OXIDATIVE STRESS INCREASES MEGALIN EXPRESSION THROUGH PI3K/AKT SIGNALING IN RENAL PROXIMAL TUBULAR CELLS

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ABSTRACT

INTRODUCTION AND AIMS: Megalin, an endocytic receptor in proximal tubular cells, plays a critical role in renal tubular protein reabsorption. Megalin is involved in albuminuria in diabetic nephropathy. We previously showed that renal megalin expression increased in type 1 diabetes mellitus in normoalbuminuric stage. However, mechanisms underlying megalin expression in type 1 diabetes mellitus remain unclear. In the present study, we evaluated whether oxidative stress affected megalin expression in HK-2 cells, which are immortalized human proximal tubular cells.

METHODS: HK-2 cells were cultured with hydrogen peroxide (0.1–0.4 mmol/l) or high glucose level (30 mmol/l) for 3–24 h, followed by treatment with *N*-acetyl-cysteine (NAC, 5 mmol/l). Megalin expression in cell lysates was determined by performing western blotting. Moreover, mRNA expression of the megalin-encoding gene was determined by performing real-time PCR. HK-2 cells were pretreated with Wortmannin, a PI3K inhibitor, and MK-2206, an Akt inhibitor, to investigate the involvement of PI3K/Akt pathway in megalin expression. Albumin uptake was evaluated by incubating HK-2 cells with 0.4 mmol/l hydrogen peroxide for 4 h, followed by incubation with 0.025 mg/ml FITC-conjugated albumin for 20 min, and by measuring fluorescence in cell lysates.

RESULTS: Treatment of HK-2 cells with hydrogen peroxide (0.1–0.4 mmol/l) significantly increased megalin expression in a dose- and time-dependent manner. Moreover, treatment with high glucose level (30 mmol/l) significantly increased megalin expression in a time-dependent manner compared with that in control cells (treated with 5 mmol/l glucose). Concurrent administration of the antioxidant NAC blocked the effects of high glucose level on megalin expression. Hydrogen peroxide treatment significantly increased FITC–albumin uptake by HK-2 cells. Furthermore, hydrogen peroxide-induced increase in megalin expression was abolished after treatment with the PI3K and Akt inhibitors Wortmannin and MK-2206, respectively.

CONCLUSIONS: These results suggest that oxidative stress increases megalin expression in and protein reabsorption by proximal tubular cells through the PI3K/Akt pathway. These alterations in proximal tubular cells may play a pathogenic role in the development of proximal tubular dysfunction.

RATIONALE

- Renal albuminuria can result from impaired albumin handling by the glomerulus or the renal tubules.
- Renal tubular damage occurs in the early stage of DM patients with normal renal function and normoalbuminuria (*Clin Sci* 84: 469-475, 1993).
- Megalin plays a critical role in proximal tubular albumin reabsorption, and altered megalin expression or function can contribute to renal tubular albuminuria.
- We previously showed that renal megalin expression increased in type 1 diabetes mellitus in normoalbuminuric stage.
- During the early stage of DM in rats, prior to development of albuminuria, the renal cortex exhibits oxidative stress that can be suppressed by renin-angiotensin system (RAS) inhibition (*Clin Sci* 124: 543-52, 2013).

METHODS

Cell Culture

Cell line: human proximal tubular cells, HK-2 (ATCC)
After serum starvation for 10 h, the culture medium was changed to DMEM containing 5 mmol/L glucose and 0.1 mg/mL human serum albumin (HSA) for 16 h. The cells were then treated with 0.1–0.4 mmol/L hydrogen peroxide or high glucose (30 mmol/L) and incubated for the specified times without FBS, but including 0.1 mg/mL HSA, to investigate megalin expression. Cells were pretreatment with 5 mmol/L N-acetyl-cysteine (NAC) (Sigma), wortmannin (Cell Signaling), or MK-2206 (Selleckchem) for 30 min before adding hydrogen peroxide or high glucose.

Determination of megalin expression

- Western blot analysis: Primary antibody: Rabbit anti-megalin (Abcam), rabbit anti-phospho-Akt (Ser473) and rabbit anti- Akt (Cell Signaling Technology), mouse anti-β-actin (Santa Cruz)
- Real-time RT-PCR: Megalin, sense 5′-TGAAATTGGCTGCGCTGTTGTGACC-3′, antisense 5′-AGCTCCATCGGGGCAGTCTCTG-3′; GAPDH, sense 5′-TGGCCTTCCGTGTTCCTACCC-3′, antisense 5′-CCGCCTGCTTCACCACCTTCT-3′

Evaluation of albumin uptake in HK-2 cells

- FITC-albumin uptake; HK-2 cells were incubated with hydrogen peroxide-containing DMEM for 4 h and 0.025 mg/ml FITC -conjugated albumin (FITC-Alb) (Sigma) for an additional 20 min. After multiple washes with PBS, fluorescence was measured at 485-nm excitation and 520-nm emission. Fluorescence was normalized to protein content after determination of the protein concentration by the Bradford method.
- DQ-red albumin; DQ-red albumin (Thermo Fisher) HK-2 cells were incubated with hydrogen for 2 h and 0.01 mg/ml DQ red albumin for an additional 30 min. Cells were washed with PBS and fixed in 4% paraformaldehyde.

Animal studies

All of the procedures in the present study were approved by the Kitasato University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were assigned to four groups (n = 5): (i) STZ group (diabetic phenotype induced by 65 mg/kg of body weight Streptzotocine administration), (ii) Sham group (vehicle), (iii) STZ + TLM group (STZ-induced diabetic rats receiving telmisartan treatment), and (iv) Sham + TLM group (vehicle-treated rats receiving telmisartan). At two weeks after STZ or vehicle administration, the kidneys were removed for westernblot analysis.

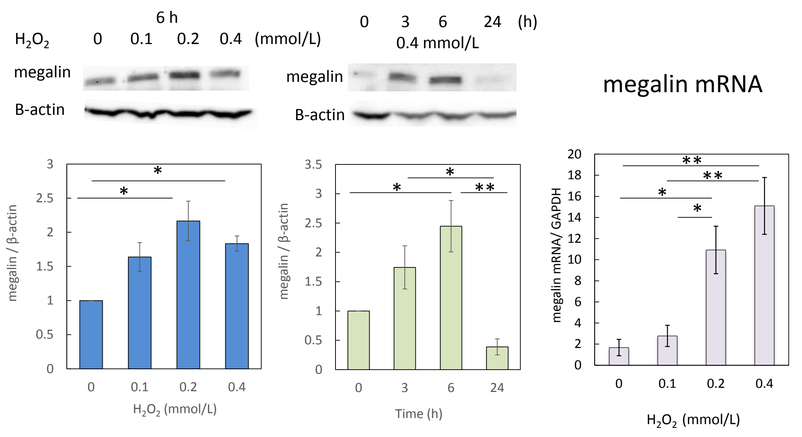
GOAL

The goal of this study was to evaluate whether oxidative stress affected megalin expression in HK-2 cells, which are immortalized human proximal tubular cells.

RESULTS

- Treatment of HK-2 cells with hydrogen peroxide (0.1–0.4 mmol/l) significantly increased megalin expression in a dose- and time-dependent manner (Figure 1).
- Treatment with high glucose level (30 mmol/l) significantly increased megalin expression in a time-dependent manner compared with that in control cells (treated with 5 mmol/l glucose). Concurrent administration of the antioxidant NAC blocked the effects of high glucose level on megalin expression (Figure 2).
- Hydrogen peroxide treatment significantly increased FITC–albumin uptake by HK-2 cells (Figure 3).
- Hydrogen peroxide-induced increase in megalin expression was abolished after treatment with the PI3K and Akt inhibitors Wortmannin and MK-2206, respectively (Figure 4, 5).

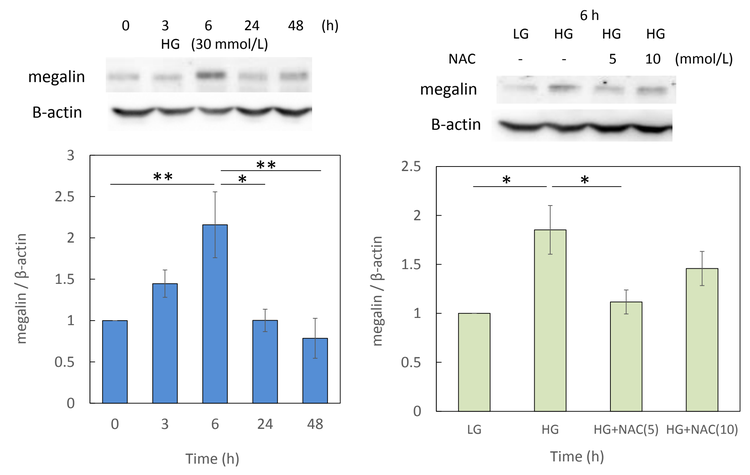
FIGURE1 Effect of hydrogen peroxide treatment on megalin expression in HK-2 cells



n = 4, Mean ±SEM, *; p < 0.05, **; p < 0.01

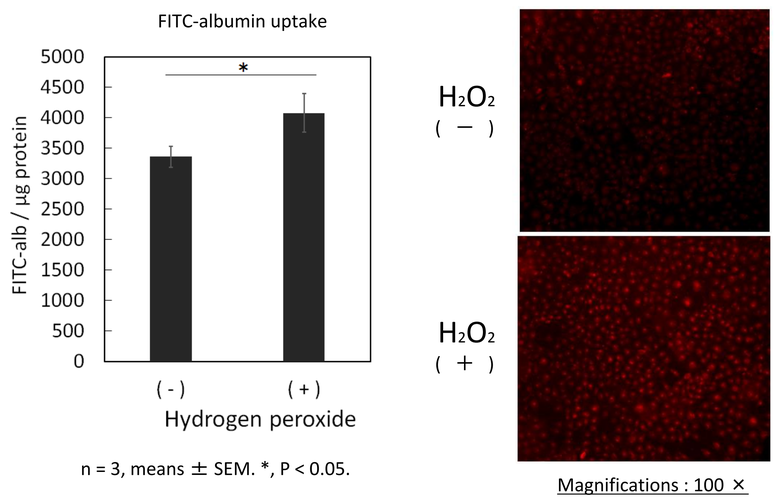
n =3 (duplicate), Mean ±SEM, *; p < 0.05, **; p < 0.01

FIGURE2 Expression of megalin in high-glucose treated cultured HK-2 cells



LG: Low Glucose, HG: High Glucose, n = 4, Mean ±SEM, *; p < 0.05, **; p < 0.01

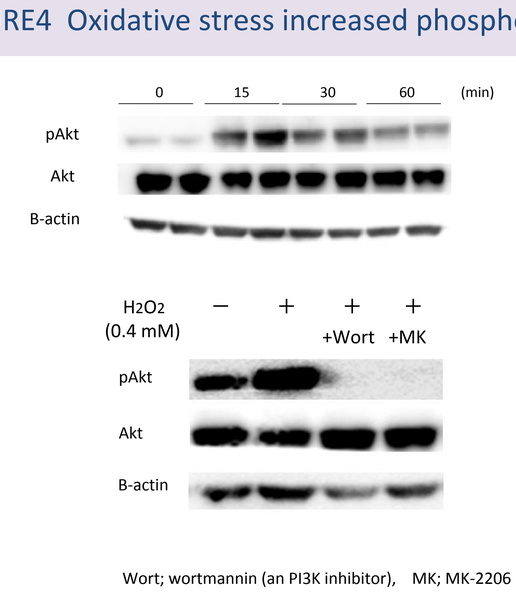
FIGURE3 Oxidative stress induces albumin uptake in proximal tubule cells



n = 3, means ± SEM. *, P < 0.05.

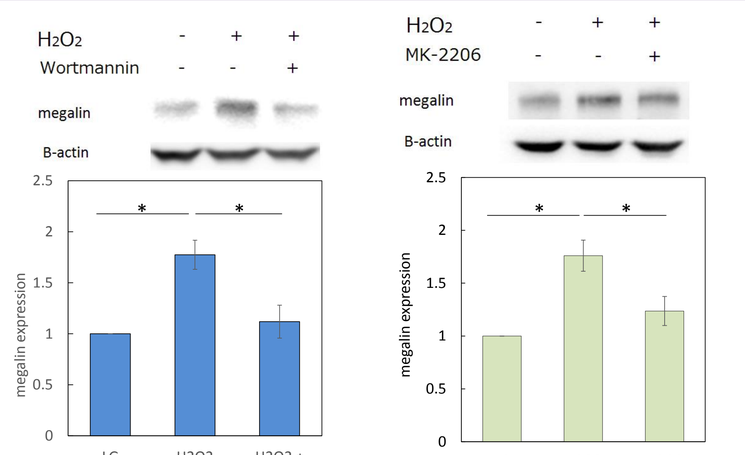
Magnifications : 100 ×

FIGURE4 Oxidative stress increased phosphorylation of Akt



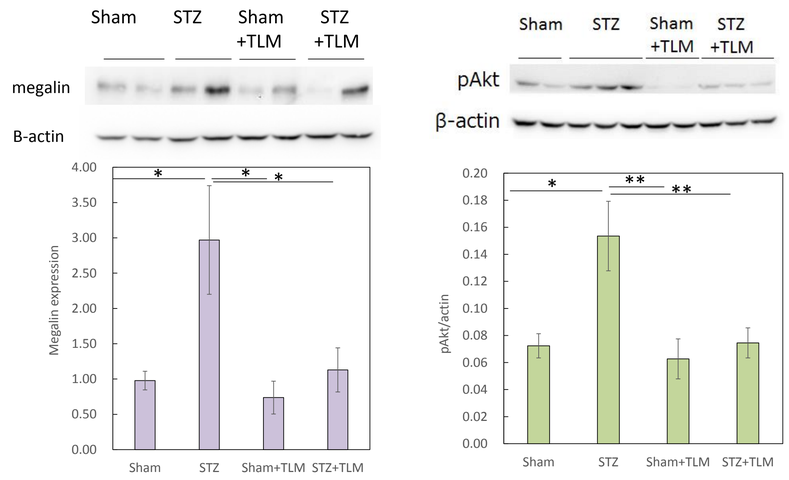
Wort; wortmannin (an PI3K inhibitor), MK; MK-2206 (an Akt inhibitor)

FIGURE5 Oxidative stress increased megalin expression via PI3K-Akt pathway



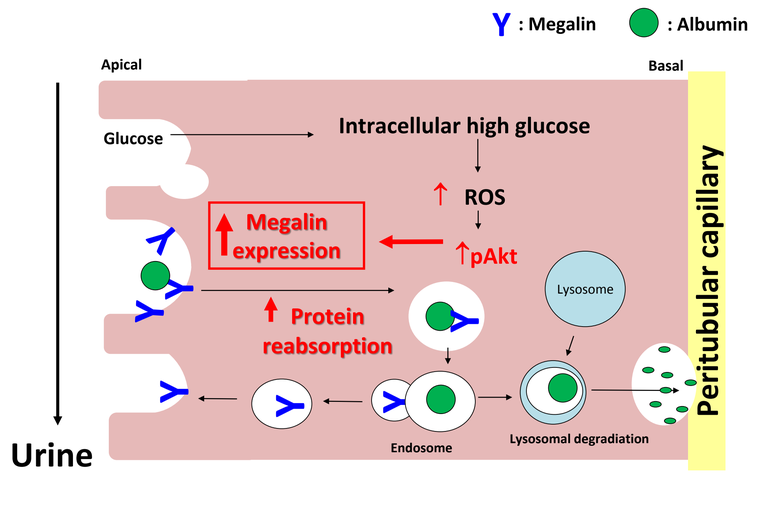
LG: Low Glucose, HG: High Glucose, n = 4, Mean ±SEM, *; p < 0.05, **; p < 0.01

FIGURE6 Expression of megalin and pAkt in the renal cortex of Streptozotocin-induced diabetic rats.



TLM; telmisartan, which is RAS inhibitor and antioxidant, n = 5, Mean ±SEM, *; p < 0.05, **; p < 0.01

Working Hypothesis



CONCLUSTIONS

Oxidative stress increased megalin expression in and protein reabsorption by proximal tubular cells through the PI3K/Akt pathway. These alterations in proximal tubular cells may play a pathogenic role in the development of proximal tubular dysfunction.

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