LY294002 Inhibits the PI3K/Akt Signaling Pathway to Block the Effect of Dexamethasone on Proteinuria Reduction: Post-Print

Authors: Zhigang Zhou, Wu Wangli, Liu Tiantian, Peng Yun, Lao Wenqin, Liang Min, Zhu Meihua

Date: 2017-12-21T00:00:00+00:00

Abstract

Objective: To investigate the PI3K/Akt signaling pathway affected by dexamethasone and LY294002, the expression of its downstream molecule Bad, and their correlation with urinary protein changes and significance in an in vivo rat model of adriamycin (ADR)-induced nephropathy. Methods: SD rats were randomly divided into four groups using a random number table: normal control (NC), adriamycin nephropathy (ADR), adriamycin + dexamethasone treatment (DEX), and adriamycin + dexamethasone + LY294002 intervention (LY294002). Twenty-four-hour urinary protein was quantified on days 7, 14, and 28. Protein expression levels of p-Akt, Akt, and Bad in renal cortex were detected by Western Blot, and Bad mRNA expression level was measured by Q-PCR. Results: The ADR group exhibited continuously increasing proteinuria, decreased p-AKT/AKT ratio, upregulated Bad protein expression, and increased Bad-mRNA expression, showing statistically significant differences compared with the NC group ($P<0.05$). The DEX group showed significantly reduced urinary protein, increased p-AKT/AKT ratio, downregulated Bad protein expression, and decreased Bad mRNA expression, with no statistically significant differences compared with the NC group ($P>0.05$). The LY294002 group demonstrated no reduction in urinary protein, decreased p-AKT/AkT ratio, upregulated Bad protein expression, and increased Bad mRNA expression, showing statistically significant differences compared with the DEX group ($P<0.05$). Conclusion: Dexamethasone reduces urinary protein in adriamycin nephropathy rats by activating the PI3K/Akt signaling pathway and downregulating the expression of its downstream molecule Bad; LY294002 intervention can inhibit this signaling pathway and block the effect of dexamethasone in reducing urinary protein. These results indicate that the PI3K/Akt signaling pathway is one of the signal transduction mechanisms by which hormones reduce urinary protein.

Full Text

Preamble

DOI: 10.3969/j.issn.1673-4254.2016.12.24
Journal: J South Med Univ, 2016, 36(12): 1727-1731
Authors: Zhou Zhigang, Wu Wangli, Liu Tiantian, Peng Yun, Lao Wenqin, Liang Min, Zhu Meihua
Affiliation: Department of Pediatrics, Second Affiliated Hospital of Guangzhou Medical University, Guangzhou 510260, China

Abstract

Objective: To investigate the involvement of the PI3K/Akt signaling pathway and its correlation with changes in urinary protein and downstream molecule Bad expression in adriamycin-induced nephropathic rats treated with dexamethasone and LY294002.

Methods: Sprague-Dawley (SD) rats were randomized using a random number table into four groups: normal control (NC), adriamycin-induced nephropathy (ADR), adriamycin + dexamethasone (DEX), and adriamycin + dexamethasone + LY294002 (LY294002). Twenty-four-hour urine samples were collected on days 7, 14, and 28 for quantitative protein analysis. On day 28, renal cortical tissues were harvested for Western blot detection of p-Akt, Akt, and Bad protein expression levels, and quantitative PCR (Q-PCR) analysis of Bad mRNA expression.

Results: The ADR group exhibited progressively increasing proteinuria, accompanied by a decreased p-AKT/AKT ratio and upregulated Bad protein and mRNA expression, all significantly different from the NC group ($P<0.05$). The DEX group showed significantly reduced proteinuria, an increased p-AKT/AKT ratio, and downregulated Bad protein and mRNA expression, with no significant differences compared to the NC group ($P>0.05$). The LY294002 group demonstrated no proteinuria reduction, a decreased p-AKT/AkT ratio, and upregulated Bad protein and mRNA expression, all significantly different from the DEX group ($P<0.05$).

Conclusion: Dexamethasone reduces urinary protein in adriamycin-induced nephropathic rats by activating the PI3K/Akt signaling pathway and downregulating downstream Bad expression. LY294002 inhibits this pathway and blocks dexamethasone' s proteinuria-reducing effect, indicating that the PI3K/Akt signaling pathway represents one of the key signaling mechanisms through which steroids reduce proteinuria.

Keywords: LY294002; PI3K/Akt signaling pathway; adriamycin-induced nephropathy; proteinuria; dexamethasone

Introduction

Proteinuria is a major clinical manifestation of kidney disease and an important indicator of progressive renal pathology. While glucocorticoids are commonly used clinically to reduce proteinuria, their precise mechanism of action remains unclear. Traditionally, their anti-proteinuric effects were attributed to anti-inflammatory and immunomodulatory actions, but recent studies have demonstrated that functional glucocorticoid receptors exist on renal podocytes, suggesting a direct protective role [1]. Molecular-level research in recent years has revealed that multiple signaling pathways critically regulate podocyte growth, phenotypic transformation, apoptosis, and autophagy [2].

The PI3K/Akt signaling pathway is a classical cell survival pathway that is widely expressed in eukaryotic cells and plays a key role in human physiology, regulating numerous processes related to cell growth and survival [3-5]. Studies have shown that this pathway exerts important protective effects in various cell types, including fibroblasts, auditory hair cells, and renal tubular epithelial cells [6]. Xavier et al. [7] found that the podocyte molecule CD2AP participates in the interaction between T RI and the PI3K regulatory subunit p85, activating the PI3K/Akt signaling pathway to protect against T -induced podocyte apoptosis. Wada et al. [8] reported that dexamethasone (DEX) inhibits podocyte apoptosis by downregulating p53, upregulating Bcl-xL, and inhibiting AIF translocation. Our previous research [9] also demonstrated the important role of the PI3K/Akt signaling pathway in protecting against podocyte apoptosis and showed that DEX can inhibit puromycin aminonucleoside (PAN)-induced podocyte injury. While previous studies have elucidated the relationship between DEX and the PI3K/Akt signaling pathway in vitro, whether DEX reduces proteinuria through this pathway in living animals has not been reported. This study investigates the phosphorylation status of Akt kinase in the PI3K/Akt signaling pathway, the expression of the downstream apoptotic protein Bad and Bad mRNA, and their correlation with urinary protein changes in an adriamycin (ADR) rat nephropathy model under the influence of DEX and LY294002 (a PI3K inhibitor).

Materials and Methods

Experimental Animals and Grouping

Specific pathogen-free (SPF) male Sprague-Dawley rats (6-8 weeks old, n=24) were purchased from the Experimental Animal Center of Sun Yat-sen University. The animals were randomly divided into four groups using a random number table: normal control (NC), adriamycin-induced nephropathy (ADR), adriamycin + dexamethasone (DEX), and adriamycin + dexamethasone + LY294002 (LY294002). All animals were housed at the Sun Yat-sen University Experimental Animal Center [Facility License: SYXK (Yue) 2011-0112].

Drug Administration Protocol

After one week of acclimatization, the ADR, DEX, and LY294002 groups received a single tail vein injection of adriamycin (5 mg/kg; D107159-25mg, Aladdin, China), while the NC group received an equivalent volume of normal saline. Dexamethasone intervention began on days 7 and 14 post-ADR injection, with the DEX and LY294002 groups receiving intraperitoneal injections of dexamethasone (1 mg/kg; D4902, Sigma, USA). The NC and ADR groups received equivalent volumes of 0.1% DMSO solvent (D4540, Sigma, USA) as vehicle control. LY294002 intervention was administered on days 7 and 14 post-ADR injection, with the LY294002 group receiving tail vein injections of LY294002 (0.3 mg/kg; L9908, Sigma, USA), while the NC, ADR, and DEX groups received equivalent volumes of 0.1% DMSO solvent via tail vein injection. Twenty-four-hour urine samples were collected on days 7, 14, and 28 post-ADR injection, and animals were sacrificed on day 28 for kidney harvest.

Experimental Procedures

24-Hour Urinary Protein Measurement Twenty-four-hour urine samples were collected in metabolic cages (BFSS19981, Nanjing Bianzhen Biotechnology Co., Ltd.) on days 7, 14, and 28 post-ADR injection. Total urinary protein was quantified using the Bradford method (Bradford Protein Assay Kit, Guangzhou Yongnuo Biotechnology Co., Ltd.) according to the manufacturer' s instructions. Samples were analyzed at 595 nm absorbance using a DR-200BS multi-functional microplate reader (Wuxi Huawei Delang Instrument Co., Ltd.) to calculate protein content.

Tissue Sampling and Preparation Renal cortical tissue (approximately 100 mg) was collected in 2 mL EP tubes and washed three times with ice-cold PBS to remove blood traces. After centrifugation and PBS removal, 300 L of RIPA lysis buffer was added, and tissues were homogenized for 5-8 minutes. The homogenate was centrifuged at 12,000 r/min for 30 minutes at 4°C. The middle layer (protein lysate) was transferred to new labeled 2 mL EP tubes and stored at -80°C until use.

Western Blot Analysis Fifty micrograms of protein per sample were separated by polyacrylamide gel electrophoresis (stacking gel: 95 V, 40 min; separating gel: 200 V, 120 min) and transferred to nitrocellulose membranes (250 mA, 1 h). Membranes were blocked with 5% skim milk, incubated overnight at 4°C with primary antibodies against p-AKT, AKT, Bad, and GAPDH (all from Cell Signaling Technology), washed twice with TBST for 7 minutes each, then incubated with secondary antibodies (Jackson) for 60 minutes at room temperature on a shaker. After three additional TBST washes (7 minutes each), membranes were incubated with ECL chemiluminescent substrate (Guangzhou Yongnuo Biotechnology Co., Ltd.) for 5 minutes in the dark and visualized using a chemiluminescence imaging system. Protein bands were quantified using

Image J software, with GAPDH as the internal reference.

Quantitative Real-Time PCR Total RNA was extracted from renal cortical tissues using Trizol reagent (Invitrogen) according to the manufacturer' s protocol. Reverse transcription was performed in a 20 L reaction system containing 5 g total RNA, 0.1 g random primers, and 200 U M-MLV reverse transcriptase (Invitrogen) using a HM-9600 PCR cycler (37°C, 3 min). The 50 L qPCR reaction system contained 5 L cDNA product, 10 L 5×SYBR Green 1 buffer, 1 L 10 mmol/L dNTPs (Promega), 1 L each of 25 mol/L forward and reverse primers (designed by Guangzhou Ribobio), and 5 U Taq DNA polymerase (Promega). Real-time fluorescence quantitative PCR was performed using a Bio-RAD MJ mini fluorescence PCR system (Bio-RAD).

Statistical Analysis

Data were analyzed using SPSS 17.0 statistical software. Inter-group comparisons were performed using one-way ANOVA, with $P<0.05$ considered statistically significant.

Results

24-Hour Urinary Protein Excretion

On days 7 and 14, urinary protein increased to varying degrees in the ADR, DEX, and LY294002 groups. The ADR and LY294002 groups showed significantly higher proteinuria compared to the NC group ($P<0.05$), while the DEX group did not differ significantly from the NC group ($P>0.05$). On day 28, all three experimental groups exhibited significantly elevated proteinuria compared to the NC group ($P<0.05$). Notably, the LY294002 group showed significantly higher proteinuria than the DEX group ($P<0.05$) .

Western Blot Analysis

The ADR group demonstrated decreased p-AKT/AKT ratio and increased Bad protein expression compared to the NC group ($P<0.05$). The DEX group showed increased p-AKT/AKT ratio and decreased Bad protein expression, with no significant differences from the NC group ($P>0.05$). The LY294002 group exhibited decreased p-AKT/AKT ratio and increased Bad protein expression, both significantly different from the DEX group ($P<0.05$) [Figure 1: see original paper].

Quantitative Real-Time PCR Analysis

Bad mRNA expression was significantly elevated in the ADR group compared to the NC group ($P<0.05$). The DEX group showed decreased Bad mRNA expression with no significant difference from the NC group ($P>0.05$). The

LY294002 group exhibited increased Bad mRNA expression, significantly different from both the NC group ($P<0.05$) and the DEX group ($P<0.05$) [Figure 2: see original paper].

Discussion

The PI3K/Akt signaling pathway promotes cell growth and proliferation while inhibiting apoptosis through phosphorylation of multiple substrates [10]. Akt, as a crucial downstream target kinase of PI3K, requires phosphorylation (p-AKT) for activation [11]. Woroniecki et al. [12] found that PI3K is rapidly phosphorylated in normal podocytes upon stimulation by signaling molecules, exerting protective effects. Our previous study demonstrated that puromycin aminonucleoside (PAN) stimulation decreases CD2AP expression and Akt phosphorylation, inhibiting the PI3K/Akt pathway and significantly increasing podocyte apoptosis [9]. Other studies have shown that the PI3K/Akt pathway inhibits pro-apoptotic gene expression (FKHRL1) and enhances anti-apoptotic gene expression (NF- B and Bcl-2) through various transcription factors, while directly phosphorylating and inactivating pro-apoptotic proteins such as Bad and caspase-9 to interrupt downstream signaling [13].

Bad (bcl-xl/bcl-2 associated death promoter) is a major downstream target protein of the PI3K/Akt pathway. Bad protein is expressed in peripheral and central neurons, lymphocytes, bone marrow hematopoietic cells, germ cells, and many epithelial cells, participating in cell apoptosis mechanisms [14-15]. Under physiological conditions, Bad protein exists in a phosphorylated state, stably exerting anti-apoptotic effects, whereas dephosphorylated free Bad protein promotes apoptosis through translocation and subsequent cellular events [16]. In our study, the ADR group exhibited decreased p-AKT/AKT ratio with elevated Bad mRNA and protein expression, accompanied by significantly increased 24-hour urinary protein excretion. These proteinuria results are consistent with those reported by Jiang et al. [17]. We hypothesize that adriamycin inhibits PI3K/Akt pathway phosphorylation, leading to increased downstream Bad mRNA and protein expression that promotes glomerular podocyte apoptosis and proteinuria, confirming successful establishment of the ADR nephropathy model.

Glucocorticoids are first-line clinical agents for reducing proteinuria in nephrotic syndrome, though their mechanisms remain unclear. While traditionally viewed as working through anti-inflammatory and immunomodulatory effects, studies have demonstrated functional glucocorticoid receptors on podocytes [18]. Dexamethasone can directly act on podocytes to stabilize CD2AP expression and distribution, facilitating interaction between CD2AP and the PI3K regulatory subunit p85, thereby activating the PI3K/Akt pathway. This activation increases GSK3 phosphorylation, decreases GSK3 activity, and downregulates downstream Bad mRNA and protein expression, repairing damaged podocytes and exerting anti-apoptotic effects [19-20]. In our DEX group, increased p-AKT/AKT ratio and decreased Bad mRNA and protein expression aligned with

these findings. Notably, proteinuria was significantly reduced compared to the NC group during weeks 1-2, suggesting that dexamethasone may reduce proteinuria in the ADR rat model through the PI3K/Akt pathway. However, the significant proteinuria increase at week 4 indicates that dexamethasone provides relief rather than "cure" for ADR-induced nephropathy, a finding that slightly differs from Chen et al. [21].

LY294002 is a specific PI3K/Akt pathway inhibitor [22] that non-specifically blocks the pathway upstream, reducing Akt phosphorylation, inducing cell cycle arrest, and increasing apoptosis [23]. Our LY294002 group results mirrored those of the ADR group, showing decreased p-AKT/AKT ratio, increased Bad mRNA expression, elevated Bad protein expression, and increased proteinuria. We propose that LY294002 blocks the PI3K/Akt pathway, preventing dexamethasone from activating this signaling cascade and thereby abolishing its proteinuria-reducing effect. This demonstrates that the PI3K/Akt signaling pathway is one of the key signaling mechanisms mediating dexamethasone' s anti-proteinuric effects.

In summary, our findings demonstrate that dexamethasone reduces proteinuria in adriamycin-induced nephropathic rats by activating the PI3K/Akt signaling pathway and downregulating downstream Bad mRNA and protein expression. LY294002 inhibits this pathway and blocks dexamethasone' s proteinuria-reducing effect, confirming that the PI3K/Akt signaling pathway represents one of the key mechanisms through which steroids reduce proteinuria. Combined with previous research, this pathway may represent an ideal therapeutic target for protecting podocytes and treating proteinuria.

References

[1] Lowenberg M, Verhaar AP, Van Den Brink GR. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression [J]. Trends Mol Med, 2007, 13(4): 158-63.
[2] 朱吉莉, 马特安, 陈星华, 等. 高糖通过 PI3K-AKT-mTOR 信号通路增加足细胞自噬 [J]. 中华肾脏病杂志, 2013, 29(7): 515-9.
[3] Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR signaling in cancer[J]. Front Oncol, 2014, 4(4): 64.
[4] 刘金坤, 郝亚娟, 黄嘉维, 等. 硫利达嗪诱导 SW480 细胞凋亡的机制 [J]. 南方医科大学学报, 2015, 35(4): 511-5.
[5] 兰凤鸣, 岳晓, 韩磊, 等. PI3K/AKT 信号通过介导 -catenin 通路的活性影响胶质瘤细胞的生长 [J]. 中华神经外科杂志, 2012, 28(3):
[6] 周文祥, 杨永丽, 杨晓, 等. PI3K/Akt/GSK3 信号通路在肾小管上皮细胞缺血再灌注损伤中的调控作用及重组人红细胞生成素预保护效应 [J]. 华中科技大学学报: 医学版, 2010, 39(3): 314-20.
[7] Xavier S, Niranjan T, Krick S, et al. TbetaRI independently activates Smad- and CD2AP-dependent pathways in podocytes[J]. J Am Soc Nephrol, 2009, 20(10): 2127-37.
[8] Wada T, Pippin JW, Marshall CB, et al. Dexamethasone prevents podocyte

apoptosis induced by puromycin aminonucleoside: role of p53 and Bcl-2-related family proteins[J]. J Am Soc Nephrol, 2005, 16(9): 2615-25.
[9] 吴汪丽, 于力, 张瑶, 等. Bad 蛋白在地塞米松抑制嘌呤霉素诱导足细胞凋亡中的表达及意义 [J]. 中华实用儿科临床杂志, 2011, 26(11):
[10] Hixon ML, Paccagnella L, Millham R, et al. Development of inhibitors of the IGF-IR/PI3K/Akt/Mtor pathway[J]. Rev Recent Clin Trials, 2010, 5(3): 189-208.
[11] 张颖, 胡舜英, 尹彤, 等. 利拉鲁肽通过 PI3K/Akt 和 MAPK/ERK 通路促进心肌微血管内皮细胞的增殖和迁移 [J]. 南方医科大学学报, 2015, 35(9): 1221-6.
[12] Woroniecki RP, Schiffer M, Shaw AS, et al. Glomerular expression transforming growth factor-beta(TGF-beta)isoforms inmice lackingCD2-associated protein[J]. Pediatric Nephrol, 2006, 21(3):
[13] Kong DX, Yamori T. Phosphatidylinositol 3-kinase inhibitors: promising drug candidates for cancer therapy[J]. Cancer Sci, 2008, 99(9): 1734-40.
[14] 徐小红, 谭建新, 冯华俊, 等. 慢性缺氧致大鼠右心室心肌细胞凋亡及其机制研究 [J]. 中国病理生理杂志, 2009, 25(1): 64-8.
[15] Yu B, Sun X, Shen HY, et al. Expression of the apoptosis-related genes BCL-2 and BAD in human breast carcinoma and their associated relationship with chemosensitivity[J]. J Exp Clinic Cancer Res, 2010, 29(1): 1-7.
[16] 何家璇, 薛荣亮, 吕建瑞, 等. 全脑缺血再灌注后 Bad 蛋白的表达与细胞凋亡的关系及 ERK 信号转导途径对其的影响 [J]. 山西医科大学学报, 2015, 46(9): 853-7.
[17] 蒋罗, 于力, 于生友, 等. 自噬与阿霉素大鼠肾组织损伤的关系 [J]. 中华肾脏病杂志, 2015, 31(5): 372-9.
[18] L wenberg M, Verhaar AP. Van den brink GR, et al. glucocorticoid signaling: a nongenomic mechanism for t-cell immunosuppression [J]. Trends Mol Med, 2007, 13(4): 158-63.
[19] 王辉阳, 于力, 于生友, 等. 地塞米松对肾小球足细胞瞬时受体电位阳离子通道蛋白 6 表达和分布的影响 [J]. 中华肾脏病杂志, 2014, 30(5): 377-83.
[20] 邹敏书, 余健, 聂国明, 等. 地塞米松抑制肾小球上皮间质转化 [J]. 医学研究杂志, 2011, 40(12): 77-80.
[21] 陈小英, 王菊霞, 郑京, 等. 泼尼松对阿霉素肾病大鼠肾组织 FAK/Pyk2 表达的影响 [J]. 南方医科大学学报, 2014, 34(8): 1149-53.
[22] Wen PY, Lee EQ, Reardon DA, et al. Current clinical development of PI3K pathway inhibitors in glioblastoma[J]. Neuro Oncol, 2012, 14(7): 819-29.
[23] Wang XJ, Feng CW, Li M, et al. Adam 17 mediates hypoxia-induced drug resistance in hepatoceellular carcinoma cell through activation of EGFR/PI3K/Akt pathway[J]. Molecul Cellul Bioch, 2013, 380(1/2): 57-66.

Note: Figure translations are in progress. See original paper for figures.