Upregulation of Ciliary Neurotrophic Factor (CNTF) and CNTF Receptor α in Rat Kidney with Ischemia-Reperfusion Injury

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Abstract. Ciliary neurotrophic factor (CNTF) is presumed to play a role as a survival factor in neuronal cells, but little is known about its role in the kidney. To investigate this, the expression of CNTF and CNTF receptor α (CNTFR α) was analyzed in the ischemic rat kidney. An ischemia/reperfusion (I/R) injury was induced by clamping both renal arteries for 45 min. Animals were killed at 1, 2, 3, 5, 7, 14, and 28 d after ischemia. The expression of CNTF and CNTFR α was monitored by reverse transcription-PCR, in situ hybridization, immunoblotting, immunohistochemistry, and electron microscopy. In sham-operated rat kidneys, CNTF expression was weak and limited to the descending thin limb of the loop of Henle. With I/R injury, CNTF mRNA and protein expressions were strikingly increased as compared with the sham-operated rat kidney, and the immunoreactivity of CNTF was mainly observed in the regenerating proximal tubules. The expression of CNTFR α mRNA was also increased after I/R injury, and its location and expression patterns were similar to the expression of CNTF. These findings suggest a possible role of CNTF as a growth factor during renal tubular repair processes after I/R injury and an autocrine or paracrine function of CNTF acting against CNTFR α.

The kidney has a remarkable capacity for restoring its structure and function after ischemic injury (1–3). Recovery is dependent on the ability of the remaining tubular cells to dedifferentiate, enter the cell cycle, proliferate, reline the damaged areas along the nephron, and then redifferentiate. This replicative repair process of renal tubule cells is mediated predominantly by the paracrine or autocrine release of growth factors, and these factors are expressed transiently in a coordinated manner during renal regeneration (4).

Several growth factors have been identified in the kidney, and these growth factors act in a coordinated manner. Of these, epidermal growth factor, insulin-like growth factor-1, and hepatocyte growth factor are well known (5). In addition, heparin-binding epidermal growth factor-like growth factor (6), fibroblast growth factor (7), and transforming growth factor-β1 (8) are associated with the repair process. Additional growth factors are believed to be involved in the repair process after ischemic injury because of the complicated nature of the process.

Ciliary neurotrophic factor (CNTF) was originally identified as a trophic molecule for the survival of embryonic chicken ciliary neurons in vitro (9). Subsequent studies have shown that CNTF is a neuronal growth factor that has a regulatory role in local neuronal healing and regeneration (10,11). The CNTF mRNA and protein are widely expressed in the brain, heart, lung, liver, kidney, and testis of the rat, in addition to preferential expression in the sciatic nerve (12,13). Apart from neuronal tissue, CNTF is most abundant in the kidney, but its role and precise localization are yet to be determined.

The effects of CNTF are mediated by a CNTF-specific ligand-binding α subunit (CNTFR α) (14,15). Functionally, CNTF receptors are critical for the developing nervous system and are needed to sustain life. Whereas disruption of the CNTF gene results in only modest changes within the central nervous system (16), mice with the CNTFR α null mutation (“knockouts”) die during the perinatal period and display profound neuron damage (17). The expression of CNTFR α mRNA is restricted primarily to nervous system and skeletal muscle, and a small amount of CNTFR α mRNA is present in the kidney (18).

From the data that have accumulated on the role of CNTF as a growth factor in nervous tissue, we hypothesized that CNTF may play a role as a growth factor in the kidney. To test this, the response of CNTF and CNTFR α to ischemia/reperfusion (I/R) injury was evaluated during regeneration of renal tubular cells after I/R injury. Our findings support the hypothesis.
Materials and Methods

Animal Preparation and Induction of Acute Renal Failure

Male Sprague-Dawley rats, weighing approximately 250 to 300 g, were housed under a 12-h light/dark cycle, and food and water were available ad libitum. The experimental protocol used in this study was approved by the Animal Ethics Review Committees of our Institution. Animals were anesthetized with an intraperitoneal injection of ketamine (2 g/kg body wt). After the abdomen was opened through a midline incision, both renal pedicles were exposed and cleaned by blunt dissection. Microvascular clamps were placed on both renal arteries to block renal blood flow. Core body temperature was maintained by placing the animals on a homeothermic table. After 45 min, the clamps were removed and blood flow was returned to the kidneys. Animals were killed at 1, 2, 3, 5, 7, 14, and 28 d after reperfusion. The sham operation was performed in a similar manner, except for the clamping of the renal vessels. Total number of animals was 84; 12 animals (6 sham and 6 I/R injury) were included at each time point. Presence of acute renal failure was determined by measuring the concentration of creatinine in serum at each experimental time point. The serum creatinine levels were measured with an autoanalyzer (Roche Diagnostics, Nutley, NJ). The serum creatinine levels (mg/L) were above 1.0 mg/dl on day 1 after I/R injury. The sham-operated rats showed no significant difference at each time point, and the average serum creatinine concentrations were approximately 0.5 mg/L.

Preservation of Kidneys

The kidneys were perfused briefly through the abdominal aorta with phosphate-buffered saline (PBS) to rinse out the blood and subsequently were fixed by in vivo perfusion with a periodate-lysine-paraformaldehyde (PLP) solution for 4 min. They were cut into sagittal slices and then immersed in PLP overnight at 4°C. After being rinsed in PBS, tissues were dehydrated in a graded series of ethanol.

Table 1. Oligonucleotide primer pairs for RT-PCR

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<th>Gene</th>
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<td>GTA GCC ATA TCC ATT GTA ATA</td>
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Figure 1. Immunoblot (A and B) and immunohistochemical detection (C and D) of proliferating cell nuclear antigen (PCNA) in rat kidney after sham operation and ischemia/reperfusion (I/R) injury. Representative immunoblots for PCNA and relative optical density in cortex (A) and medulla (B). *, P < 0.05 versus sham. In the sham-operated rat kidney (C), there were few PCNA-positive cells. After ischemia, numerous PCNA-positive cells were observed in the renal tubular cells (D). Optical densities represent the mean ± SEM for six animals. Magnification, ×200.
and embedded in wax (polyethylene glycol 400 disterate; Poly-science, Inc., Warrington, PA). For immunohistochemistry using a pre-embedding method, PLP-fixed tissues were cut on a vibratome (Lancer Vibratomes Series 1000; Technical Products International, St. Louis, MO) to a thickness of 50 μm.

Immunohistochemistry for Proliferating Cell Nuclear Antigen and CNTF

Wax sections were dewaxed and hydrated through the ethanol series and were immunostained with the primary antibody to proliferating cell nuclear antigen (PCNA; PC-10, DAKO, Glostrup, Denmark) using Vectastain ABC kit (Vector Laboratories, Burlington, CA) according to the manufacturer’s instructions. Diaminobenzidine was used as chromogen. After immunostaining, sections were counterstained with hematoxylin.

Immunohistochemistry using a pre-embedded method was performed as described previously (19). The 50-μm-thick vibratome sections were washed with 50 mM NH₄Cl in PBS. Before incubation with primary antibody, the tissue sections were incubated for 3 h with PBS containing 1% bovine serum albumin, 0.05% saponin, and 0.2% gelatin (solution A). Tissue sections were then incubated overnight at 4°C with monoclonal antibody against CNTF (diluted to 1:10; Chemicon International Inc., Temecula, CA) in PBS containing 1% bovine serum albumin (solution B). After washes with solution A, the tissue sections were incubated for 2 h with peroxidase-conjugated goat anti-mouse IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:50 in solution B. The tissues were then rinsed, first in solution A and subsequently in 0.05 M Tris buffer (pH 7.6). For the detection of horseradish peroxidase, the sections were incubated in 0.1% 3,3’-diaminobenzidine in 0.05 M Tris buffer for 5 min, after which H₂O₂ was added to a final concentration of 0.01. After the sections were washed with 0.05 M Tris buffer, they were dehydrated in a graded series of ethanol and embedded in Epon-812. The embedded 50-μm-thick sections were examined, and 1 μm of semi-thin sections were cut and photographed on Olympus Photomicroscope (Tokyo, Japan) equipped with differential-interference contrast.

For the immunoelectron microscopy, some of the immunostained vibratome sections were postfixed with 1% osmium tetroxide and embedded in Epon-812. Ultrathin sections were stained with lead citrate and observed with a transmission electron microscope (1200EX, JOEL, Tokyo, Japan).

Semiquantitative Reverse Transcription-PCR for CNTF mRNA and CNTFRα mRNA

Total RNA was isolated from Tri Reagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions. First-strand cDNA was reverse-transcribed from the RNA using random hexanucleotide primers (Life Technologies, Gaithersburg, MD) and Moloney murine leukemia virus reverse transcriptase (Life Technologies) as previously prescribed (20). The cDNA was then amplified by PCR for 25 to 30 cycles. Ten μl of each reaction cup was run on a 1.5% agarose gel, which contained 1 μg/ml ethidium bromide. Reverse transcription-PCR (RT-PCR) products were quantified by densitometry of a photograph of ethidium bromide–stained agarose. The number of PCR cycles was optimized to measure the amount of mRNA in the linear range, and an analysis of cycle sequencing revealed that the sequence was identical to position 77-627 in rat CNTF cDNA and 240-664 in rat CNTFRα cDNA. In the semiquantitative measurement, CNTF and CNTFRα primers were coamplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, and the CNTF/GAPDH or CNTFRα/GAPDH product ratios were calculated and considered an index of CNTF or CNTFRα mRNA expressions. Six animals were used for PCR in each time point, and three measurements were used per animal. The primers used for CNTF, CNTFRα, and GAPDH amplification are listed in Table 1.
Immunoblotting of CNTF and PCNA

Tissues were homogenized in a buffer containing 10 mmol/L Tris Cl (pH 7.6), 150 mmol/L NaCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate, 1% (vol/vol) aprotinin, 2 mmol/L Na₃VO₄, and freshly added leupeptin (1 μg/ml), pepstatin (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mmol/L). Homogenates were centrifuged at 16,000 × g for 15 min at 4°C, and protein concentrations were determined on supernatants using the Bradford method protein microassay (Bio-Rad, Hercules, CA). Homogenates were boiled for 5 min in Laemmli sample buffer.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 15% polyacrylamide gels and were electroblotted onto Bio-Blot nitrocellulose (Costar, Cambridge, MA). Nonspecific binding was blocked by incubating the blots for 1 h in 5% (wt/vol) nonfat milk. CNTF protein was detected with a monoclonal anti-CNTF antibody (Chemicon International Inc.), and PCNA protein was detected with anti-PCNA antibody (PC10, Dako, Copenhagen, Denmark) by incubation overnight. Primary antibody incubation was followed by six washes with Tris-buffered saline containing 0.005% Tween 20 (TBS-T). The blots were then incubated with

Figure 4. Light micrographs of a 50-μm-thick vibratome section (A, C through F) and a 1-μm-thick section (B) illustrating immunostaining for CNTF in kidneys from sham-operated rats (A and B) and from rats with I/R injury on days 1 (C), 3 (D), 7 (E), and 28 (F). (A and B) Strong CNTF immunostaining in the inner stripe of the outer medulla (ISOM) is evident. High magnification micrographs of the transition between the outer stripe of the outer medulla (OSOM) and ISOM (B); descending thin limb (*) showed strong CNTF immunostaining, but proximal tubules (PT) were negative. (C through F). From 1 d after ischemic injury, induced CNTF immunostaining started to appear between OSOM and the cortex (Cx). CNTF immunostaining in OSOM and Cx remained strong to 7 d after ischemic injury and decreased gradually. Magnifications: ×15 in A; ×480 in B; ×18 in C through F.
peroxidase-conjugated antibody, goat anti-mouse IgG for CNTF and mouse anti-rat IgG for PCNA for 30 min. Antibody-reactive protein was detected using an enhanced chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK). Densitometric analysis was performed using Imagemaster VDS software (Pharmacia Biotech, Piscataway, NJ). Six animals were used for PCR in each time point, and three measurements were used per animal.

In Situ Hybridization for CNTFR α

The riboprobe for CNTFR α used in the present study was kindly provided by Dr. H. D. Hofmann (21). After dewaxing, the sections were treated in 0.2 N HCl for 20 min and incubated in 20 µg/ml pepsin (0.1 N HCl) for 20 min at room temperature, which was followed by three washes with PBS. Prehybridization and hybridization steps were carried out at 53°C for 1 h and 15 h, respectively. The prehybridization buffer was composed of 50% formamide, 4 × SSC, 10% dextran sulfate, 1 × Denhardt’s solution, and 1 µg/µl salmon sperm DNA. The hybridization buffer was identical with the prehybridization buffer except that salmon sperm DNA was substituted by 150 ng/µl CNTFR α riboprobe. After posthybridization washing, sections were incubated with antidigoxigenin antiserum conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), and histochemical detection was then performed using the 4-Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl-phosphate mixture (Boehringer Mannheim).

Statistical Analyses

Data reported are mean ± SEM, and all statistical analyses were calculated with SYSTAT for Macintosh v. 5.2 (SYSTAT Inc., Chicago, IL). The sham-operated rats showed no significant variations at
Expression of CNTF mRNA and Protein in Rat Kidneys with Sham Operation and I/R Injury

CNTF mRNA expression in the cortex and medulla began to increase on days 1 to 2 and was maximal on days 5 to 7. It then gradually decreased (Figure 2). Expression of CNTF protein (Figure 3) was similar to CNTF mRNA expression. In the cortex (Figure 3A), a very faint band for CNTF protein was detected in sham-operated rat kidney. With I/R injury, CNTF protein began to increase on day 1 and achieved a maximum on day 7. The relative optical densities in each lane, taking day 1 as a 100% reference point, were as follows: day 1, 100%; day 2, 791%; day 3, 622%; day 5, 791%; day 7, 1600%; day 14, 278%; and day 28, 239%. The pattern of CNTF expression in the medulla was similar to that of the cortex (Figure 3B). The relative optical densities of CNTF protein were as follows: day 1, 99%; day 2, 305%; day 3, 250%; day 5, 265%; day 7, 433%; day 14, 272%; and day 28, 295%.

Localization of CNTF Protein in Rat Kidneys with Sham Operation and I/R Injury

In rat kidneys with sham operations, CNTF immunoreactivity was observed only in the descending thin limb of the loop of Henle (Figure 4, A and B). With I/R injury, CNTF expression began to appear in the outer part of the outer stripe of the outer medulla (OSOM) (Figure 4C) and progressively expanded into the cortex and the OSOM up to 7 d (Figure 4, D and E). Thereafter, CNTF expression in the cortex and OSOM decreased gradually (Figure 4F). The CNTF-positive cells in the OSOM were predominantly proximal tubule cells, which were squamous or cuboidal in shape and had less developed apical microvilli (Figure 5, A and B). In contrast, the exfoliated cells showed no CNTF immunoreactivity (Figure 5A).

Immunoelectron microscopy showed densely immunostained cells with no or poorly developed apical microvilli (Figure 5C). These cells seemed to be regenerating large and bright nuclei with prominent nucleoli, many mitochondria and free ribosomes, and intact intercellular junctions and basal lamina.

Expression of CNTFR α mRNA in Rat Kidneys after Sham Operation and I/R Injury

The expression pattern of CNTFR α mRNA was similar to that of the CNTF protein. The expression of CNTFR α mRNA in the medulla (Figure 6) was increased on day 1 (0.24 ± 0.05 versus 0.55 ± 0.10; P < 0.05) and on day 2 (versus 0.65 ± 0.11; P < 0.05) as compared with the sham-operated rat. This increase of CNTFR α mRNA was observed up to day 7 (versus 0.50 ± 0.11; P < 0.05), but thereafter its levels were progressively decreased (day 14, versus 0.38 ± 0.05; day, 28 versus 0.24 ± 0.05).

Localization of CNTFR α mRNA in Rat Kidneys with Sham Operation and I/R Injury

In agreement with results from RT-PCR, the hybridization signal of CNTFR α mRNA was detectable in sham-operated rat kidneys (Figure 7, A through C). With I/R injury, CNTFR α mRNA expression progressively increased in both the renal cortex and the medulla up to day 7 (Figure 7, D through F). The S3 segment of the proximal tubules in the OSOM showed the most prominent increase in signal (Figure 8B). In the cortex, the initial part of the proximal tubules, continuous with the glomerular urinary pole, and the S2 segment of the proximal tubules in the medullary ray showed strong signal intensity (Figures 7F and 8A). Thereafter, in situ signals of CNTFR α mRNA decreased gradually, but a moderate level of CNTFR α
mRNA was observed in the thick ascending limb of the inner stripe of the outer medulla (ISOM) on day 28 (Figures 7G and 8C).

**Discussion**

Our study demonstrates striking changes of CNTF expression in rat kidneys after I/R injury. Low levels of CNTF in rat kidneys were significantly increased in the regenerating proximal renal tubular cells after I/R injury, and this increase occurred in parallel with the recovery of normal renal structures. This finding suggests that CNTF may have a potential role as a growth factor in the repair process of renal tubular cells after ischemic injury. To the best of our knowledge, the present study shows for the first time the induction of CNTF in rat kidney after I/R injury.

The presence of CNTF and CNTFR α in rat kidney is still controversial. Stöckli et al. (22) reported that CNTF mRNA is undetectable in the kidney, but Ohta et al. (12,13) reported that mRNA was observed in the thick ascending limb of the inner stripe of the outer medulla (ISOM) on day 28 (Figures 7G and 8C).

![Figure 7. Expression of CNTFR α mRNA in kidneys from sham-operated rats (A through C) and I/R injury on days 1 (D), 3 (E), 7 (F), and 28 (G). In low magnification (A), no clear hybridization signal was observed. At high magnification (B and C), weak signals were detected in the distal tubules (DT) and the descending thin limb of Henle’s loop (DTL). There were no signals in the proximal tubules (PT). Induction of in situ signals of CNTFR α mRNA was observed in the outer medulla (OM) on day 1 (D), and its signals were increased and expanded into the cortex on day 7 (E and F). Thereafter, in situ signals of CNTFR α mRNA decreased gradually. Note persistent moderate levels of CNTFR α mRNA on day 28 (G). The asterisk indicates proximal tubules showing strong hybridization signal in a medullary ray. Magnifications: ×15 in A; ×300 in B and C; ×18 in D through G.](image-url)
CNTF mRNA and protein are present. For CNTFα, Davis et al. (23) first reported that it is expressed exclusively within the nervous system and skeletal muscle. These discrepant results seem to be related to the low level of CNTF and CNTFα in the kidney. In the present study, the presence of both CNTF and CNTFRα was confirmed with RT-PCR and/or immuno-blotting, and their specific locations were established with the use of immunohistochemistry or in situ hybridization.

The anatomic location of CNTF-producing cells is important if we are to understand the possible role of CNTF in rat kidney with I/R. In general, ischemic renal injury in rat induces growth factors at the distal (S3) segment of the proximal tubule (1,24). This site is the most vulnerable to I/R injury (25). Our study showed a significant increase in CNTF immunoreactivity in the S3 segment of the proximal tubule, a site at which CNTF normally is not expressed. Further study of proximal tubules using electron microscopy revealed that CNTF-positive cells were morphologically similar to regenerating tubular cells. Conversely, there was no immunoreactivity of CNTF in the detached or exfoliated tubular cells. These immunohistochemical and electron microscopic studies provide evidence that the primary source of induced CNTF is regenerating proximal tubular cells and that necrotic cells do not produce CNTF in rat kidney with I/R injury.

The proliferative activity of tubular cells is maximal at 2 to 3 d and on day 7 in rat kidneys after I/R injury (26). This biphasic pattern was confirmed in this study using PCNA immunoblotting (Figure 1). During this period, several growth factors seem to be working in a coordinated manner. The expressions of epidermal growth factor (day 1) and hepatocyte growth factor (6 to 12 h) are maximal during the early phase, and insulin-like growth factor-1 is detectable at 3 to 7 d (5). In the present study, CNTF expression was maximal on day 7 (16-fold in the cortex, 4.3-fold in the medulla as compared with the control group). This finding suggests that CNTF regulates processes that occur later, such as redifferentiation of kidney epithelia.

Figure 8. In situ hybridization localization of CNTFRα in kidneys 3 (A and B) and 28 d (C) after ischemic injury. (A) Part of the renal cortex. Note the strongly stained initial part of the proximal tubule (PTi) and the unstained part of the proximal tubule (PT) which is just distal of PTi. (B) S3 segments showing strong hybridization signal. Note that the cells with the protruding shapes (arrows) and extruded cells found in the lumen showed no hybridization signals. (C) Part of the outer medulla. Moderate hybridization signal intensity was observed in the thick ascending limbs of Henle’s loop. Magnification, ×300.

Distribution of the CNTF receptor is also important as CNTF signal depends on CNTF binding to CNTFRα, and this process is essential for the trophic effect of CNTF to occur (27). Our study shows the close relationship between CNTF and CNTFRα in location as well as expression pattern after I/R injury. Both CNTF and CNTFRα were strongly expressed on the regenerating proximal tubular cells or were expressed on tubules located close by (CNTF in the descending thin limb of Henle’s loop; CNTFRα in the thick ascending limb). In addition, a similar expression pattern of CNTF and CNTFRα was observed during the regeneration period. This suggests that interaction between CNTF and CNTFRα is mediated by the autocrine or paracrine mechanism during the course of regeneration after injury.

Our study demonstrates the striking increase of CNTF in the rat kidney after I/R injury, but the mechanism that regulates CNTF expression is unknown. In the absence of injury, neurotrophic protein cannot be secreted, because there is no signal sequence that could allow its secretion according to the classical vesicular mechanism (22). Moreover, several studies failed to demonstrate the release of significant amounts of the protein by cultured CNTF-expressing cells (28,29). This has fostered the belief that CNTF can be released only from damaged cells. Recently, evidence was presented for release of CNTF through other secretory pathways that do not require conventional signal sequences. Cultured astrocytes could be stimulated by exogenous cytokines, for example, by interleukin-1 and tumor necrosis factor-α, to secrete CNTF (30). Interestingly, these two cytokines are implicated in the pathogenesis of I/R injury and upregulated in renal tubular cells with I/R injury (31,32). Therefore, we speculate that inflammatory
cytokines produced after I/R injury may be responsible for the CNTF production in regenerating renal tubular cells. In vitro study will be needed to define the roles of these cytokines in regulating CNTF expression in normal and injured conditions.

In summary, our study demonstrates the upregulation of CNTF and CNTFR α at regenerating renal tubular cells after I/R injury. This finding suggests a role for CNTF as a growth factor in renal tissue repair.

Acknowledgments

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References