods in both conditions (data not shown).

Activation of α₂M by CH₃NH₂. In addition to proteases, primary amines such as serotonin and CH₃NH₂ are known to activate α₂M present in plasma (Eccleston and Howard, 1985). To determine whether rhNGF is interacting with activated α₂M, 125I-rhNGF was incubated in human plasma containing 200 mM CH₃NH₂ for 4 h at 37°C. Figure 4 shows that in the presence of CH₃NH₂, the dominant peak was the 125I-rhNGF/HMW complex, with an SEC HPLC profile similar to the one obtained after incubation of labeled rhNGF in human plasma for more than 4 days at 37°C. To assess the specificity of NGF in forming these 125I-rhNGF/HMW complexes, 125I-rhNGF was coincubated with an excess of cold rhNGF (100 μg/ml) in CH₃NH₂-treated human plasma. The presence of excess unlabeled rhNGF efficiently prevented the formation of the 125I-rhNGF/HMW complex (Fig. 5). To confirm the interactions of 125I-rhNGF with plasma α₂M, 125I-rhNGF was incubated in pure activated α₂M. The SEC HPLC chromatogram was superimposable to a profile of 125I-rhNGF in CH₃NH₂-treated human plasma.

Assay Detection of rhNGF in Various Matrices. To further elucidate the time and concentration dependence of rhNGF recovery in human plasma, the immunoreactivity and bioactivity of rhNGF were measured after incubation in various matrices. Levels of ELISA-detected rhNGF in human plasma were constant (~80% recovery at all timepoints) and did not decline up to 72 h at 37°C (Fig. 6). However, in the presence of CH₃NH₂-treated human plasma or pure activated α₂M, only 40 and 33% of rhNGF was detectable at time 0, respectively. The concentration of detectable rhNGF decreased dramatically to 3.88 μg/ml ± 0.19 (~15%) in activated plasma and 4.78 μg/ml ± 0.67 (~19%) in pure activated α₂M after 2 h of incubation. A similar bioactivity profile of rhNGF under these incubation conditions was also observed (Fig. 7A). After 8-h incubation, ~84% of the rhNGF (21.01 μg/ml) in control human plasma was bioactive and decreased slightly throughout the time course. In contrast, the recovery of rhNGF (4.14 μg/ml) declined to ~16% in activated human plasma or ~23% (5.81 μg/ml) in pure activated α₂M after 8 h of incubation. Therefore, the expected interaction of rhNGF with α₂M in CH₃NH₂-treated human plasma or pure activated α₂M rendered the rhNGF/α₂M complex undetectable by ELISA and interfered with the rhNGF-mediated activation of trkA receptor.

To directly demonstrate the correlation between the formation of 125I-rhNGF/HMW complexes and a loss of its neurotrophin activity, 750 ng/ml rhNGF or 1.3 μg/ml 125I-rhNGF were incubated in fresh human plasma or in CH₃NH₂-treated human plasma for 3 h at 37°C. The four samples were subjected to SEC HPLC, and fractions of the eluant from each sample were collected at 1 ml/min. Bioactive rhNGF concentration in fractions obtained from incubations containing unlabeled rhNGF was measured by KIRA, and the radioactivity of fractions obtained from incubations containing labeled rhNGF was quan-
titated by gamma counting. Figure 7B shows superimposed SEC HPLC profiles of bioactive and radioactive rhNGF in CH$_3$NH$_2$-treated and untreated human plasma. In fresh untreated plasma, the fractions containing bioactive rhNGF coeluted at the position of the $^{125}$I-rhNGF dimer. This confirmed our previous data, which showed that NGF in normal human plasma was fully bioactive and the dominant SEC peak was the $^{125}$I-rhNGF dimer. In contrast, the presence of CH$_3$NH$_2$ in plasma rendered the rhNGF inactive as seen by a substantially smaller rhNGF peak measured by KIRA. This activation of human plasma also resulted in a shift in the $^{125}$I-rhNGF radioactivity peak to the $^{125}$I-rhNGF/HMW complex species detected in fractions 6, 7, 8, and 9 where no detectable rhNGF activity was found.

**Inhibition and Reversal of CH$_3$NH$_2$-Activated α$_2$M in Human Plasma.** Reagents such as 2,4-dinitrophenyl thiocyanate and iodine (Van Leuven et al., 1983; Bjork et al., 1985) have been demonstrated to modify the scission of the thiol ester on α$_2$M that is essential for the activation of α$_2$M. In this study, it was demonstrated that DMSO was also capable of inhibiting and reversing the activation of α$_2$M in human plasma. SEC HPLC confirmed that $^{125}$I-rhNGF initially treated with CH$_3$NH$_2$ formed a dominant $^{125}$I-rhNGF/HMW complex peak. However, the addition of increasing concentrations of DMSO (1, 2.5, 5, 10, and 25%, v/v) decreased the amount of the $^{125}$I-rhNGF/HMW complex with a gradual recovery of the $^{125}$I-rhNGF dimer peak (Fig. 8). Twenty-five percent DMSO was capable of completely reversing the interaction of $^{125}$I-rhNGF with α$_2$M. The SEC profile under these conditions was identical with that of $^{125}$I-rhNGF incubated in untreated human plasma. To determine whether the NGF released by DMSO from α$_2$M also recovered its immunoreactivity, rhNGF was incubated in human plasma treated with CH$_3$NH$_2$. After the activation step, 0, 5, 10, or 25% DMSO was added. In Fig. 9, ELISA quantitations indicated that most of the rhNGF in control plasma was recovered (82.0% ± 0.0), whereas only 24.0% ± 0.0 of the rhNGF was recovered in CH$_3$NH$_2$-treated plasma. However, the mean percent rhNGF recovery in treated plasma increased with increasing concentrations of DMSO. All of the immunoreactive rhNGF

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**Fig. 4.** Effect of CH$_3$NH$_2$ on the interactions of rhNGF with plasma components. $^{125}$I-rhNGF (1.3 µg/ml) was incubated in citrated human plasma with and without 200 mM CH$_3$NH$_2$ for 4 h at 37°C. Twenty-six nanograms of labeled NGF was analyzed on a TSK-GEL G4000 SWXL column. Peak of radioactivity shifts to an earlier elution time after treatment with CH$_3$NH$_2$.

**Fig. 5.** rhNGF interacts with pure α$_2$M and CH$_3$NH$_2$-treated human plasma.

$^{125}$I-rhNGF (1.3 µg/ml) or $^{125}$I-rhNGF (1.3 µg/ml) with an excess of cold rhNGF (100 µg/ml) was incubated in the following: 1) citrated human plasma with and without 200 mM CH$_3$NH$_2$; and 2) pure α$_2$M (2 mg/ml) with and without 200 mM CH$_3$NH$_2$ for 4 h at 37°C. Twenty-six nanograms of labeled rhNGF was analyzed on a TSK-GEL G2000 SWXL column. Column buffer diluent for rhNGF: 10 mM sodium acetate and 140 mM NaCl, pH 5.5 containing 1 mg/ml human serum albumin and 0.5 mg/ml protamine sulfate.

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**Fig. 6.** Recovery of NGF incubated in CH$_3$NH$_2$-treated fresh human plasma, pure α$_2$M, and control untreated fresh human plasma.

rhNGF (25 µg/ml) was incubated in citrated human plasma with and without 200 mM CH$_3$NH$_2$ and in pure α$_2$M (1.5 mg/ml) for increasing time intervals at 37°C. Aliquots were taken at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 48, and 72 h. Samples were analyzed by rhNGF ELISA. Values are mean concentration ± S.D. of four determinations.
was recovered with 25% DMSO. Figure 10 summarizes the effects of CH$_3$NH$_2$ and DMSO in human plasma. The elution time and radioactive peak size of 125 I-rhNGF incubated in CH$_3$NH$_2$-treated plasma containing 25% DMSO was identical with that of 125 I-rhNGF in untreated control plasma as well as 125 I-rhNGF coincubated with excess unlabeled rhNGF in CH$_3$NH$_2$-treated plasma.

Interactions of NGF with Plasma Components In Vivo. The interactions of NGF in vivo were studied by SEC HPLC analysis of the plasma obtained at various timepoints after i.v. dosing of 125 I-rhNGF (274 mCi/rat) in rats. At 10 min postdose, the dominant peak was 125 I-rhNGF dimer but its proportion to the 125 I-rhNGF/HMW complex peak was larger than that observed after incubation with plasma in vitro (Fig. 11A, one SEC chromatogram is shown). By 30 min postdose, the sizes of these two peaks were equivalent and at 60 min postdose, the HMW complex peak was substantially larger than the 125 I-rhNGF dimer. Interestingly, the peak of free I-125 increased substantially with time, suggesting in vivo processing of the protein and/or of the I-125 moiety. Furthermore, by 60 min the levels of detectable radioactivity also declined, possibly a result of degradation and/or clearance of 125 I-rhNGF from the circulation. The SEC HPLC profiles of the plasmas at each timepoint were similar ($n = 3$).

A, rhNGF (25 μg/ml) was incubated in citrated human plasma ± 200 mM CH$_3$NH$_2$ and in pure αM (1.5 mg/ml) for increasing time intervals at 37°C. Aliquots were taken at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 48, and 72 h. Samples were analyzed by rhNGF KIRA. Values are mean bioactive concentration ± S.D. of four determinations. B, rhNGF (750 ng/ml) was incubated ± 200 mM CH$_3$NH$_2$ in citrated human plasma for 3 h at 37°C. Samples were applied to a G3000 SWXL column and eluted with 0.4 M KHPO$_4$, pH 6.5. Fractions were collected at 1.0 ml/min and analyzed by KIRA. Bioactive rhNGF (ng/ml) is represented by ■ (rhNGF incubated in activated plasma) and □ (rhNGF incubated in control plasma). 125 I-rhNGF (1.3 μg/ml) was also incubated in column buffer and CH$_3$NH$_2$-treated citrated human plasma for 3 h at 37°C. Samples were analyzed by SEC HPLC as described above, and radioactivity in the fractions was quantitated by a gamma counter. Radioactivity (cpm) is represented by ■ (125 I-rhNGF in activated plasma) and □ (125 I-rhNGF in column buffer).

min postdose, the sizes of these two peaks were equivalent and at 60 min postdose, the HMW complex peak was substantially larger than the 125 I-rhNGF dimer. Interestingly, the peak of free I-125 increased substantially with time, suggesting in vivo processing of the protein and/or of the I-125 moiety. Furthermore, by 60 min the levels of detectable radioactivity also declined, possibly a result of degradation and/or clearance of 125 I-rhNGF from the circulation. The SEC HPLC profiles of the plasmas at each timepoint were similar ($n = 3$).

Intravenous dosing of 125 I-rhNGF (~89 μCi/mouse) in mice revealed similar results. There was limited presence of the 125 I-rhNGF/HMW complex in plasma 5 min postdose (Fig. 11B, one SEC HPLC profile is shown). By 60 min postdose, the 125 I-rhNGF/HMW complex peak was equivalent to the 125 I-rhNGF dimer peak. Likewise, there was an increase in the area under the peak of free I-125. Figure 11C shows the ratio of integration of each peak from all SEC HPLC chromatograms at each timepoint ($n = 3$). There was little variability.
in the relative proportion of the $^{125}$I-rhNGF/HMW complex and $^{125}$I-rhNGF dimer peaks. These results indicated that the association of $^{125}$I-rhNGF with $\alpha_2$M occurred at a faster rate than was observed in vitro.

Discussion

We have assessed the stability and interactions of rhNGF in plasma in vitro and in vivo. The nature of the interactions between rhNGF and plasma proteins were also characterized, as well as the effects of various chemical modifications on these interactions. There was minimal degradation of rhNGF in human plasma in vitro. ELISA and PC12 survival activity assays determined that the recovery of rhNGF was greater than 90% for as long as 4 days of incubation, indicating a highly stable molecule at concentrations of at least 10 $\mu$g/ml. There was a slight decrease in rhNGF recovery with time. At decreasing concentrations (200 and 20 ng/ml) in plasma and with prolonged incubation time, the recovery of rhNGF by ELISA further declined, suggesting an apparent disappearance or masking of rhNGF from the assay detection. SEC HPLC analysis of $^{125}$I-rhNGF showed that longer incubation periods resulted in a shift of the free $^{125}$I-rhNGF dimer profile into a $^{125}$I-rhNGF/HMW complex. We confirmed that this HMW peak consisted of $^{125}$I-rhNGF bound to $\alpha_2$M, a known major plasma component and protease inhibitor (Swenson and Howard, 1979; Sottrup-Jenson et al., 1984). It has been shown that $\alpha_2$M is capable of binding to cytokines and growth factors including neurotrophins (Liebl and Koo, 1993; Wolf and Gonias, 1994). Keck et al. (1994) have postulated that growth factors including NGF bind only to the activated form of $\alpha_2$M, which occurs after the cleavage of its thiol ester bonds. After proteolytic degradation and thiol ester cleavage, $\alpha_2$M undergoes a conformational change or “activation”, which traps and blocks the protease activity. A similar conformational activation change has been postulated in the specific binding of nonprotease growth factors including NGF to $\alpha_2$M (Sottrup-Jensen et al., 1984). It has been shown that $\alpha_2$M is capable of binding to cytokines and growth factors including neurotrophins (Liebl and Koo, 1993; Wolf and Gonias, 1994). Keck et al. (1994) have postulated that growth factors including NGF bind only to the activated form of $\alpha_2$M, which occurs after the cleavage of its thiol ester bonds. After proteolytic degradation and thiol ester cleavage, $\alpha_2$M undergoes a conformational change or “activation”, which traps and blocks the protease activity. A similar conformational activation change has been postulated in the specific binding of nonprotease growth factors including NGF to $\alpha_2$M (Sottrup-Jensen et al., 1984). We have confirmed that treatment of fresh citrated human plasma with 200 mM CH$_3$ NH$_2$ substantially increased the interaction of rhNGF with $\alpha_2$M, shifting the $^{125}$I-rhNGF radioactivity peak to a HMW complex after SEC HPLC analysis. This interaction was prevented by coincubation with excess rhNGF (100$\times$). The percentage of naturally occurring activated $\alpha_2$M in plasma is very low (Chu and Pizzo, 1994). However, levels of activated $\alpha_2$M may change in several pathological conditions, including septic shock, pancreatitis, and traumatic injuries (Abbink et al., 1991; Pixley et al., 1993; Scully et al., 1993). A protein similar to $\alpha_2$M is also produced during pregnancy (Sand et al., 1985). The capacity of this protein to interact with NGF remains to be elucidated. In addition, Keck et al. (1994) have shown that incubation of 2.5S mouse NGF in fresh plasma in the presence of 1% CO$_2$ to keep the pH close to 7 prevented binding of NGF to $\alpha_2$M. We have demonstrated that rhNGF bioactivity and immunological ELISA detection were significantly decreased when incubated in...
CH₃NH₂-activated α₂M present in human plasma or pure activated α₂M. It has been shown that 2,4-dinitrophenyl thiocyanate blocks the CH₃NH₂ activation of α₂M (Van Leuven et al., 1983; Cunningham et al., 1990). We have identified an additional reagent that prevents and completely reverses the activation of α₂M in human plasma and the subsequent binding of rhNGF to α₂M. Treatment with 25% DMSO after incubation of rhNGF in activated human plasma resulted in full recovery of rhNGF measured by ELISA. The SEC HPLC profile indicated a shift from a mostly 125I-rhNGF/HMW complex peak to the 125I-rhNGF dimer form. Therefore, DMSO effectively released rhNGF bound to α₂M, allowing full recovery of rhNGF measured by ELISAs and IRAs (data not shown).

We also conducted a time profile characterization of the formation of 125I-rhNGF/HMW complexes in vivo. Intravenous administration of 125I-rhNGF in mice and rats was followed by SEC HPLC analysis of the plasma at increasing times postdose. Results indicated that within 10 min after dosing, 125I-rhNGF interacted with α₂M, forming 125I-rhNGF/HMW complexes. These complexes were identical in molecular mass to those observed in vitro. However, the formation of 125I-rhNGF/HMW complexes seemed to occur at a faster rate in vivo. There is no data regarding the formation of HMW complexes in other nonrodent animal models in vivo. Therefore, the presence and nature of NGF interactions in humans remains to be determined. This experimental approach could not clearly distinguish selective clearance of each radioactive species (125I-rhNGF/HMW determined. This experimental approach could not clearly distinguish presence and nature of NGF interactions in humans remains to be determined. Therefore, the interactions of NGF with a protein isolated from mouse salivary gland. Ann NY Acad Sci 128:1013–1021.


