Intracellular Kinases Mediate Increased Translation and Secretion of Netrin-1 from Renal Tubular Epithelial Cells

Calpurnia Jayakumar, Riyaz Mohamed, Punithavathi Vilapakkam Ranganathan, Ganesan Ramesh*

Vascular Biology Center, Georgia Health Sciences University, Augusta, Georgia, United States of America

Abstract

Background: Netrin-1 is a laminin-related secreted protein, is highly induced after tissue injury, and may serve as a marker of injury. However, the regulation of netrin-1 production is not unknown. Current study was carried out in mouse and mouse kidney cell line (TKPTS) to determine the signaling pathways that regulate netrin-1 production in response to injury.

Methods and Principal Findings: Ischemia reperfusion injury of the kidney was induced in mice by clamping renal pedicle for 30 minutes. Cellular stress was induced in mouse proximal tubular epithelial cell line by treating with pervanadate, cisplatin, lipopolysaccharide, glucose or hypoxia followed by reoxygenation. Netrin-1 expression was quantified by real time RT-PCR and protein production was quantified using an ELISA kit. Cellular stress induced a large increase in netrin-1 production without increase in transcription of netrin-1 gene. Mitogen activated protein kinase, ERK mediates the drug induced netrin-1 mRNA translation increase without altering mRNA stability.

Conclusion: Our results suggest that netrin-1 expression is suppressed at the translational level and MAPK activation leads to rapid translation of netrin-1 mRNA in the kidney tubular epithelial cells.

Introduction

Netrin-1 is a laminin-related secreted protein that is widely expressed in many tissues, including kidney [1,2]. Netrin-1 was shown to regulate neuronal migration [3], inflammation during ischemia reperfusion injury of the kidney [1,4,5], lung [6], whole animal hypoxia [7], cisplatin induced kidney injury [8], angiogenesis during development [9], and in adult heart [10]. In addition, netrin-1 is also shown to increase kidney epithelial proliferation and migration [11] and cancer development and progression [12,13]. Netrin-1 is known to bind to three distinct families of receptors, the DCC family (DCC and neogenin), the UNC5 family (UNC5A-D), and DSCAM, to mediate its biological effects in different tissues. The highest levels of netrin-1 mRNA were seen in the kidney, among the many organs studied so far; however netrin-1 protein expression is minimal in the kidney [1,2]. Localization studies had determined that netrin-1 expression is restricted to vascular endothelial cells, and little or no expression was seen in the tubular epithelial cells. However, within hours after injury of the tubules, netrin-1 protein is induced and excreted into urine [14,15]. Therefore, netrin-1 was identified as an early diagnostic biomarker of acute kidney injury (AKI) in mice as well as in humans [14,16]. However, the mechanism through which renal insult induced netrin-1 protein expression was unknown. Clinically AKI is characterized by a rapid reduction in kidney function resulting in a failure to maintain fluid, electrolyte and acid-base homoeostasis [17]. There are many causes of acute kidney injury in human which may include, ischemia of the kidney, drug administration such as cisplatin and gentamycin, and infection.

We investigated the regulation of netrin-1 at the transcriptional and translational levels in mouse kidney tubular epithelial cells in vitro. Pervanadate was used to induce oxidative stress and MAPK activation. Pervanadate is a powerful phosphatase inhibitor that leads to the accumulation of phosphorylated proteins and the activation of kinases that are normally retained in inactive forms by dephosphorylation [18,19]. Our results showed that netrin-1 is rapidly translated in tubular epithelial cells, which is regulated by stress-activated MAPK pathways.

Materials and Methods

Renal ischemia reperfusion

C57BL/6J mice (8–9 weeks of age, The Jackson Lab, Bar Harbor, ME) were anesthetized with sodium pentobarbital (50 mg/kg body weight, intra-Peritoneally) and were placed on a heating pad to maintain body temperature at 37°C. Both renal pedicles were identified through dorsal incisions and clamped for 30 minutes. Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline, intraperitoneally. The mice were kept in a warm incubator until they regained consciousness. Urine and kidney tissue were collected 6 hrs after reperfusion and processed for ELISA and RNA isolation. The institutional animal care and use committee of the Georgia Health...
Translational Regulation of Netrin-1

Our earlier studies identified netrin-1 as an early diagnostic biomarker of acute kidney injury. Netrin-1 protein was highly induced after many forms of renal injury and excreted in urine. However, the regulation of netrin-1 expression is unknown. To determine whether netrin-1 induction is due to increase in transcription, mice were subjected to 30 minutes of ischemia and then reoxygenated. Excretion of netrin-1 protein in urine and mRNA expression in kidney were quantified. Results shown in Figure 1, ischemia reperfusion induced a large increase in netrin-1 excretion in urine at 3 hrs (not shown) and 6 hrs after reperfusion. However, netrin-1 mRNA levels were significantly reduced at 6 hrs after reperfusion, suggesting that increased secretion of netrin-1 was associated with rapid degradation of netrin-1 mRNA in tubular epithelial cells.

Pervanadate increases netrin-1 production in renal proximal tubule cells

As seen in Figure 1, netrin-1 excretion is increased in urine within hours after reperfusion but the expression of kidney netrin-
1 mRNA is down-regulated. However, the mechanism for increased netrin-1 production was unknown. Since oxidative stress and MAPK activation are prominent events after ischemia reperfusion and other forms of acute kidney injury, we used pervanadate to investigate the influence of cellular stress on netrin-1 production in mouse proximal tubular epithelial cells (TKPTS). As shown in Figure 2, addition of pervanadate increased netrin-1 protein expression in a concentration-dependent and time-dependent manner.

Pervanadate activates MAPKs in renal proximal tubule cells

Pervanadate is known to activate MAP kinases in HeLa and smooth muscle cells [18,19]. Since MAPKs are involved in the regulation of translation [20], we examined the effect of pervanadate on the activity of the three MAP kinase pathways in TKPTS cells by Western blot analysis. As shown in Figure 3A, pervanadate increased the phosphorylation of all three MAPKs in a concentration-dependent manner.

Inhibition of MAP kinases reduces pervanadate-induced netrin-1 production

Pervanadate increases both netrin-1 expression (Figure 2) and MAPK activity (Figure 3A) in TKPTS cells. To determine if the activation of MAPK pathways mediates the increase in netrin-1 expression, TKPTS cells were treated with 50 μM pervanadate in the presence or absence of specific inhibitors of p38 MAP kinase.
(10 μM SB203580), ERK (10 μM U0126), or JNK (20 μM SP600125). As shown in Figure 3B, pervanadate induced a 2-fold increase in the secretion of netrin-1 protein. Inhibition of p38 and ERK reduced netrin-1 protein to near control levels. The JNK inhibitor SP600125 did not reduce netrin-1 protein levels. Interestingly, addition of the antioxidant dimethyl thiourea did not inhibit pervanadate-induced netrin-1 production, suggesting that netrin-1 production is via oxidative stress-independent mechanism. Also, hydrogen peroxide alone did not significantly alter the netrin-1 production whereas orthovanadate alone induced a marginal increase in netrin-1 production (Figure 3B).

To determine if the inhibition of MAP kinase inhibitors is due to an alteration in netrin-1 mRNA levels, netrin-1 transcripts were quantified in the presence of MAP kinase inhibitors. As shown in Figure 3C, pervanadate significantly reduced netrin-1 mRNA as compared to control. Addition of the MEK2 inhibitor U0126 suppressed pervanadate-induced netrin-1 degradation and significantly increased the amount of netrin-1 mRNA as compared to control. Addition of p38 MAP kinase inhibitor partially prevented pervanadate-induced netrin-1 mRNA degradation. Inhibitors alone did not affect netrin-1 mRNA nor protein levels. These results suggest that the ERK-mediated translation increase is also associated with enhanced degradation of netrin-1 mRNA in tubular epithelial cells.

Pervanadate does not increase netrin-1 mRNA stability

The expression levels of many secreted proteins are determined, in part, by their regulated degradation. We examined if the pervanadate-induced increase in netrin-1 protein was related to a stabilization of netrin-1 mRNA. The results in Figure 3 suggested that addition of pervanadate may enhance degradation of netrin-1 mRNA. To examine this issue more directly, we determined the effect of pervanadate on the degradation of netrin-1 mRNA in TKPTS cells. Cells were treated with pervanadate and/or 10 μg/ml actinomycin D was added to arrest further transcription. The levels of netrin-1 were determined at discrete time points over the next 24 hours (Figure 4). In cells treated with actinomycin D

![Figure 3. MAPK pathway mediates pervanadate-induced increase in netrin-1 production in TKPTS cells.](image)

![Figure 4. Pervanadate did not increase netrin-1 mRNA stability.](image)
alone, netrin-1 mRNA decayed modestly, to ~60% of original levels, by 60 minutes. In contrast, netrin-1 mRNA levels decayed rapidly, to ~20% of the original levels by 60 minutes in cells treated with actinomycin D and pervanadate. These results indicate that pervanadate did not increase mRNA stability, thereby increasing netrin-1 production. Rather, pervanadate may affect translation of netrin-1 mRNA.

Suppression of new protein synthesis enhances netrin-1 mRNA degradation

Stabilization and/or transcription of netrin-1 mRNA may involve the binding of certain proteins to the mRNA or gene. To determine if the slow degradation of netrin-1 with actinomycin D involves the synthesis of new protein, RNA stability was studied after translational arrest. TKPTS cells were treated with cycloheximide (Figure 4). In cells that received only actinomycin D, netrin-1 mRNA levels decay at a modest rate whereas in cycloheximide-treated cells, netrin-1 mRNA decay was enhanced, suggesting that new protein synthesis is required for the slow degradation of mRNA and/or transcription.

Drug-induced increase in netrin-1 secretion is also mediated by ERK MAPK

Our \textit{in vivo} study shows that drugs, such as endotoxin and cisplatin that are known to induce acute kidney injury increased netrin-1 production from kidney tubules and are excreted in the urine [16]. However, the regulation of enhanced netrin-1 production was unknown. To determine the mechanism of drug induced netrin-1 production, TKPTS cells were treated with endotoxin and cisplatin as described in Materials and Methods. As shown in Figure 5a, cisplatin induced increased netrin-1 secretion significantly by 6 hr, whereas endotoxin-induced increase occurs in 2 hr. Addition of MEK2 inhibitor U0126 inhibited both the cisplatin and endotoxin-induced increase in netrin-1 production.

Hyperglycemia suppresses netrin-1 secretion but high protein induces netrin-1 secretion through ERK MAPK

Like acute kidney injury, chronic kidney diseases such as diabetic nephropathy also show enhanced production of netrin-1 and increased levels in urine (unpublished observation). However, whether hyperglycemia itself can induce enhanced netrin-1 production from renal tubular epithelial cells is unknown. To determine whether hyperglycemia induces netrin-1, an increased concentration of glucose was added to tubular epithelial cell cultures. As shown in Figure 6A, increased glucose concentration has an opposite effect on netrin-1 production, suggesting that hyperglycemia itself is not a signal for the enhanced netrin-1 production seen \textit{in vivo}. Mannitol alone did not have any influence on netrin-1 production.

Diabetic nephropathy is associated with increased proteinuria which may induce netrin-1 production. To determine whether increased amount of protein can induce netrin-1, TKPTS cells were treated with different concentrations of albumin (low endotoxin and fatty acid free) for 24 hrs. As shown in Figure 6B, increased albumin concentration enhanced netrin-1 production, which was completely suppressed by MEK2 inhibitors, suggesting that ERK MAPK mediates albumin-induced netrin-1 production.

Hypoxia and reoxygenation-induced increase in netrin-1 production is also mediated by ERK MAPK

As shown Figure 1, ischemia reperfusion induced increased excretion of netrin-1 in urine. However, the mechanism was unknown. To determine the role of intracellular kinase in enhanced production of netrin-1, TKPTS cells were subjected to hypoxia and reoxygenation as described in Materials and Methods. As shown in Figure 7, hypoxia reoxygenation increased netrin-1 protein secretion, which was significantly inhibited by MEK2 inhibitor U0126.

Discussion

Netrin-1 is an early diagnostic biomarker of acute kidney injury and cancers [14,15,21]. In the kidney, netrin-1 protein is mostly expressed in vascular endothelial cells. In response to ischemia...
reperfusion, endothelial expression is down-regulated and tubular epithelial expression is up-regulated. However, the mechanism of regulation of netrin-1 production during injury of the kidney or other tissue was unknown. In this report, we examined the mechanism of netrin-1 production in mouse proximal tubular epithelial cells (TKPTS) in vitro and ischemia reperfusion injury of the kidney in vivo. Our results show that netrin-1 protein is highly induced but the induction is not due to increased transcription of netrin-1 gene. Rather, netrin-1 expression is regulated at the translational level by MAPKs, independent of mRNA stability. An ERK mediated increase in netrin-1 production was also seen in vitro with drugs that are known to cause acute kidney injury.

Our in vivo studies show a disconnect between increased protein and mRNA levels, suggesting that netrin-1 expression may be regulated at the posttranslational level. Translation of mRNA is regulated by several pathways. One of the important pathways is mitogen activated protein kinases (MAPK). Pervanadate is known to increase MAPK activation and suppresses phosphatase activity. Consistent with previous studies [18,19], pervanadate addition to renal tubular epithelial cells increased the activation of all three MAP kinases (ERK, P38 and JNK). Moreover, pervanadate increased the secretion of netrin-1. We also showed that inhibition of MAPK pathways suppressed the pervanadate-induced increase in netrin-1 production. However, only ERK and to some extent p38 pathways play a role in netrin-1 production whereas JNK inhibition did not alter the pervanadate-induced increase in netrin-1 production. The ERK pathway inhibitor reduced netrin-1 production more than p38 inhibitors. These results suggest the pervanadate-mediated increase in netrin-1 production in renal epithelial cells is mediated by the ERK and p38 pathways. To our knowledge, this is the first study to document the role of MAPK in netrin-1 production.

Pervanadate addition did not increase netrin-1 mRNA levels in TKPTS cells suggesting that increased production of netrin-1 in renal epithelial cells is not due to increased transcription. Therefore, MAPK may affect netrin-1 production at posttranscriptional levels. The stability of mRNA is an important element in the regulation of certain genes, including many involved in inflammatory responses [20]. Netrin-1 mRNA may be inherently unstable, which is supported by our data that either suppression of transcription or translation induced a rapid degradation of netrin-1 mRNA. Moreover, we determined that pervanadate enhanced the degradation of netrin-1 mRNA in TKPTS cells. These results suggest that the pervanadate-induced increase in netrin-1 production is not due to increased netrin-1 mRNA stability.
The dual role of pervanadate or pervanadate-induced signaling in netrin-1 mRNA degradation while simultaneously increasing netrin-1 protein production is puzzling. Moreover, addition of the MAPK inhibitor U0126 suppressed netrin-1 protein production but increased netrin-1 mRNA content. It is possible that the ERK pathway may promote both translation and degradation of netrin-1 mRNA. Therefore, inhibition of ERK patheways leads to accumulation of netrin-1 mRNA. However, it is also possible that rapid translation of netrin-1 mRNA may be associated with rapid degradation of netrin-1 mRNA in tubular epithelial cells independent of the ERK pathway. Our in vitro studies also support this view that increased excretion of netrin-1 protein after ischemia reperfusion injury was associated with a reduction in netrin-1 mRNA in the kidney. The mechanism for this opposing effect on protein and mRNA is not clear. Whether, the 3' mRNA in the kidney. The mechanism for this opposing effect on protein and mRNA is not clear. Whether, the 3' and 5' untranslated regions of mRNA play a role in translation and stability is unclear. Scanning of 5' and 3' untranslated regions (UTRs) of netrin-1 mRNA show two unique sequences that are known to regulate translation and mRNA stability; an internal ribosome entry site (IRES) in the 5' UTR and a K-box motif in the 3' UTR. The role of these sequences in the regulation of netrin-1 translation and degradation is unknown and needs further study. To our knowledge, this is the first example of increased translation associated with increased degradation of mRNA in response to stress and hypoxia.

Our studies also show that the pervanadate-induced increase in translation of netrin-1 mRNA is not an artificial stress signal. The agents or stimuli that are known to induce acute kidney injury and the associated increase in netrin-1 mRNA in vivo also induced netrin-1 mRNA in vitro in TKPTS cells, which was again mediated through ERK MAPK pathways. In addition, drugs or hypoxia induced an increase in netrin-1 production without increasing netrin-1 transcription levels, significantly suggesting that regulation is at the translational level (data not shown).

In summary, we have determined that pervanadate, cisplatin, lipopolysaccharide (LPS), high protein levels and hypoxia induce netrin-1 secretion from proximal tubular epithelial cells. However, the increase in netrin-1 secretion was not associated with an increase in netrin-1 mRNA levels, suggesting that the regulation is at posttranscriptional levels. MAP kinases, particularly ERK, and p38 MAPKs, mediate the actions of drugs and hypoxia on netrin-1 protein production. The MAP kinase mediated increase in netrin-1 production is not through an increase in mRNA stability, suggesting that regulation is at the level of translation. These results are relevant to the pathogenesis of acute kidney injury and chronic kidney disease since netrin-1 is used as an early diagnostic biomarker and has also been shown to play an important role in the pathophysiology of acute and chronic kidney diseases. Identification of the proteins involved in netrin-1 mRNA translation and the sequences to which they bind will require further investigation.

Acknowledgments
We wish to thank Rhea-Beth Markowitz, PhD, for her help in editing the manuscript.

Author Contributions
Conceived and designed the experiments: GR. Performed the experiments: CJ GR. Analyzed the data: CJ GR. Contributed reagents/materials/analysis tools: CJ RM PVR. Wrote the paper: CJ GR.

References
1. Wang W, Brian RW, Ramesh G (2008) Netrin-1 and kidney injury. I. Netrin-1 protects against ischemia-reperfusion injury of the kidney. Am J Physiol Renal Physiol 294: F739–F747.
2. Li NY, Komatsuzaki K, Fraser FP, Tseng AA, Proshlan P, et al. (2005) Netrin-1 inhibits leukocyte migration in vitro and in vivo. Proc Natl Acad Sci USA 102: 14279–14284.
3. Colamarino SA, Tessler-Lavigne M (1995) The axonal chemosattractant netrin-1 is also a chemorepellent for trochaear motic axons. Cell 81: 621–629.
4. Tadagavadi RK, Wang W, Ramesh G (2010) Netrin-1 regulates Th1/Th2/Th17 cytokine production and inflammation through UNC5B receptor and protects kidney against ischemia-reperfusion injury. J Immunol 185: 3750–3758.
5. Grenz A, Dalton JH, Bauerle JD, Badulak A, Rabyard D, et al. (2011) Partial Netrin-1 Deficiency Aggravates Acute Kidney Injury. PLoS ONE 6: e18412.
6. Mirakaj V, Thix CA, Laucher S, Mielke C, Morote-Garcia JC, et al. (2010) Netrin-1 dampens Pulmonary Inflammation during Acute Lung Injury. Am J Respir Crit Care Med 180: 670–676.
7. Rosenberger P, Schwab JM, Mirakaj V, Mauckovsky E, Mager A, et al. (2009) Hypoxia-inducible factor-dependent induction of netrin-1 damps inflammation caused by hypoxia. Nat Immunol 10: 195–202.
8. Rajasundari A, Fays L, Melden P, Ramesh G (2011) Netrin-1 overexpression in kidney proximal tubular epithelium ameliorates cisplatin nephrotoxicity. Lab Invest.
9. Navankasthunus S, Whitehead KJ, Suli A, Sorensen LK, Lim AH, et al. (2008) The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. Development 135: 659–667.
10. Ahmed RPH, Haider KH, Shujia J, Afzal MR, Ashraf M (2010) Sonic Hedgehog Gene Delivery to the Rodent Heart Promotes Angiogenesis via iNOS/Netrin-1/PKC Pathway. PLoS ONE 5: e8576.
11. Wang W, Reeves WB, Ramesh G (2009) Netrin-1 increases proliferation and migration of renal proximal tubular epithelial cells via the UNC5B receptor. Am J Physiol Renal Physiol 296: F723–F729.