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Exogenous Pentraxin-3 Inhibits the
Reactive Oxygen Species-Mitochondrial
and Apoptosis Pathway in Acute Kidney
Injury

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Exogenous Pentraxin-3 Inhibits the Reactive Oxygen Species-Mitochondrial and Apoptosis Pathway in Acute Kidney Injury

Directed by Professor Woong Kyu Han

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

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June 2017

This certifies that the Master's Thesis of
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ABSTRACT

Exogenous Pentraxin3 Inhibits the Reactive Oxygen Species- Mitochondrial and Apoptosis Pathway in Acute Kidney Injury

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(Directed by Professor Woong Kyu Han)

Purpose: Pentraxin-3 (PTX3) is a long pentraxin group and has been studied for inflammatory diseases and various organs. We found that PTX3 has a positive effect on PTX3 in proliferating kidney cell proliferation during ischemia and proinflammatory acute kidney injury (AKI). The aim of this study was to develop an experimental model of AKI in vitro and to analyze the protective mechanism of exogenous recombinant PTX3.

Materials and Methods: We used HK-2 which was a renal tubular cell line. Calcium ionophores (A23187) induced injury by increasing intracellular calcium concentration and exogenous recombinant PTX3, was treated on the injured cells.

Also, we confirmed the expression of caspase-3 and PARP which are involved in intracellular ROS and apoptosis.

Results: Fluorescence microscopy revealed a significant increase in the influx of intracellular calcium due to A23187, resulting in renal cell damage, and increased calpain activity. After that, the activity of ROS rapidly increased after treatment with A23187 and decreased after treatment with exogenous recombinant PTX3. The activity of caspase-3 and PARP decreased after treatment with exogenous recombinant PTX3.

Conclusion: PTX3 reduces ROS and calpain activity and stabilizes mitochondrial membrane potential. Also, PTX3 reduces apoptosis by decreasing caspase-3 and PARP. As a result, PTX3 blocks ROS, Calpain activity, mitochondria and apoptosis pathway, and consequently protects renal tubular cells.

Key words: Pentraxin-3, ROS, Calpain, Mitochondria, Apoptosis

Exogenous Pentraxin-3 inhibits the ROS-Mitochondrial and apoptosis
pathway in the acute kidney injury

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I. INTRODUCTION

The pentraxins (PTXs) are a superfamily of multifunctional multimeric proteins divided into short (C-reactive protein and serum amyloid P component) and long (PTX3) forms.¹ PTX3 is rapidly induced by several stimuli in different cell types.² Peripheral blood leukocytes and myeloid dendritic cells release PTX3 in response to proinflammatory cytokines (interleukin [IL]-1 and tumor necrosis factor- α [TNF- α]), to agonists of Toll-like receptors (TLRs), and to stimulation with microbial components.² Different signaling pathways can affect PTX3 production depending on the cell and/or stimulus types. PTX3 binds the C1q complex and activates the classical complement pathway.² PTX3 also participates in the clearance of apoptotic cells and several microorganisms.¹ Furthermore, PTX3 has

been known to exert counteracting effects against interferon-gamma (IFN- γ) and IL-10 in inflammatory reactions. PTX3 has been identified as a key factor in the host defense against certain fungal, bacterial, and viral infections.

PTX3 levels are very low in the serum and tissues of normal subjects but rapidly increase in response to inflammatory stimulation in a wide range of diseases, including infectious, autoimmune, and degenerative disorders.^{3,4} Results from clinical tests suggest that elevated PTX3 levels may serve as a sensitive marker for determining early diagnoses and prognoses of certain severe illnesses, such as acute myocardial infarction.^{5,6} The role of PTX3 in inflammatory conditions was investigated *in vivo* using transgenic mice carrying multiple copies of PTX3.⁷ Lipopolysaccharide exposure induces the overexpression of PTX3 in these mice, which significantly improves their survival. In contrast, PTX3-deficient animals exhibit greater myocardial damage associated with an increased neutrophil infiltration in a model of cardiac ischemia/reperfusion injury.⁸

Levels of PTX3 levels are also increased in acute respiratory distress syndrome, in cardiovascular disease, in a variety of atherosclerotic diseases, and in kidney disease. Interestingly, it was reported that PTX3 affects the counterbalancing mechanism for mononuclear phagocytes in TNF-induced acute kidney injury (AKI).⁹ The authors found that PTX3 inhibited leukocyte adhesion and transmigration and relieved sterile renal inflammation. Eventually, PTX3 contributed to the recovery of injured tubular cells.

Clinical cases of AKI frequently result from renal ischemia-reperfusion injury (IRI), with an incidence exceeding 50% after major cardiac, hepatobiliary, or aortic surgery.^{10,11} Renal IRI, which results from cardiopulmonary bypass, partial nephrectomy, and renal transplantation, is an emerging clinical problem, as acute renal injury can result in end-stage renal disease.¹² Ischemic AKI is frequently complicated by multiorgan dysfunction, systemic inflammation, sepsis, and death.¹⁰ Renal cells are damaged during AKI and may undergo apoptosis, or programmed cell death, which is an evolutionarily conserved and highly regulated process involving a series of molecular events.^{13,14}

Biochemical and metabolic alterations that occur during IRI include the generation of reactive oxygen species (ROS), decreasing ATP levels, increasing inflammatory mediators, and rapid restoration of a physiological pH, which in turn increases intracellular sodium concentrations and leads to an overload of intracellular and mitochondrial calcium. Reperfusion injury is mediated by the interaction of these factors, which opens the mitochondrial permeability transition pore and initiates cell death pathways.¹⁵ ROS induce oxidative stress and affect various cell signaling pathways, including apoptotic pathways, after long-term and irreversible accumulation.¹⁶ Indeed, ROS can increase intracellular calcium levels and influence mitochondrial function, leading to the activation of a pro-apoptotic protein.^{14,16} Ischemia, inflammatory damage, and ROS-induced injury lead to cell death via activation of either the caspase-3 pathway or a

recently identified caspase-independent pathway mediated by the activation of poly(ADP-ribose) polymerase-1 (PARP-1).^{13,17}

With this in mind, we speculated that PTX3 might regulate postischemic tissue inflammation and injury. We utilized a model of ischemic AKI induced by Ca²⁺ overload and hypoxia to determine whether administration of exogenous recombinant PTX3 protects against ischemic AKI *in vitro* and to examine the mechanism for recovery.

II. MATERIALS AND METHODS

1. Cell culture

HK-2 cells were cultured in keratinocyte serum-free medium (Gibco, Grand Island, NY, USA) supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL human recombinant epidermal growth factor, 10% fetal bovine serum, and 1% penicillin/streptomycin in an atmosphere of 5% CO₂/95% O₂ at 37°C. Cells were subcultured every 7 d using 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin. The medium was replaced with fresh medium every 2 d.

2. Cell viability

HK-2 cells (2×10^4 cells/mL) in 96-well culture plates were treated with various doses of recombinant human PTX3 (Sino Biological, Inc., China) for 24 h. Cell viability was assessed using the Cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. Briefly, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt was added to each well, and wells were incubated for 1 h. The formation of a water-soluble formazan product in the medium was determined using a Beckman Coulter Microplate reader at 450 nm. Treatment with TGF- β was used as a positive control. For hypoxic treatment, HK-2 cells were incubated overnight in a hypoxic chamber (Tapei/Espec, Osaka, Japan); for

ischemic treatment, cells were incubated with the Ca^{2+} ionophore, A23187 (Sigma).

3. Intracellular calcium measurement

HK-2 cells in 12-well culture plates were loaded with a fluo-4/NW Ca^{2+} indicator (Molecular Probes, Belgium) and incubated at 37°C for 45 min according to the manufacturer's instructions. The fluorescent images were acquired using a fluorescent microscope (Olympus America, Melville, NY).

4. Calpain activity assay

To detect the activity of calpain in cells, we used a calpain activity fluorometric assay kit (Biovision, CA, US) according to the manufacturer's instructions. Briefly, a calpain substrate, Ac-LLY-AFC, was added to the supernatants of cell lysate extracts, which were incubated at 37°C for 1 h in the dark. The cleaved substrates were excited at 400 nm and analyzed at 505 nm using a fluorometer (Varioskan Flash 3001; Thermo Fisher Scientific, Vantaa, Finland).

5. ROS assay

HK-2 cells in 12-well culture plates were stained with DCF (Molecular Probes, Belgium) and incubated at 37°C for 45 min according to the manufacturer's

instructions. The fluorescent images were acquired using a fluorescent microscope (Olympus America, Melville, NY).

6. MMP assay

HK-2 cells in a glass-bottom dish (glass diameter, 10 mm) were treated with the mitochondrial probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine iodide (JC-1; Cayman Chemical, Ann Arbor, MI) and incubated at 37°C for 20 min. Healthy cells with mainly JC-1 aggregates were detected with excitation at 540 nm and emission at 570 nm, and apoptotic or unhealthy cells with mainly JC-1 monomers were detected with excitation at 485 nm and emission at 535 nm using a confocal microscope (LSM Meta 700; Carl Zeiss, Oberkochen, Germany).

7. TUNEL staining

Apoptotic cells were detected by TUNEL staining using a TACS 2 TdT-FITC *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Images were taken on a confocal microscope (LSM Meta 700; Carl Zeiss, Oberkochen, Germany) and analyzed with LSM Image Browser software.

8. Western blotting

Total cellular protein extracts were prepared on ice using a PRO-PREP protein extract solution (Intron, Seoul, Korea). Cell lysates were loaded onto a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane for 1 h. Membranes were incubated 4°C overnight with cleaved PARP, active caspase-3, PTX3, or GAPDH primary antibodies (Abcam) diluted 1:1000 with 5% bovine serum albumin in Tris-buffered saline-Tween 20 (TBS-T). After incubation, the membranes were washed with TBS-T, and secondary antibodies (1:10000; horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) were applied at room temperature for 1 h. Labeled bands were detected using a West Pico chemiluminescence kit (Thermo Scientific, Rockford, IL, USA).

9. Statistical analysis

Quantitative values are expressed as means \pm S.E.M. Statistical differences were determined using the Student t-test. A *p*-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS software version 23.0 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA).

III. RESULTS

1. Inflammatory signals induce PTX3 production

Stimulation with TNF- α and IL-1 β is reported to strongly induce the production of PTX3.¹⁸ To verify this, Western blotting was performed under reducing conditions with supernatants from HK-2 cells stimulated with TNF- α or IL-1 β (0–300 ng/mL). The results show that under inflammatory conditions, the PTX3 protein was detectable at the correct molecular weight and was increased in proportion with the inflammatory response (Fig. 1).

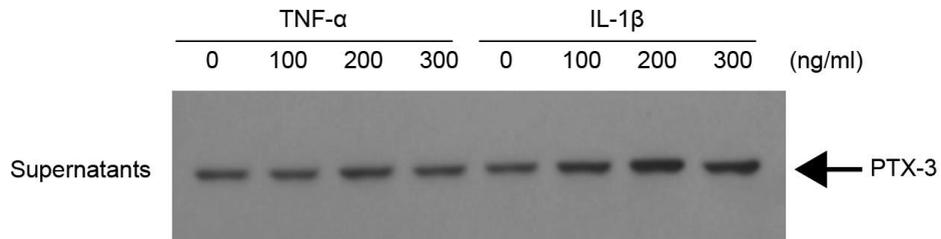


Figure 1. PTX3 was detected by Western blotting of supernatants from cells stimulated with inflammatory signals. Expression of PTX3 by epithelial-derived cell line human kidney 2 (HK-2) was stimulated with medium, TNF- α (0 to 300 ng/mL) or IL- β (0 to 300 ng/mL). Quantification of the expression of PTX3 was increased in proportion to the concentration of TNF- α and IL- β .

2. Low dose of PTX3 increases cell viability

The viability of HK-2 cells increased significantly with the addition of a low dose (0.1 nM) of PTX3 (Fig. 2A) (* $p < 0.05$). However, increasing doses (up to 5 nM) did not further increase cell viabilities (Fig. 2A, Fig. 2B). The viabilities of cells treated with high doses (up to 500 nM) of PTX3 were also compared with those of untreated cells and cells treated with 50 ng/ml TGF- β , as negative and positive controls, respectively. The highest cell viability was observed in cells treated with 1 nM PTX3 (Fig. 2B). At concentrations higher than 10 nM, cell viabilities declined (Fig. 2B) (* $p < 0.05$).

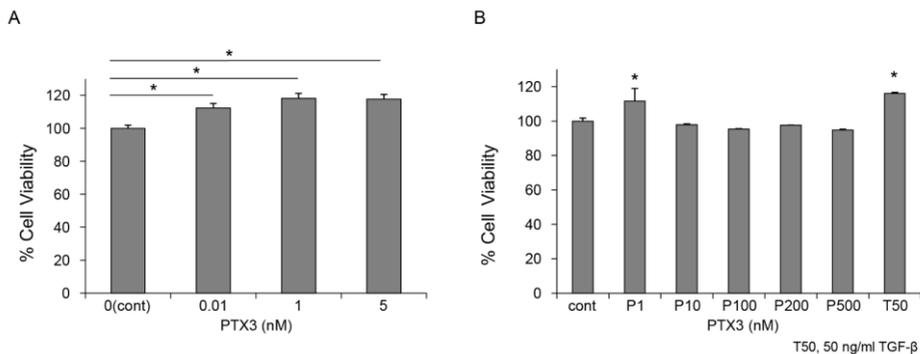


Figure 2. The viability of HK-2 cells with exogenous PTX3. Treatments with low (A) and high (B) doses of exogenous PTX3 (P) were compared. Con, control; T50, 50 ng/mL TGF- β (positive control). Data are means \pm s.e.m. from each HK-2 group, * $p < 0.05$.

3. PTX3 protects cells from ischemic/hypoxic injury

The ischemic injury was modeled in HK-2 cells by treatment with the calcium ionophore A23187 (0.3 $\mu\text{g}/\text{mL}$) or by exposure to a hypoxic chamber. The viabilities of cells treated with A23187 were significantly decreased compared to those of the control groups at 24 and 48 h (Fig. 3A)(* $p < 0.05$). However, the viability after 48 h of cells that also received PTX3 was significantly higher than the group that received only A23187 (Fig. 3A)(* $p < 0.05$). The viabilities of cells coadministered 1, 5, and 10 nM PTX3 did not differ. Cell viabilities were significantly reduced by exposure to the hypoxic chamber (Fig. 3B). However, treatment with 5 nM and 10 nM exogenous PTX3 after hypoxic injury significantly increased cell viabilities compared to hypoxic injury only (Fig. 3B)(* $p < 0.05$). Cell viabilities after hypoxia did not differ between cells treated with 1, 5, and 10 nM PTX3.

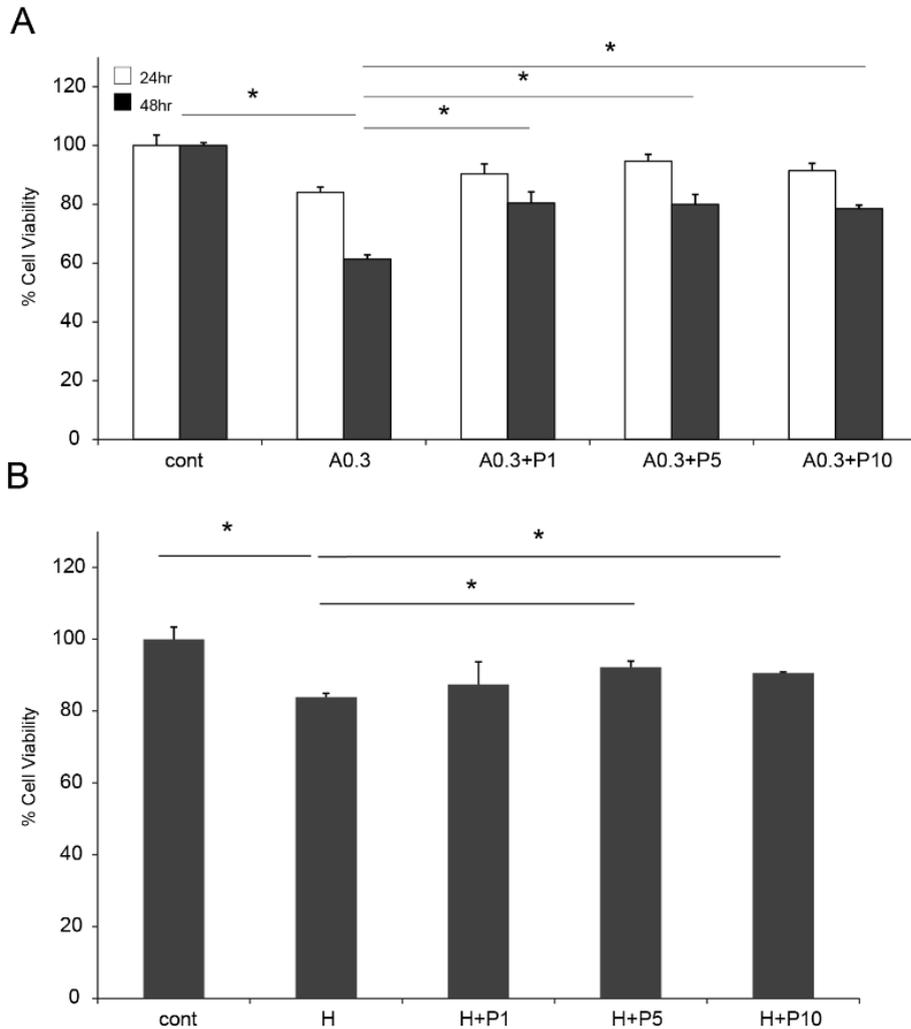


Figure 3. Exogenous PTX3 improves the viability of HK-2 cells after ischemic and hypoxic injuries. Cell viabilities were measured with and without PTX3 at 1, 5, or 10 nm (P1, P5, and P10, respectively) (A) 24 and 48 h after treatment with 0.3 μ g/mL A23187 (A0.3) and (B) after exposure to a hypoxic environment (H). We show that PTX3 provides significant cell viability increasing against HK-2

cell injury after 48hr. Values are expressed as means \pm s.e.m of at least three independent experiments, $*p < 0.05$.

4. PTX3 reduces $[Ca^{2+}]_i$ in ischemic cells

In HK-2 cells treated with A23187, intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and calpain activity were determined after exposure to exogenous PTX3. By using microscopy to measure fluo-4 fluorescence, we found that treatment with A23187 increases $[Ca^{2+}]_i$ in HK-2 cells (Fig. 4A). However, $[Ca^{2+}]_i$ decreased in cells also treated with exogenous PTX3 (5 nM). We also assessed the activity of calpain, an indicator of apoptotic cell death, by fluorimetry. As measured by relative fluorescence units (RFU), calpain activity in HK-2 cells increased with increasing A23187 concentrations (Fig. 4B)($*p < 0.05$). Furthermore, these increases of calpain activity were attenuated by treatments with 5 nM PTX3 (Fig. 4B)($*p < 0.05$).

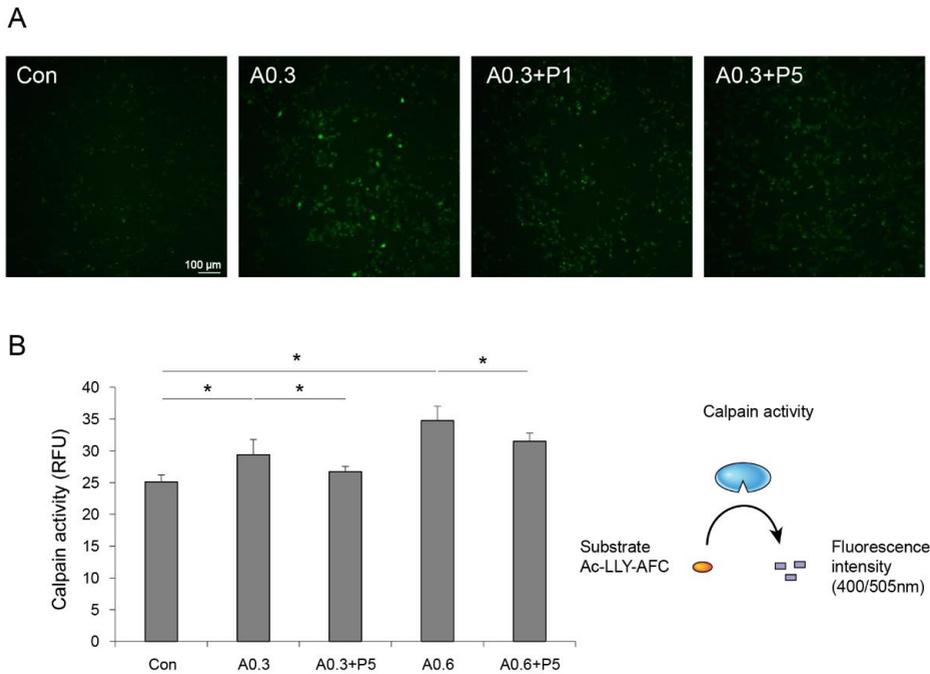


Figure 4. Exogenous PTX3 attenuates calcium responses to ischemic injury. (A) Intracellular calcium indicated by fluo-4 fluorescence was enhanced in HK-2 cells treated with 0.3 $\mu\text{g}/\text{mL}$ A23187 and reduced by the addition of 1 and 5 nM PTX3 (P1 and P5, respectively). (B) Calpain activity as measured by fluorimetry increased in cells treated with 0.3 $\mu\text{g}/\text{mL}$ and 0.6 $\mu\text{g}/\text{mL}$ A23187 (A0.3 and A0.6, respectively), which was attenuated by treatment with PTX3. $*p < 0.05$ versus control group subjected to administrate A23187.

5. PTX3 reduces the production of ROS in ischemic cells

To evaluate the generation of intracellular ROS, HK-2 cells treated with A23187 and exogenous PTX3 were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCF). The fluorescence intensity was significantly lower in A23187-treated cells that were exposed to 1 and 5 nM PTX3 compared with the intensity in cells treated with A23187 alone (Fig. 5A). As measured by relative fluorescence units (RFU), DCF staining in HK-2 cells increased with A23187 treated group (Fig. 5B)(* $p < 0.05$). Moreover, the intensity of DCF staining was attenuated by the higher PTX3 dose, significantly (Fig. 5B)(* $p < 0.05$).

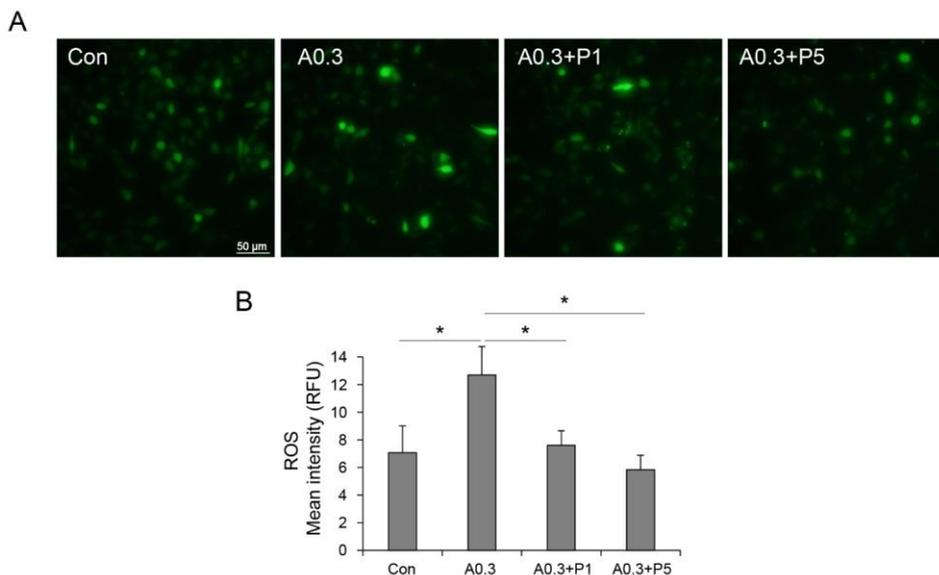


Figure 5. (A) DCF staining for ROS increased with A23187 treatment and was attenuated with exposure to 1 and 5 nM PTX3 (P1 and P5, respectively). (B) ROS activity as measured by fluorimetry increased in cells treated with 0 nM/mL and 5 nM/mL PTX3 (A0.3 and A0.3+P5, respectively), which was attenuated by

treatment with PTX3. $*p < 0.05$ versus A23187 (0.3 $\mu\text{g}/\text{mL}$) subjected to administrate PTX3.

6. PTX3 retains MMP in ischemic cells

The effect of ROS generation on the mitochondrial membrane potential (MMP) in HK-2 cells was measured via JC-1 fluorescence using confocal microscopy. A high MMP is depicted as red fluorescence from aggregates of JC-1, whereas a low MMP is depicted as green fluorescence from JC-1 monomers. As shown in Figure 5B, control HK-2 cells exhibited strong red fluorescence, indicating the cells had a high MMP. However, the red fluorescence decreased after exposure to A23187 (0.3 $\mu\text{g}/\text{mL}$), with the corresponding green fluorescence indicating a low MMP. Moreover, the intensity of the green fluorescence increased while the red fluorescence decreased when the concentration of A23187 was increased to 1 $\mu\text{g}/\text{mL}$. The addition of exogenous PTX3 enabled A23187-treated cells to retain their MMP, exhibited as greater red fluorescence with reduced green fluorescence, corresponding to a high MMP (Fig. 6A).

The JC-1 fluorescence ratio was significantly lower in A23187-treated cells that were exposed to 0.3 and 0.6 $\mu\text{g}/\text{mL}$, compared with the intensity in cells untreated (Fig. 6B)(** $p < 0.05$).

As shown from quantitative data, the Red/Green-fluorescence cells ratio were found to be 15.48%, 26.57%, 29.42% and 6.82% at A23187 0.3 μ mL only, 0.3 μ mL (A23187) + 1nM (PTX3) , 0.3 μ mL (A23187) + 5nM (PTX3) and A23187, 1 μ mL only treatment, respectively (Fig. 6B)(* p <0.05).

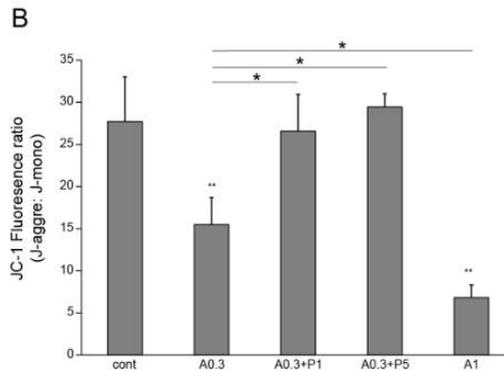
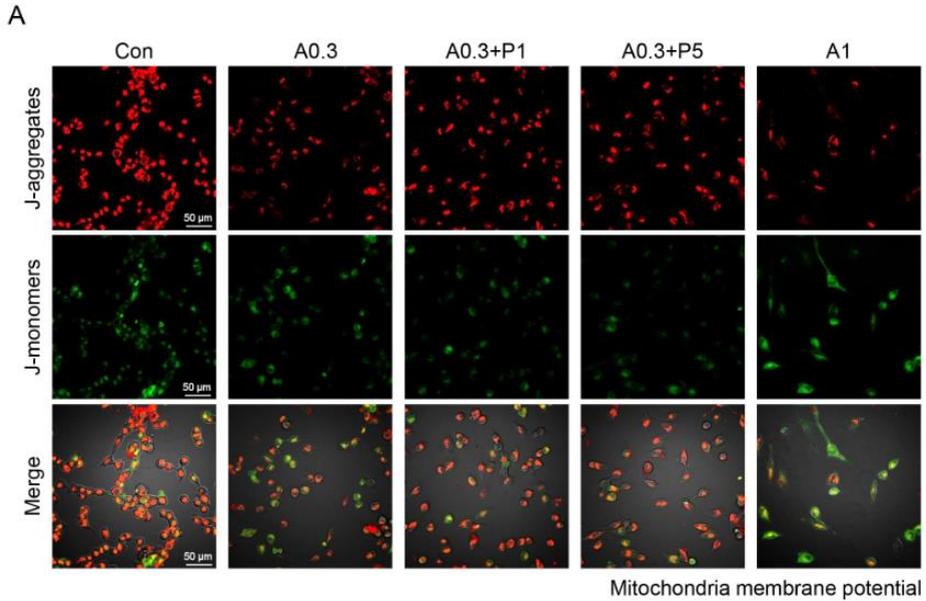


Figure 6. (A) Mitochondrial membrane potentials were visualized by the

intensity of fluorescing JC-1 aggregates (red) and monomers (green), which indicate high and low potentials, respectively, after treatment with 0.3 or 1 $\mu\text{g}/\text{mL}$ A23187 (A0.3 and A1, respectively) and exposure to PTX3. (B) Numerical data were expressed as % Red/Green fluorescence cells which were increased with increasing doses of PTX3. Data is representative of five independent experiments and expressed as means \pm s.e.m, $*p < 0.05$ as compared with their respective A23187 (0.3 $\mu\text{g}/\text{mL}$) only. $**p < 0.001$ as compared control group and A23187 administrated group (0.3 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$).

7. PTX3 reduces apoptosis in ischemic cells

We performed terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) to identify apoptotic cells. Apoptosis of HK-2 cells was induced by A23187 (Fig. 7A). However, treatment of these ischemic cells with 1 or 5 nM PTX3 attenuated this response. These results were verified by quantification of the fluorescence intensities, which showed that cells treated with A23187 had the highest average fluorescence intensity and that this was reduced by exposures to increasing concentrations of PTX3 (Fig. 7B)($*p < 0.05$).

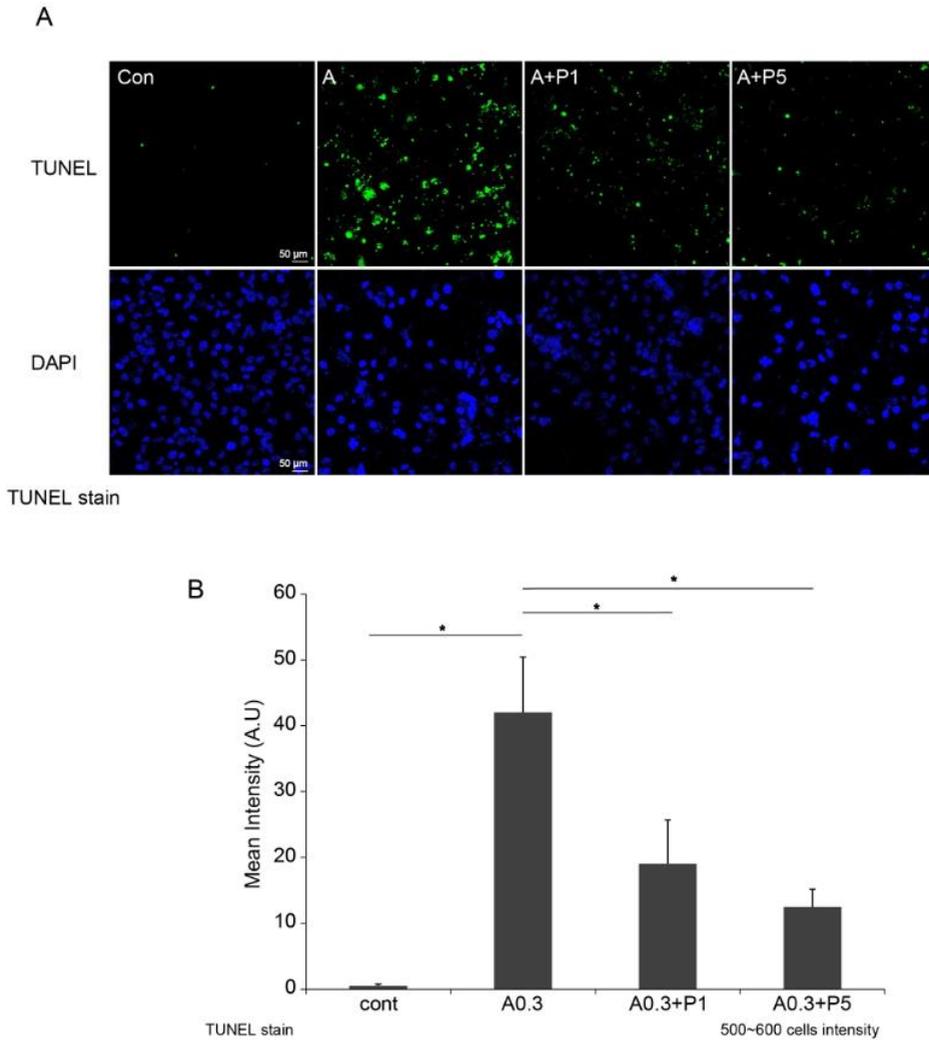


Figure 7. Treatment of HK-2 cells with 0.3 $\mu\text{g}/\text{mL}$ A23187 (A0.3) induces apoptosis as determined by TUNEL assays. Representative micrographs (A) and quantification (B) of TUNEL staining revealed that apoptotic processes were attenuated in ischemic cells exposed to 1 and 5 nM PTX3 (P1 and P5,

respectively). Numerical data were expressed as mean intensity apoptotic cells respective to their control. Data expressed means \pm s.e.m, $*p < 0.05$ as compared with their respective A23187 (0.3 μ g/mL) only.

8. PTX3 reduces the activity of caspase-3 and PARP-1 in ischemic cells

ROS-induced injury can lead to cell death via caspase-3 and caspase-independent (mediated by PARP-1 activation) pathways.¹⁷ To clarify which pathway plays a role in our model of ischemia, we performed Western blotting for caspase-3 and the DNA repair protein PARP-1. The treatment of HK-2 cells with 0.3 μ g/mL A23187 increased the level of active caspase-3, which is consistent with the initiation of apoptosis (Fig. 8). However, this increase in active caspase-3 was attenuated in cells exposed to PTX3. We also observed that A23187 treatment resulted in cleavage of PARP-1, which was similarly attenuated in cells exposed to PTX3. Thus, A23187 stimulates caspase-3 and caspase-independent pathways, both of which are suppressed after exposure to PTX3.

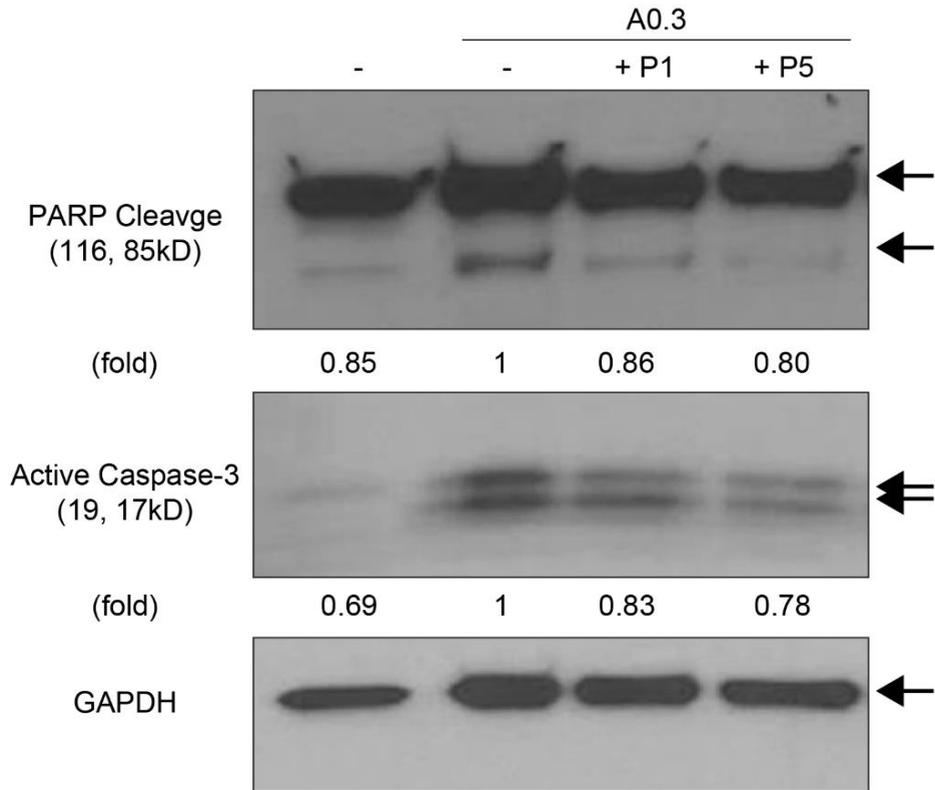


Figure 8. Active caspase-3 and PARP cleavage were detected by Western blotting of supernatants from untreated cells and cells treated with 0.3 $\mu\text{g}/\text{mL}$ A23187. The corresponding increases in PARP cleavage, and active caspase-3 were attenuated in cells treated with 1 or 5 nM PTX3 (P1 and P5, respectively). GAPDH was used as a loading control.

IV. DISCUSSION

Acute IRI is one of the most common acute kidney injuries that occurs during partial nephrectomy or other ischemic lesions. However, the influence of secondary inflammatory responses on kidney cells has not yet been clarified. In AKI, TLR-4 was shown to act as a major maladaptive high-mobility group protein B1 receptor and to be involved in the positive regulation of PTX3.¹⁹ Accordingly, it appears that PTX3 may have a proinflammatory role. In this respect, PTX3 may be considered as an adverse factor to IRI.¹⁹ However; it was also hypothesized that PTX3 plays an anti-inflammatory role in IRI of kidney cells on the basis of its cardioprotective effect in acute myocardial infarction.²⁰ Furthermore, PTX3 reportedly counteracts TNF-induced proinflammatory cytokines in kidney cells.^{1,9} Previously reported data document that the acute-phase protein PTX3 is induced systemically and inside postischemic kidneys, which limits leukocyte adhesion and transmigration.²¹ This mechanism prevents overactivation of the immune system and helps resolve sterile inflammation as a prerequisite for rapid and effective tubular repair. Thus, two contradictory findings in the acute injury of renal cells indicate that PTX3 may play a role in kidney cell damage. Previous reports also showed that innate immune effector cells largely contribute to postischemic renal inflammation and AKI. Oxidative stress induces the expression of PTX3 inside the kidney, which suppresses

leukocyte recruitment to the ischemic injury.^{9,22} This induction represents an endogenous mechanism to limit unnecessary IR injury. However, a later role for PTX3 in suppressing renal inflammation remains to be defined. We conducted this study to determine how PTX3 influences renoprotection and cell signaling after IRI.

We first confirmed that proinflammatory signals (TNF- α and IL-1 β) induce the expression of PTX3 in a kidney cell line. Thus, PTX3 secreted from injured tubular cells could potentially affect a specific region during injury. We also found that very high concentrations of PTX3 were not toxic (as determined by cell viability). To establish an *in vitro* model of ischemic injury, HK-2 cells were treated with the calcium ionophore A23187 (0.3 $\mu\text{g}/\text{mL}$), and these cells were then exposed to various concentrations of PTX3. After 48 hours, cells exposed to PTX3 exhibited higher cell viability than those exposed to ischemic (or hypoxic) injury alone.

Our results confirm the protective effect of PTX3 and indicate that PTX3 suppresses Ca²⁺ influx and subsequent calpain activity. High intracellular concentrations of calcium induce cytochrome c release and apoptosome formation and activate calpain, and caspase-3.²³ Calpains can affect mitochondrial function and are essential for apoptosis in human microvascular endothelial cells.²⁴ Much of the mitochondrial calpain research performed to date has focused on its proapoptotic role in the cleavage of caspase-3.²⁵ Our results

show that ischemia caused by A23187 increased $[Ca^{2+}]_i$, as expected, and that exposure to PTX3 attenuated this. Consequently, calpain activity was also decreased, as was the production of ROS, in cells exposed to PTX3.

The ROS-induced change in mitochondrial function was observed in ischemic HK-2 cells as a decrease in the fluorescence from JC-1 aggregates, indicative of an increased MMP. However, the exposure of these cells to PTX3 stabilized the MMP. Moreover, MMP was stabilized as PTX3 increased. The changes in MMP were mirrored by the increase in apoptosis in ischemic cells, with protection proffered by exposure to PTX3.

One of the signaling cascades most commonly involved in apoptosis is the activation of a highly specialized family of caspases. Activated caspases initiate cell death by cleaving and activating effector caspases²⁶ and PARP-1, the cleavage of which is considered a hallmark of apoptosis.^{27,28} Ischemic injury in HK-2 cells induced by A23187 resulted in increases in the active form of caspase-3 and cleavage of PARP-1. Interestingly, these apoptotic indications were attenuated in cells exposed to PTX3. These results demonstrate that A23187 induced apoptosis in HK-2 cells through activation of caspase-3 and PARP and that PTX3 protects cells against calcium-mediated mitochondrial apoptosis pathways.

Although the function of PTX3 has been reported to be related to immunity, we identified calpain activity and MMP in kidney cells as targets of PTX3 function

(Fig. 9). Future studies are needed to determine if PTX3 performs similarly in other models of IRI and whether recombinant PTX3 protects against apoptosis *in vivo*. Such experiments would broaden considerably the translational aspect of recombinant PTX3 and might provide further mechanistic insight into the beneficial properties of PTX3 observed against IRI.

V. CONCLUSION

Our results show that exogenous recombinant PTX3 protects renal cells against IRI and apoptosis. PTX3 reduces the generation of ROS, suppressed calpain and caspase-3 activity, and stabilizes the MMP. Our studies may lead to new therapeutic approaches using a drug that reduces apoptotic signals in renal cells after IRI.

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ABSTRACT (IN KOREAN)

급성신장손상을 유발한 실험 모델에서 exogenous recombinant PTX-3의
조절이 손상된 세포의 회복에 미치는 영향

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목적: Pextraxin3 (PTX3)는 long pentraxin group의 일종으로 기존에 염증성 질환 및 각 장기의 질환에 연구가 되어 왔다. 본 연구자들은 선행연구를 통하여 PTX3가 ischemia(hypoxia)와 proinflammatory acute kidney injury (AKI)시에 신장세포 proliferation에서 PTX3의 긍정적인 효과에 대해서 알게 되었다. 따라서 본 연구자의 이번 연구의 목적은 추가적인 중요 연구로 in vitro에서 AKI experimental model을 개발하고 이를 통해서 exogenous recombinant PTX3의 효과를 분석 하고자 하였다.

대상 및 방법: 신장 세포주 HK-2를 사용하여세포내 칼슘의 농도를 증가시켜 injury를 유발하는 Ca ionophore A23187를 0.1, 0.3, 0.6, 1 µg/ml을 첨가하고 각각의exogenous recombinant PTX3를 농도별로 추가로 첨가한다. 세포 독성에 대해서 MTT assay를 시행하여 관찰하였다. 또한

A23187를 처리한 이후에 세포내 ROS 및 세포고사에 관여하는 caspase-3 및 PARP의 발현도 Western blot을 이용하여 관찰하였다.

결과 : 공초점 편광 현미경을 관찰시에 A23187로 인하여 세포내 Calcium의 influx의 현격한 증가를 관찰하였으며 이로 인하여 신세포 손상이 야기됨을 확인하였다. 이후 ROS의 활성은 A23187를 처리한 이후에 급격히 증가하였으며 exogenous recombinant PTX3를 처리한 이후에 감소하는 양상이 관찰되었다. caspase-3 및 PARP의 활성도는 exogenous recombinant PTX3를 처리한 이후에 감소하는 양상이 관찰되었다.

결론: 본 연구에서는 급성 신세포 손상에서 exogenous recombinant PTX3를 처리한 이후 ROS 활성의 감소 및 caspase-3의 활성을 낮추는 것을 관찰하였다. 이로 인하여 신세포의 고사의 비율을 낮추고 회복에 긍정적인 영향을 준다는 것을 확인하였다

핵심되는 말 : 신장 세포, Pentraxin, 급성 신 손상