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厦门大学

硕 士 学 位 论 文

环氧合酶-2 介导镉诱导肾损伤的内质网应激调控机制研究

Cyclooxygenase-2 Mediates Cadmium-induced Kidney Injury via Endoplasmic Reticulum Stress

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摘 要

目的：镉及其化合物对哺乳动物的肝、肾、骨骼等脏器具有毒性损害作用。环氧合酶-2 (cyclooxygenase-2, COX-2)是花生四烯酸代谢形成前列腺素家族(prostaglandins, PGs)成员通路中的关键限速酶，其在调控细胞增殖、转移、凋亡和血管形成中发挥重要作用。已有报道镉经由 ROS 诱导大鼠肾细胞中 COX-2 表达升高而引起肾损伤，同时也有报道自噬在镉诱导的肾损伤中扮演重要的作用，但是 COX-2 和自噬在镉暴露诱导肾毒性损伤中的作用及其机制尚待阐明。本研究拟探讨 COX-2 的表达是否参与镉诱导的肾毒性损伤及其调控机制。

方法：(1)体内试验，采用 8~10 周雄性 ICR 小鼠，分为 4 组，包括对照组(0.9%生理盐水)和 $CdCl_2$ 暴露组(0.2、1、5 mg/kg)，连续 7 天腹腔注射，采用 HE 染色观察肾组织的损伤情况，qRT-PCR 检测肾组织中 *mMT1*、*mMT2*、*mPTGS2*、*mGRP78*、*mATF4*、*mCHOP*、*mIRE1α* 和 *mATF6* 的 mRNA 水平，免疫组化检测 COX-2、GRP78 和 LC3 的表达，Western Blot 观察 COX-2、内质网(ER)应激和自噬相关蛋白的表达水平，血清生化检测肾功能指标。**(2)**体外试验：**a)**采用人胚肾 HEK 细胞给予不同剂量 $CdCl_2$ (0~160 μmol/L)分别处理 12 和 24 h，采用 MTT 法检测细胞活力。**b)**采用 40 μmol/L $CdCl_2$ 建立损伤模型，qRT-PCR 检测细胞中 *hMT1B*、*hPTGS2*、*hGRP78*、*hATF4* 和 *hCHOP* 的 mRNA 水平，Western Blot 检测 COX-2、LC3、GRP78、ATF4、CHOP 和 p-eIF2α 的蛋白水平，评价 $CdCl_2$ 对 HEK 细胞染毒导致的自噬和 ER 应激结局效应。**c)**采用激光共聚焦检测 COX-2、LC3、CHOP 和 ATF4 蛋白的表达。**d)**采用 *PTGS2* 小干扰 RNA (siRNA)或 *PTGS2* 敲低细胞株以及临床干预药物 COX-2 抑制剂塞来昔布，联合 $CdCl_2$ (40 μmol/L)处理 12 h，验证 COX-2 与自噬的调控关系。**e)**采用溶酶体抑制剂氯喹(chloroquine, CQ)，或自噬诱导剂雷帕霉素(rapamycin, rapa)联合 $CdCl_2$ (40 μmol/L)处理 12 h，检测自噬在此细胞模型中的作用。**f)**构建 HEK-Fluc-Gluc/-SEAP 报告基因细胞检测 ER 应

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激的发生，采用 ER 应激抑制剂或 siRNA 联合 $CdCl_2$ (40 μmol/L)处理 12 h，验证 COX-2 与 ER 应激的调控关系。**g)**采用人肾近曲小管上皮 HK-2 细胞给予不同剂量 $CdCl_2$ (0~160 μmol/L)分别处理 12 和 24 h，采用 MTT 法检测细胞活力，qRT-PCR 检测细胞中 *hMT1B*、*hMT2A*、*hPTGS2*、*hGRP78* 和 *hATF4* 的 mRNA 水平，Western Blot 检测 COX-2、LC3、GRP78、ATF4 和 p-eIF2α 的蛋白水平，进行 $CdCl_2$ 对 HK-2 细胞染毒导致的自噬和 ER 应激等结局效应的验证。

结果：(1)体内试验表明，与对照组相比，0.2 和 1 mg/kg $CdCl_2$ 暴露组小鼠体重没有显著性差异，在 5 mg/kg 高剂量组降低($P < 0.05$)；肝体比随剂量的增加而增大，但肾体比没有显著性差异；$CdCl_2$ 暴露组血清尿素氮(BUN)和肌酐(CRE)的比值降低；HE 染色结果显示，$CdCl_2$ 暴露组(1 和 5 mg/kg)出现肾小球萎缩和肾小管肿大、酸性颗粒增多，提示 $CdCl_2$ 可以诱导肾损伤和肾功能紊乱；$CdCl_2$ 暴露组小鼠肾组织中 COX-2、GRP78、ATF4、CHOP、p-eIF2α 和 LC3 蛋白的表达都增加，且免疫组化结果证实 COX-2、GRP78、CHOP 和 LC3 蛋白水平的增高。**(2)**体外试验研究结果表明：**a)** $CdCl_2$ 处理组 HEK 细胞活力抑制率呈浓度依赖性增高(12 和 24 h 的 IC_{50} 分别为 69.7 和 51.6 μmol/L)；与对照组相比，$CdCl_2$ 处理组细胞中 *hMT1B* mRNA 水平增高，*hPTGS2* mRNA 水平在 3、6 和 12 h 均增高，且 6 h 时最高，COX-2 蛋白水平随 $CdCl_2$ 浓度的增加而增高，代谢产物 PGE2 的产量也随浓度的增加而升高。**b)**自噬相关蛋白 LC3-II 和 p-AKT 水平升高，p62 (自噬底物蛋白)和 p-mTOR 的水平降低。**c)** CQ 或 rapa 干预处理，CQ 可部分挽救 $CdCl_2$ 诱导 HEK 细胞生长活力的抑制；rapa 虽不能拯救细胞活力，但能使自噬相关蛋白 LC3-II 和 p-AKT 水平升高，p-mTOR 的水平降低。**d)** COX-2 抑制剂 CAY10404、NS398 或塞来昔布处理 HEK 细胞后可抵消 $CdCl_2$ 诱导的 COX-2 和 LC3-II 蛋白的表达升高；而且，在 siRNA 或稳定敲低 *PTGS2* 的细胞中表现的类似结果对其进行了验证。**e)**相比对照组，$CdCl_2$ 处理 HEK 细胞中 *hGRP78*、*hATF4*、*hCHOP* 的 mRNA 和蛋白水平均显著增高($P < 0.05$)；同时，HEK-Fluc-Gluc/SEAP 细胞中报告基因结果显示，$CdCl_2$ 处理后 Gluc 和 SEAP 的蛋白分泌量减少。**f)** ER 应激抑制剂 4-PBA 或 siRNA (si*GRP78*/si*CHOP*)干预处理细胞后也可抵消 GRP78、ATF4、CHOP、p-eIF2α、COX-2 和自噬标志蛋白 LC3-II 的高表达；而且，胞浆胞核蛋白检测发现，4-PBA 和 *ATF4* siRNA 可抑制 ATF4 的核转位，且抑制胞浆中 COX-2 的表达。**g)** HK-2 细胞给予不同浓度 $CdCl_2$ (0~40 μmol/L)处理后，与对

照组相比，$CdCl_2$ 处理组细胞中 *hMT1B* 和 *hMT2A* 的 mRNA 水平呈浓度-依赖性增高，*hGRP78* 和 *hATF4* 的 mRNA 水平几乎没有改变，而 *hPTGS2* mRNA 水平降低；WB 结果显示，GRP78、p-eIF2α、ATF4、LC3-II 和 COX-2 的蛋白水平都升高，导致细胞的生长活力受到抑制。

结论：$CdCl_2$ 急性暴露可诱导肾细胞自噬和 ER 应激发生、介导 COX-2 表达和毒性转归；阐明了经由 ER 应激 eIF2α-ATF4 通路介导的 COX-2 表达参与调控 $CdCl_2$ 诱导肾损伤的分子机制；提示镉诱导的肾损伤中 COX-2 可能是一个潜在的干预靶点。

关键词：镉　环氧合酶 2 (COX-2)　内质网(ER)应激　自噬　肾毒性损伤

Abstract

Objectives: As a toxic heavy metal, cadmium (Cd) and its compound has toxic effect on liver, kidney, bone and other organs in mammals. As one of the key rate-limiting enzyme of arachidonic acid metabolism and prostaglandins (PGs) synthesis, cyclooxygenase-2 (COX-2) has been described to play an important role in regulation of cell proliferation, transformation, apoptosis, and angiogenesis. Recent studies show that cadmium exposure to rat increase reactive oxygen species (ROS) level in kidney tissue with COX-2 overexpression. And other studies show that autophagy plays an essential role in Cd-induced kidney injury. However, the mechanisms of Cd-induced kidney injury accompanied by autophagy and COX-2 are still obscure. In this study, we aimed to determine the role and mechanisms of Cd-induced nephrotoxicity involved in COX-2.

Methods: (1) *In vivo*, eight to ten weeks-old male ICR mice were randomly separated into four groups, control (0.9% physiological saline) and three $CdCl_2$ exposure groups (0.2, 1, or 5 mg/kg), were intraperitoneally injected every day for one week. *mMT1*, *mMT2*, *mPTGS2*, *mGRP78*, *mATF4*, *mCHOP*, *mIRE1α*, and *mATF6* mRNA levels in kidney tissue were tested by qRT-PCR; the expression of COX-2, GRP78 and LC3 proteins was tested with immunohistochemistry; COX-2, endoplasmic reticulum (ER) stress and autophagy related proteins were detected using western blot (WB); hematological renal functions were analyzed by biochemical detection. **(2)** *In vitro*: **a)** HEK cells were exposed to various concentrations (0~160 μmol/L) of $CdCl_2$ for 12 and 24 h, cell viability was evaluated by MTT. **b)** In order to evaluate autophagy and ER stress caused by $CdCl_2$, the HEK cells was chosen to expose to 40 μmol/L $CdCl_2$, *hMT1B*, *hPTGS2*, *hGRP78*, *hATF4*, and *hCHOP* mRNA levels were tested by qRT-PCR; the expression of COX-2, LC3, GRP78, ATF4, and CHOP proteins was detected by WB. **c)** The immunofluorescence of COX-2, ATF4, CHOP, and LC3 proteins was performed with a laser scanning confocal microscope. **d)** In order to verify the relationship between COX-2 and autophagy, HEK cells were treated with *PTGS2* siRNA or celecoxib, a clinical drug COX-2 inhibitor, combining with $CdCl_2$ (40 μmol/L) for 12 h. **e)** HEK cells were treated with CQ (a lysosome inhibitor) or rapa (an autophagy inducer) combining with $CdCl_2$ (40 μmol/L) for 12 h

to study the role of autophagy in $CdCl_2$-indcued toxicity. **f)** ER dysfunction was detected by constructing HEK-Fluc-Gluc/-SEAP reporter cell lines, HEK cells were treated with ER stress inhibitor or siRNA combining with $CdCl_2$ (40 μmol/L) for 12 h to verify the relationship between ER stress and COX-2. **g)** In order to evaluate autophagy, ER stress, and cytotoxicity caused by $CdCl_2$ in HK-2 cells, the cells were exposed to various concentrations (0~160 μmol/L) of $CdCl_2$ for 12 and 24 h, cell viability was evaluated by MTT; *hMT1B*, *hMT2A*, *hPTGS2*, *hGRP78*, and *hATF4* mRNA levels in HK-2 cells were tested by qRT-PCR; the expression of COX-2, LC3, GRP78, ATF4, and p-eIF2α proteins was detected by WB.

Results: (1) The body weights did not change in the 0.2 or 1 mg/kg $CdCl_2$ groups, while it showed a significant decline in the 5 mg/kg $CdCl_2$ group compared to control ($P < 0.05$). The ratio of liver to body was significantly increased in the 1 and 5 mg/kg $CdCl_2$ exposure groups. There was no significant alteration of kidney to body weight in any group. The ratio of BUN/CRE was decreased in all three $CdCl_2$ exposure groups. Shrinkage of glomeruli and the degeneration of tubules were observed in the 1 and 5 mg/kg $CdCl_2$ groups compared with that in control group using hematoxylin and eosin (HE) staining, which indicated that $CdCl_2$ damaged the kidney tissues. In mice kidney tissues, COX-2, GRP78, ATF4, CHOP, p-eIF2α, and LC3 proteins were sharply increased in all $CdCl_2$ exposure groups. Immunohistochemical analysis showed that COX-2, ATF4, CHOP, and LC3 protein was significantly increased in kidney tubules upon $CdCl_2$ exposure. **(2)** *In vitro*: **a)** the cell growth rate was decreased in a concentration- and time-dependent manner after $CdCl_2$ treatment compared with control (0.9% saline), and the 50% growth inhibition concentration (IC_{50}) at 12 and 24 h was 69.7 and 51.6 μmol/L, respectively. The *hMT1B* mRNA was increased in a time-dependent manner in HEK cells exposed to $CdCl_2$ compared with control. The *hPTGS2* mRNA was increased at 3, 6, and 12 h. COX-2 protein was significantly increased in a concentration- and time-dependent manner after $CdCl_2$ treatment. PGE2 was also increased in $CdCl_2$-treated cells. **b)** $CdCl_2$ treatment resulted in p-AKT elevation and p-mTOR decrease in HEK cells. Along with these changes, the appearance of LC3-II and degradation of p62 was increased in a concentration- and time-dependent manner after $CdCl_2$ treatment. **c)** CQ, rescued cell growth inhibition induced by $CdCl_2$. Moreover, rapa couldn't rescued cell growth viability, but it could enhance $CdCl_2$-induced autophagy, as evidenced by the decrease of p-mTOR and increase of p-AKT and LC3-II. **d)**

Inhibition of COX-2 with CAY10404, NS398, or celecoxib counteracted COX-2 overexpression and autophagy caused by the $CdCl_2$ in kidney cells. Meanwhile, we found the same effects in *PTGS2* siRNA or *PTGS2* knockdown groups. **e)** $CdCl_2$ significantly elevated *hGRP78*, *hATF4* and *hCHOP* mRNA and proteins levels in HEK cells compared to control ($P < 0.05$). $CdCl_2$ also decreased the secretion of Gluc and SEAP in a concentration-dependent manner in HEK-Fluc-Gluc and HEK-Fluc-SEAP cells compared with control, respectively. **f)** Blocking ER stress with 4-phenylbutyrate (4-PBA) or siRNA (si*GRP78*/si*CHOP*) partially counteracted GRP78, ATF4, CHOP, p-eIF2α, COX-2, and LC3-II overexpression induced by $CdCl_2$. In addition, ATF4 was translocated into the nucleus and *PTGS2* was transcriptionally activated upon $CdCl_2$ treatment. These were inhibited by 4-PBA or *ATF4* siRNA in HEK cells. **g)** The qRT-PCR results showed that *hMT1B* and *hMT2A* mRNA were increased in a concentration-dependent manner in HK-2 cells. The *hGRP78* and *hATF4* mRNA was not changed, while the *hPTGS2* mRNA was decreased after $CdCl_2$ treatment compared with control. Nevertheless, the WB results showed that GRP78, p-eIF2α, ATF4, LC3-II, and COX-2 proteins were increased in HK-2 cells with different concentrations of $CdCl_2$ (0~40 μmol/L), resulting in cell viability decease.

Conclusion: Acute cadmium exposure induced autophagy, ER stress, and COX-2 overexpression resulting in kidney injury. The results of the current study suggest a novel molecular mechanism that: the ER stress eIF2α-ATF4 pathway mediated COX-2 overexpression contributes to $CdCl_2$-induced kidney autophagy and injury. The present study implies that COX-2 may be a potential target for intervention against $CdCl_2$-induced nephrotoxicity.

Keywords: cadmium; cyclooxygenase-2 (COX-2); endoplasmic reticulum (ER) stress; autophagy; nephrotoxicity

目 录

Table of Contents

英文缩略语词汇表

Abbreviations and Acronyms

缩略词	英文全称	中文全称
4-PBA	4-phenylbutyrate	4-苯基丁酸
AA	arachidonic acid	花生四烯酸
AKT	RAC-α serine/threonine-protein kinase	RAC-α 丝氨酸/苏氨酸蛋白激酶 B
ATF4	activating transcription factor 4	活化的转录因子 4
ATF6	activating transcription factor 6	活化的转录因子 6
ATG	autophagy-related gene	自噬相关基因
BUN	blood urea nitrogen	血尿素氮
Cd	cadmium	镉
CHOP	CCAAT-enhancer-binding protein homologous protein	C/EBP 同源蛋白
COX-2	cyclooxygenase-2	环氧合酶-2
CQ	chloroquine	氯喹
CRE	creatinine	肌酐
CREB	cAMP response element binding protein	cAMP 反应元件结合蛋白
DTT	DL-Dithiothreitol	二硫苏糖醇
DMF	dimethyl formamide	二甲基甲酰胺
eIF2α	eukaryotic initiation factor 2α-subunit	真核细胞翻译起始因子 2α
ELISA	enzyme-linked immunosorbent assay	酶联免疫吸附试验
ER	endoplasmic reticulum	内质网
ERK	extracellular signal-regulated kinase	胞外信号调节激酶
Fluc	firefly luciferase	萤火虫荧光素酶
Gluc	*Gaussia* luciferase	长腹水蚤荧光素酶
GRP78	glucose-regulated protein 78	葡萄糖调节蛋白 78
GSK3αβ	glycogen synthase kinase 3αβ	糖原合酶激酶 3αβ

缩略词	英文全称	中文全称
HE	hematoxylin and eosin	苏木精和伊红
HEK	human embryonic kidney	人胚胎肾细胞
IC_{50}	50% growth inhibition concentration	半数生长抑制浓度
IRE1α	inositol-requiring enzyme 1α	肌醇依赖激酶 1α
JNK	Jun N-terminal kinase	Jun 氨基末端激酶
LC3	microtubule-associated protein 1 light chain 3	微管相关蛋白 1 轻链 3
MAPK	mitogen-activated protein kinase	丝裂原活化蛋白激酶
MEK	mitogen-activated protein/ERK kinase	丝裂原活化蛋白/ERK 激酶
mTOR	mammalian target of rapamycin	雷帕霉素靶蛋白
MT	metallothionein	金属硫蛋白
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide	溴化 3-(4，5-二甲基噻唑-2)-2，5-二苯基四氮唑
NC	negative control	阴性对照
NF-κB	nuclear factor-κB	核因子 κB
NIH	National Institutes of Health	美国国立卫生研究院
PBS	phosphate buffer solution	磷酸盐缓冲液
PAGE	polyacrylamide gel electrophoresis	聚丙烯酰胺凝胶电泳
PERK	protein kinase RNA-like endoplasmic reticulum kinase	蛋白激酶 RNA 样内质网激酶
PGE2	prostaglandin E2	前列腺素 E2
PTGS2	prostaglandin-endoperoxide synthase 2	前列腺素内过氧化物合成酶 2
PTEN	phosphatase and tensin homolog deleted on chromosome 10	10 号染色体缺失的磷酸酶及张力蛋白同源物
PI3K	phosphatidylinositol3-kinase	磷脂酰肌醇 3-激酶
PPRE	peroxisome proliferator response element	过氧化物酶体增殖剂反应元件
Pin1	prolyl-isomerase	脯氨酰异构酶
rapa	rapamycin	雷帕霉素

缩略词	英文全称	中文全称
ROS	reactive oxygen species	活性氧簇
SEAP	secreted alkaline phosphatase	分泌型碱性磷酸酶
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis	十二烷基硫酸钠聚丙烯酰胺凝胶电泳
siRNA	small interference RNA	小干扰 RNA
Sp1	specificity protein 1	特异性蛋白 1
Tg	thapsigargin	毒胡萝卜素
TSC	tuberous sclerosis complex	结节性硬化复合物
UPR	unfolded protein response	未折叠蛋白反应
zVAD-FMK	Z-Val-Ala-Asp-fluoromethylketone	zVAD 氟甲基酮