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Protective Effect of Trophic Factor Supplementation on Cold Ischemia/ Rewarming Injury to Kidney Cells

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Abstract : The aim of this study was to investigate whether trophic factor supplementation (TFS) enhance the survival of kidney cell during cold ischemic storage and rewarming. The effect of TFS on the phosphorylation of p44/42 and p38 mitogen activated protein kinases (MAPK) signaling pathway was determined by Western blot. Apoptotic changes after cold ischemic storage and rewarming was determined by 4',6' - diamino -2- phenylindole (DAPI) staining. The cell viability was evaluated by live assay. TFS significantly decreased p44/42 and p38 MAPK activity induced by cold ischemic injury and rewarming ($p < 0.05$). The number of apoptotic cells was decreased after 5 minute rewarming in the presence of TFS. TFS significantly increased the cell viability after 5 minute rewarming ($p < 0.05$). Therefore, it was concluded that trophic factor supplementation protects kidney tubule cells from cold ischemic and rewarming injury via the inhibition of p44/42 and p38 MAPK activation and reducing apoptotic change.

***Key words* :** trophic factors, kidney cells, cold ischemia.

Introduction

Organ preservation is essential from procurement to transplantation. Cold ischemic injury (CII) during kidney preservation has been reported as a major risk factor in causing delayed graft function, allograft nephropathy, and apoptosis after transplant (2,7,8,12).

Apoptosis is a form of programmed cell death morphologically characterized by cell shrinkage and condensation of both nuclear chromatin and cytoplasm (10,11). CII can result in apoptotic cell death by disruption of the outer mitochondrial membrane and release of apoptogenic molecules such as cytochrome c. These events subsequently lead to the cleavage of procaspase 9 and 3, and eventually apoptotic cell death.

Previously, it has been shown that trophic factor supplementation (TFS) of University of Wisconsin (UW) organ preservation solution (TFS-UW) with a mixture of nerve growth factor (NGF), substance P, insulin-like growth factor I (IGF-I) and bovine neutrophil peptide 1 (BNP-1) reduced CII during cold storage of kidneys and early apoptotic changes in vascular endothelial cells (3,5). It also has been reported that TFS limited p44/42 and p38 mitogen activated protein kinases (MAPK) activity induced by CII and subsequent rewarming (4). Therefore, we hypothesize that TFS, which showed substantial reductions in CII, may have a protective effect on cell death induced by CII. The aim of the present study was to investigate whether TFS enhance the survival of kidney cell during cold ischemic storage and rewarming under culture conditions which simulate kidney preservation and reperfusion injury after engraftment.

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Materials and Methods

Madin-Darby canine kidney (MDCK) cells, an immortalized cell line isolated from distal convoluted tubule, were grown in Dulbecco's minimum essential media (DMEM) medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were seeded in 6 well plates and grown to approximately 70% confluence (Control). After washing with phosphate buffer solution (PBS), cells were stored at 4°C under nitrogen for 4 days in UW solution or UW solution supplemented with trophic factors (TFS-UW); NGF (20 μg/L), substance P (2.5 μg/L), IGF-I (10 μg/L) and BNP-1 (1 μg/L). Cells were then rewarmed in their normal cell medium for 1 and 5 minutes.

Cells were washed in PBS, lysed in M-PER lysis buffer (Pierce, Rockford, IL) containing protease inhibitor (Calbiochem, La Jolla, CA) and 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) and centrifuged at 4°C for 10 minutes at 10,000 g. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Protein samples were separated on a 10% SDS-polyacrylamide gel and then transferred to an Immobilon membrane.

Membranes were incubated with antibodies against phospho-p44/42 (Cell Signaling Technology, Beverly, MA) and phospho-p38 (Cell Signaling Technology, Beverly, MA). Secondary antibodies conjugated with horseradish peroxidase (Promega, Madison, WI) were detected by the ECL system (Amersham, Arlington Heights, IL).

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% bovine serum albumin, and nuclei were stained with 0.8% 4',6' -diamino -2- phenylindole (DAPI; Roche Diagnostics, Indianapolis, IN) in PBS for 10 minutes at room temperature. Then, DAPI stained nuclei were observed using immunofluorescence microscope.

A assessment of cell viability was made with a commercially available fluorescent live probe (Molecular Probes, Eugene, OR). Cells were grown and stained green with calcein-AM (CAM) in the 96 well plates. Then, the cell viability was evaluated using fluorescence plate reader (Synergy HT; Bio-tek Instruments Inc.).

All statistical analyses were performed using the Student's t-test. $p < 0.05$ was considered statistically significant.

Results

When MDCK cells were stored under cold ischemic conditions for 4 days in either UW solution or TFS-UW, there was an increase in p44/42 and p38 MAPK compared to control cells ($p < 0.05$, Fig 1). Upon 1 and 5 minute rewarming, TFS resulted in a reduction in phophorylated p44/42 and p38 MAPK relative to that seen in unmodified UW solution at 5 minute rewarming for both p44/42 and p38 MAPK ($p < 0.05$) (Fig 1).

To find out the effect of TFS on apoptotic changes induced by cold ischemia and rewarming, we performed DAPI staining. As shown in Fig 2, the number of apoptotic cells was decreased after 5 minute rewarming in the presence of TFS compared with that of cells stored in UW solution and rewarmed for 5 minutes (Fig 2).

To examine the effect of TFS on cell viability, the intensity of living cells stained with green color was measured by live assay. The intensity of cold stored for 4 days and rewarmed group was remarkably lower than that of untreated normal cells. As the rewarming time increased, the cell viability was slightly increased. There was a significant increase after 5 minute rewarming in the presence of trophic factor supplementation compared with cells stored in UW solution and rewarmed for 5 minutes (Fig 3).

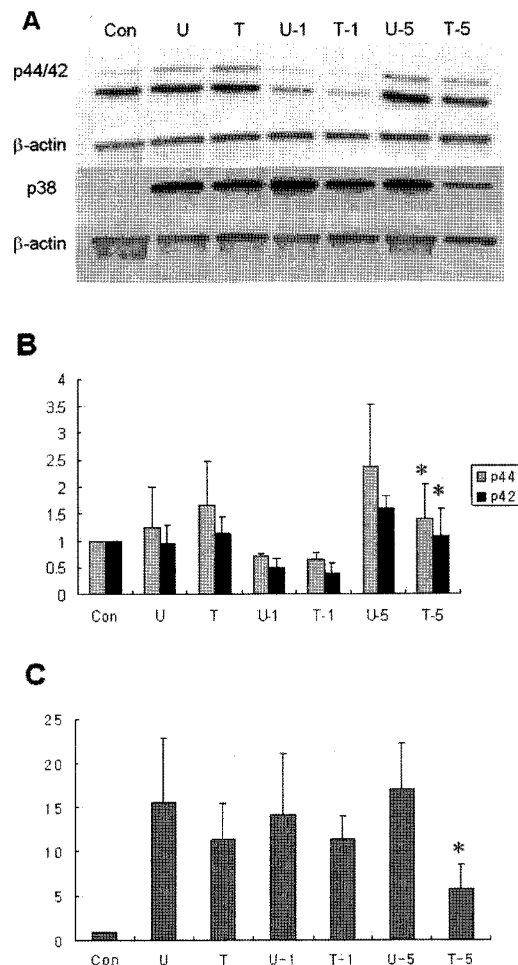

Fig 1. The effect of trophic factor supplementation on phosphorylation of p44/42 activity and p38 activity in Madin-Darby canine kidney (MDCK) cells (A). The values of p44/42 (B) and p38 (C) were normalized to control cells maintained under standard culture conditions. The presence of trophic factors in UW solution (relative to unmodified UW solution) was associated with an decrease in the activation state of these signaling molecules after cold ischemic storage for 4 days and rewarming for 1 and 5 minutes. There were significant decreases after 5 minute rewarming in the presence of trophic factor supplementation (*; $p < 0.05$ compared with that of cells stored in UW solution and rewarmed for 5 minutes) (B and C). Con = untreated normal cells; U = cells stored in UW solution for 4 days; T = cells stored in TFS-UW for 4 days; U-1 = cells stored in UW solution for 4 days followed by 1 minute rewarming; T-1 = cells stored in TFS-UW solution for 4 days followed by 1 minute rewarming; U-5= cells stored in UW solution for 4 days followed by 5 minute rewarming; T-5= cells stored in TFS-UW solution for 4 days followed by 5 minute rewarming.

Discussion

Cold ischemic injury during organ preservation leads to cell damages such as necrosis and apoptosis as well as significant alteration of the pattern of MAPK signaling activity in endothelial cells and kidney tubule cells (4,9). It also has been shown that the presence of TFS in UW solution reduced early apoptotic changes in vascular endothelial cells and tempered p44/42 and p38 MAPK activity induced by CII and subsequent rewarming (3,4). Based upon these results, we hypothesized that TFS may have a beneficial effect on MDCK cells' survival by modulation of the apoptosis and the

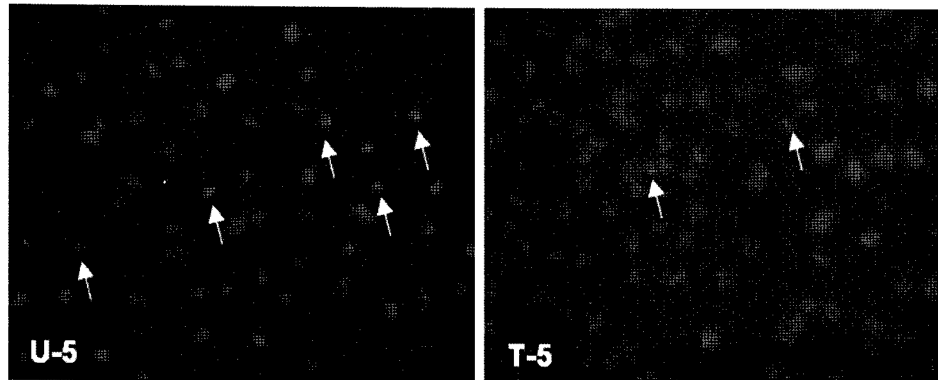

Fig 2. The effect of trophic factor supplementation on DAPI staining in MDCK cells. The number of condensed and fragmented apoptotic cells (arrow) was decreased after 5 minute rewarming in the presence of trophic factor supplementation compared with that of cells stored in UW solution and rewarmed for 5 minutes (× 200).

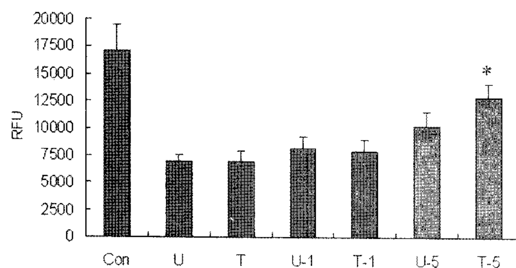

Fig 3. Effect of trophic factor supplementation on MDCK cells' viability. The intensity was decreased after cold ischemic storage for 4 days. As the cells were rewarmed for 1 and 5 minutes, the intensity gradually increased. There was a significant increase after 5 minute rewarming in the presence of trophic factor supplementation (*; $p < 0.05$ compared with cells stored in UW solution and rewarmed for 5 minutes). RFU = relative fluorescence units.

p44/42 and p38 MAPK pathways activated by cold hypoxic stress.

It has been reported that p44/42 and p38 MAPK pathway were activated by hypothermia (8). Park et al (6) showed that the MAPK activities, including JNK, p44/42 and p38, were also enhanced after ischemica in vivo and chemical anoxia in vitro. Consistent with these results, we found that the activation of p44/42 and p38 MAPK signaling was induced by cold ischemic storage in MDCK cells. It has been shown that p38 was involved in the process of endothelial cell apoptosis among MAPK pathways (1) and that the inhibition of p38 MAPK activation by exposure to VEGF or specific p38 MAPK inhibitor prevented endothelial cell apoptosis via reducing caspase-3 activity (13). In the present study, the activation of the p44/42 and p38 MAPK pathways was significantly decreased after 5 minute rewarming in the presence of TFS compared with that of cells stored in UW solution and rewarmed for 5 minutes. This finding suggests that TFS may have a beneficial effect in ameliorating the cell damage or death induced by CII via modulation of these signaling pathways.

When the effect of TFS on apoptotic change induced by CII was determined, the number of apoptotic cells was decreased after 5 minute rewarming in the presence of TFS compared with that of cells stored in UW solution and rewarmed for 5 minutes. These results are in agreement with the previous results suggesting that the number of caspase 3 acitve cells was significantly decreased by TFS (3).

Finally, it was directly confirmed that TFS significantly increased the intensity of viable cells after 5 minute rewarming compared with that of cells stored in UW solution and rewarmed for 5 minutes.

Taken together, it was concluded that trophic factor supplementation protects kidney tubule cells from cold ischemic and rewarming injury via the inhibition of p44/42 and p38 MAPK activation and reducing apoptotic change and may be useful to improve kidney preservation for transplantation.

References

1. Hyman KM, Seghezzi G, Pintucci G, Stellari G, Kim JH, Grossi EA, Galloway AC, Mignatti P. Transforming growth factor-beta1 induces apoptosis in vascular endothelial cells by activation of mitogen-activated protein kinase. Surgery. 2002; 132: 173-179.
2. Kruman II, Gukovskaya AS, Petrunyaka VV, Beletsky IP, Trepakova ES.Apoptosis of murine BW 5147 thymoma cells induced by cold shock. J Cell Physiol. 1992; 153: 112-117.
3. Kwon YS, Foley JD, Murphy CJ, McAnulty JF. The effect of trophic factor supplementation on cold ischemia-induced early apoptotic changes. Transplantation. 2007; 83: 91-94.
4. Kwon YS, Foley JD, Russell P, McAnulty JF, Murphy CJ. Prevention of cold ischemia/rewarming-induced ERK 1/2, p38 kinase and HO-1 activation by trophic factor supplementation of UW solution. Cryobiology. 2008; 57: 72-74.
5. McAnulty JF, Reid TW, Waller KR, Murphy CJ. Successful six-day kidney preservation using trophic factor supplemented media and simple cold storage. Am J Transplant. 2002; 2: 712-718.
6. Park KM, Chen A, Bonventre JV. Prevention of kidney ischemia/reperfusion-induced functional injury and JNK,

p38, and MAPK kinase activation by remote ischemic pretreatment. J Biol Chem. 2001; 276: 11870-11876.
7. Rauen U, Polzar B, Stephan H, Mannherz HG, de Groot H. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. FASEB J. 1999; 13: 155-168.
8. Roberts JR, Rowe PA, Demaine AG. Activation of NF-kappaB and MAP kinase cascades by hypothermic stress in endothelial cells. Cryobiology. 2002; 44: 161-169.
9. Roux PP, Blenis J, ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004; 68: 320-344.
10. Savill J, Mooney A, Hughes J. Apoptosis and renal scarring. Kidney Int Suppl. 1996; 54: 14-17.
11. Shankland SJ, Wolf G. Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. Am J Physiol Renal Physiol. 2000; 78: 515-529.
12. Tanaka T, Miyata T, Inagi R, Kurokawa K, Adler S, Fujita T, Nangaku M. Hypoxia-induced apoptosis in cultured glomerular endothelial cells: involvement of mitochondrial pathways. Kidney Int. 2003; 64: 2020-2032.
13. Yilmaz A, Kliche S, Mayr-Beyrle U, Fellbrich G, Waltenberger J. p38 MAPK inhibition is critically involved in VEGFR-2-mediated endothelial cell survival. Biochem Biophys Res Commun. 2003; 306: 730-736.

Trophic factor supplementation에 의한 cold ischemia/rewarming 손상으로 부터의 신장 세포 보호

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초 록 : 본 연구는 trophic factor supplementation (TFS)이 cold ischemic storage와 rewarming 동안에 신장 세포의 생존에 미치는 효과를 알아보기 위해 실시했다. p44/42와 p38 mitogen activated protein kinases (MAPK) 활성이 TFS에 의해 영향을 받는지를 Western blot을 통해 알아보았다. Apoptotic changes를 알아보기 위해 4',6' - diamino- 2 - phenylindole (DAPI) 염색을 실시했다. 세포생존도를 알아보기 위해 live assay를 실시하였다. 그 결과, TFS는 cold ischemic storage와 rewarming 동안 증가된 44/42와 p38 MAPK activity를 유의성 있게 감소시켰다 ($p < 0.05$). 또한, cold ischemic storage와 rewarming에 의한 apoptotic cell 수가 TFS에 의해 감소함을 관찰했다. 마지막으로 TFS는 유의성 있게 세포 생존도를 증가시켰다 ($p < 0.05$). 따라서, TFS는 p44/42와 p38 MAPK 활성을 감소시키고 apoptotic change를 억제함으로써 cold ischemia와 rewarming injury로부터 신장 세포를 보호하는 것으로 생각된다.

Key words : trophic factors, 신장 세포, cold ischemia