PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2017, 44(11): 999~1006
www.pibb.ac.cn

Hypoxia Induced C3a Receptor Expression in Tubular Epithelial Cell Through HIF-1α/NF-κB Pathway*

ZHAO Wen-Jin[1], CHEN De-Jun[2], LI Li-Juan[1], ZHENG Jing-Min[1]**

([1] *National Clinical Research Center of Kidney Diseases, Jinling Hospital, Nanjing University School of Medicine, Nanjing* 210002, China;
[2] *Nephrology Department, Zhejiang Taizhou Hospital, Enze Medical Center, Linhai* 317000, China)

Abstract The anaphylatoxin C3a specific receptor C3aR plays a critical role in the renal diseases, but little is known about the regulation of C3aR expression. Tubulointerstitial hypoxia is common in kidney diseases and is an important pathogenic factor contributing to renal injuries. To investigate whether hypoxia is involved in the regulation of C3aR expression in tubular epithelial cell, in the present study, we investigated the effect of hypoxia on C3aR expression and the underlying mechanism. The human proximal tubular epithelial cells (HK-2) were cultured in hypoxic condition mimicked by addition of NaN_3. The mRNA expression of C3aR was analyzed by quantitative real-time PCR; the protein level of C3aR was evaluated by Western blotting and immunofluorescence. The levels of HIF-1α and NF-κB in nucleus, as well as the effect of HIF-1α or NF-κB inhibition on the expression of C3aR were also examined. We found that hypoxia induced upregulation of C3aR expression both in mRNA and protein level. The protein levels of HIF-1α and NF-κB in nucleus were increased in hypoxic condition. Pre-incubation with HIF-1α or NF-κB inhibitor, both inhibited the C3aR mRNA and protein expression induced by hypoxia. In addition, supplementation with HIF-1α inhibitor decreased the nuclear translocation of NF-κB. These results indicated that hypoxia could induce the expression of C3aR in tubular epithelial cells through HIF-1α/NF-κB pathway.

Key words hypoxia, C3aR, HIF-1α, NF-κB, expression
DOI: 10.16476/j.pibb.2017.0110

Complement activation has been linked to many kidney disorders [1–2]. The complement system is activated through three pathways: the classical, alternative, and mannose-binding-lection pathway. All the three pathways are converged on the cleavage of C3, which generates C3a and C3b. While C3b participates in the subsequent cascade reaction and finally form membrane attack complex, C3a is released into the microenvironment. Through interacting with its receptor C3aR, C3a can recruit and activate leukocytes and stimulate them to secrete inflammatory cytokines[3]. In addition to being expressed in bone marrow-derived cells like mast cells, neutrophils and monocytes, C3aR has also been reported to be expressed by some non-lymphoid tissues, such as brain, lung, liver and kidney [4–7]. In different tissues and pathological conditions, signaling through C3aR exhibits various biological functions[4–6, 8–9].

In the kidney, C3aR has been reported to be expressed by renal tubular epithelial cells and glomerular podocytes[7, 10]. The expression of C3aR has been reported to be upregulated, and excessive signaling through C3aR was thought to contribute to kidney injuries, including tubulointerstitial injuries[11–14]. However, little is known about the mechanisms through which C3aR is regulated in kidney.

*This work was supported by a grant from The National Natural Science Foundation of China (81370828).
**Corresponding author.
Tel: 86-25-80863792, E-mail: zzjjmm7713@ sina.com
Received: July 3, 2017 Accepted: July 27, 2017

As tubulointerstitial hypoxia is common in kidney diseases and is an important pathogenic factor contributing to renal injuries[15-19], by using an *in vitro* model, the present study investigated the influence of hypoxia in the expression of C3aR in renal tubular epithelial cells and explored the underlying mechanism.

1 Materials and methods

1.1 Cell culture and experimental design

The human proximal tubular epithelial cell line (HK-2 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37℃ in a 5% CO_2 atmosphere. After reaching 70% confluence, cells were kept in serum free medium for 12 h and then incubated under normoxic or hypoxic conditions. Each experiment was repeated three times. Cells under passage 15-18 were used for the experiments. Hypoxic condition was mimicked by addition of sodium azide(NaN_3, inhibitor of mitochondrial oxidative phosphorylation) at different concentrates(0.5, 1 and 2 mmol/L respectively) for different time (3, 4 and 5 h respectively)[6]. Cells in control group were grown in medium without NaN_3. To block the role of hypoxia-inducible factor (HIF), HK-2 cells were pre-incubated with 30 μmol/L of sc-205346 (Santa Cruz Biotechnology, CA, USA) for 30 min. To inhibit NF-κB, the cells were pretreated with 10 μmol/L of BAY 11-7082 (Beyotime Biotechnology, Nantong, Jiangsu, China). As NaN_3 is hypertoxic to the cells, the cells were exposed to NaN_3 for certain time followed by recovery in serum-free medium for 12 h, then the protein level of C3aR in the cells were examined.

1.2 RT-PCR

Total RNA of cultured cells was extracted using the TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. cDNA was generated using the PrimeScript™ RT Master Mix kit (Takara, Dalian, Shandong, China) and analyzed by real-time PCR with an ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Calspan, CA, USA) using 1 μl cDNA, 250 nmol/L primers and UltraSYBR Mixture (CWBio, Taizhou, Jiangsu, China). 18S RNA was used as an endogenous control. The specificity of the PCR products was established by melting curve analysis and by 1.5% agarose gel electrophoresis to verify the size. The following gene-specific primers were used: 18S forward, 5′ TTT CTC GAT TCC GTG GGT GG 3′ and reverse primer, 5′ AGC ATG CCA GAG TCT CGT TC 3′; C3aR forward, 5′ TGA AGC CTT CAG CTA CTG TCT CAG 3′ and reverse primer, 5′ GGA CAA TGA TGG AGG GGA TGA G 3′. $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative mRNA expression level for each target gene.

1.3 Immunofluorescence

HK-2 cells were grown on cover slips. Cells were fixed in 4% formalehyde for 15 min. After washed with PBS for three times, cells were permeabilized in 0.5% Triton X-100 for 10 min. Then, the cells were blocked with 20% FBS for 1h at room temperature. After that, cells were incubated with rabbit anti-human C3aR polyclonal antibody (1∶100, Santa Cruz Biotechnology, CA, USA), rabbit anti-human NF-κB p65 antibody (1∶500, Beyotime Biotechnology, Nantong, Jiangsu, China) or rabbit anti-human HIF-1α polyclonal antibody(1∶200, Beyotime Biotechnology, Nantong, Jiangsu, China) at 4 ℃ overnight. After washing, the cells were incubated with Cy3-labled goat anti-rabbit IgG antibody (CWBio, Taizhou, Jiangsu, China, 1∶200) or Alexa Fluor 488 labeled donkey anti-rabbit IgG antibody (Invitrogen, USA, 1∶2 000) for 30 min at room temperature. After washing with PBS, the nuclei were stained with 4′ 6-diamidino-2-phenylindole. Then, the slides were mounted in glycerol solution and photographed under a fluorescence microscope.

1.4 Western blotting

Total protein was extracted from cultured cells at 4 ℃ using a protein extraction buffer (Beyotime, Nantong, Jiangsu, China) containing a protease inhibitor cocktail (Proteinase Inhibitor Cocktail Tablets, Roche, Basel, Switzerland). Nuclear and cytoplasmic protein was extracted from cells using Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech, Shanghai, China). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels) and transferred to a polyvinylidene difluoride membrane (Merck Millipore, USA). After blocked with 5% skim milk powder, the membrane was probed with a primary antibody, such as rabbit anti-human C3aR polyclonal antibody (1∶1 000), rabbit anti-human HIF-1α polyclonal

antibody (1∶500), rabbit anti-human NF-κB p65 polyclonal antibody(1∶500), rabbit anti-human β-actin polyclonal antibody (1∶5000, EnoGene, Nanjing, Jiangsu, China) and rabbit anti-human Histone H3 antibody(1∶1000, Beyotime Biotechnology, Nantong, Jiangsu, China) at 4℃ overnight and then incubated with peroxidase-conjugated secondary antibodies (HRP-labeled goat anti-rabbit IgG antibody, 1∶20 000, TRAns, Beijing, China) for 1 h at room temperature. The immunolabeled proteins were detected by chemiluminescence using the Chemiluminescent HRP substrate (Merker Millipore, Massachusetts, USA).

1.5 Statistical analysis

Data were expressed as (mean ± *SD*) and were analyzed using Student's *t*-test. The statistical analysis was performed using SPSS 10.0 (SPSS, Chicago, IL, USA). All statistical tests were two-tailed, and $P < 0.05$ were considered significant.

2 Results

2.1 Hypoxia induced C3aR expression in HK-2 cells

The effect of hypoxia on C3aR expression was evaluated using a model of chemical hypoxia induced by NaN_3. As shown in Figure 1a and 1b, exposure to NaN_3 induced a time and dose dependent increase in C3aR mRNA expression. After 12 h of recovery, C3aR mRNA levels declined in comparison with anoxic cells but were still elevated compared with normoxia (Figure 1c). Western blotting (Figure 1d) and immunofluorescence (Figure 1e) analysis showed that C3aR protein levels were higher in HK-2 cells subjected to 4 h ischemia followed by 12 h recovery as compared with non-ischemic control HK-2 cells.

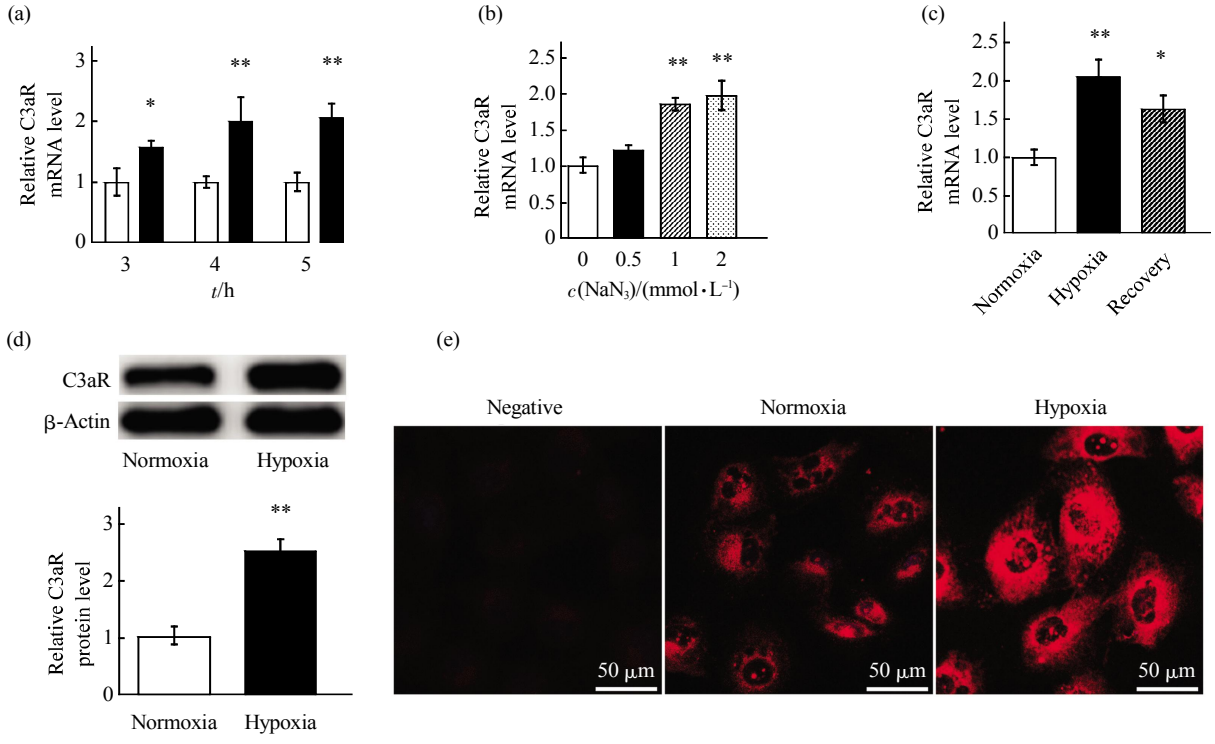

Fig. 1　Hypoxia induced the expression of C3aR in HK-2 cells

The hypoxia condition was mimicked by treatment of HK-2 cells with NaN_3 at different concentrates (0.5, 1 and 2 mmol/L respectively) for different time (3, 4 and 5 h respectively). (a) Results of quantitative PCR showing that NaN_3 induced the expression of C3aR mRNA in HK-2 cells in a time-dependent manner (1 mmol/L NaN_3 was used in the assay). □: Normoxia; ■: Hypoxia. (b) Results of quantitative PCR showing that NaN_3 induced the expression of C3aR mRNA in HK-2 cells in a concentration-dependent manner (the cells were exposed to NaN_3 for 4 h in the assay). (c) Results of quantitative PCR showing that 12 h recovery declined C3aR mRNA levels in the HK-2 cells in comparison with anoxic HK-2 cells but were still elevated compared with the cells in normoxia. (d) Results of Western blotting demonstrating that hypoxia treatment increased the level of C3aR protein in HK-2 cells. (e) Results of immunofluorescence showing increased C3aR level in HK-2 cells treated with hypoxia. *P <0.05, **P<0.01.

2.2 Hypoxia induced translocation of HIF-1α and NF-κB into nucleus

To investigate the effect of hypoxia on the HIF-1α and NF-κB, cells were incubated with 1 mmol/L of NaN_3 for 4 h. The nuclear levels of HIF-1α and NF-κB were examined by immunofluorescence and Western blotting. As shown in Figure 2, both immunofluorescence and Western blotting analysis demonstrated the increased HIF-1α and NF-κB levels in the nucleus of HK-2 cells treated with NaN_3 when compared with controls.

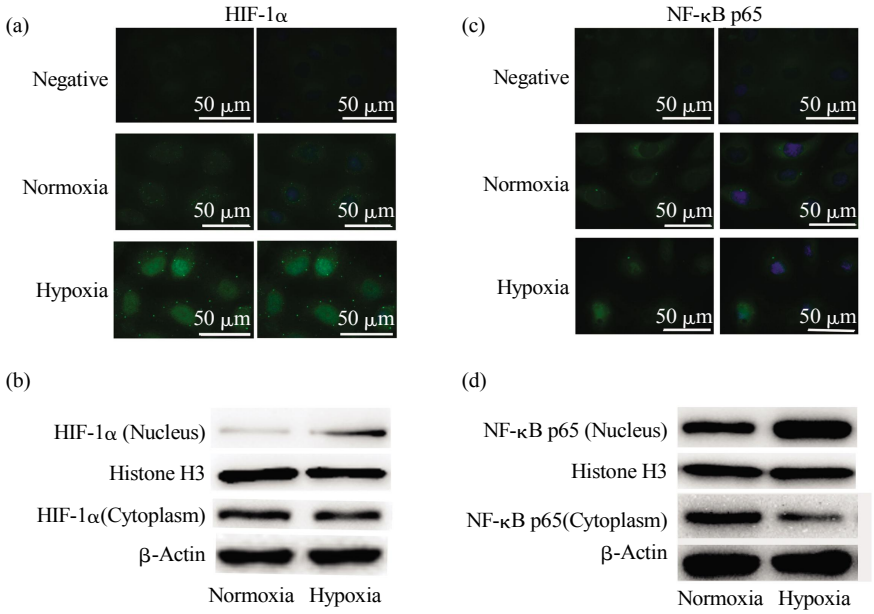

Fig. 2 Hypoxia enhanced the translocation of HIF-1α and NF-κB p65 into nucleus

The hypoxia condition was mimicked by treatment of HK-2 cells with NaN_3. HK-2 cells were treated with 1 mmol/L NaN_3 for 4 h, the nuclear translation of HIF-1α and NF-κB were examined by immunofluorescence and Western blotting. (a) Representative pictures of immunofluorescence showing increased nuclear translocation of HIF-1α in HK-2 cells treated with hypoxia when compared with controls. (b) Representative picture of Western blotting demonstrating higher level of HIF-1α in the nucleus of HK-2 cells treated with hypoxia. (c) Representative pictures of immunofluorescence showing increased nuclear translocation of NF-κB p65 in HK-2 cells treated with hypoxia when compared with controls. (d) Representative picture of Western blotting demonstrating the increased level of NF-κB p65 in the cell nucleus of HK-2 cells treated with hypoxia.

2.3 HIF-1α inhibitor attenuated the upregulation of C3aR and translocation of NF-κB induced by hypoxia

To investigate whether HIF-1α was involved in the up-regulation of C3aR induced by hypoxia, cells were pretreated with HIF-1α inhibitor sc-205346 for 30 min before exposure to 1 mmol/L NaN_3 for 4 h. Results of Western blotting showed that pretreatment of the cells with sc-205346 significantly decreased the translocation of HIF-1α (Figure 3a). In the meanwhile, pretreatment with HIF-1α inhibitor suppressed the effect of hypoxia on the expression of C3aR both in mRNA and protein levels (Figure 3b–d). To test the effect of HIF-1α on NF-κB regulation, the level of NF-κB in the nucleus was also investigated. As shown in Figure 3e and f, HIF-1α inhibitor reduced hypoxia-induced translocation of NF-κB.

2.4 The upregulation of C3aR in hypoxia condition was attenuated by NF-κB inhibitor

Further, to define the role of NF-κB in the induction of C3aR expression by hypoxia, HK-2 cells were pre-incubated with the NF-κB inhibitor BAY (10 μmol/L) before exposure to NaN_3. As shown in Figure 4a, pre-treatment of HK-2 cells with BAY markedly reduced the level of NF-κB p65 in nucleus. In the meantime, pre-treatment of HK-2 cells with BAY significantly reduced the C3aR mRNA(Figure 4b) and protein levels induced by hypoxia(Figure 4c and d).

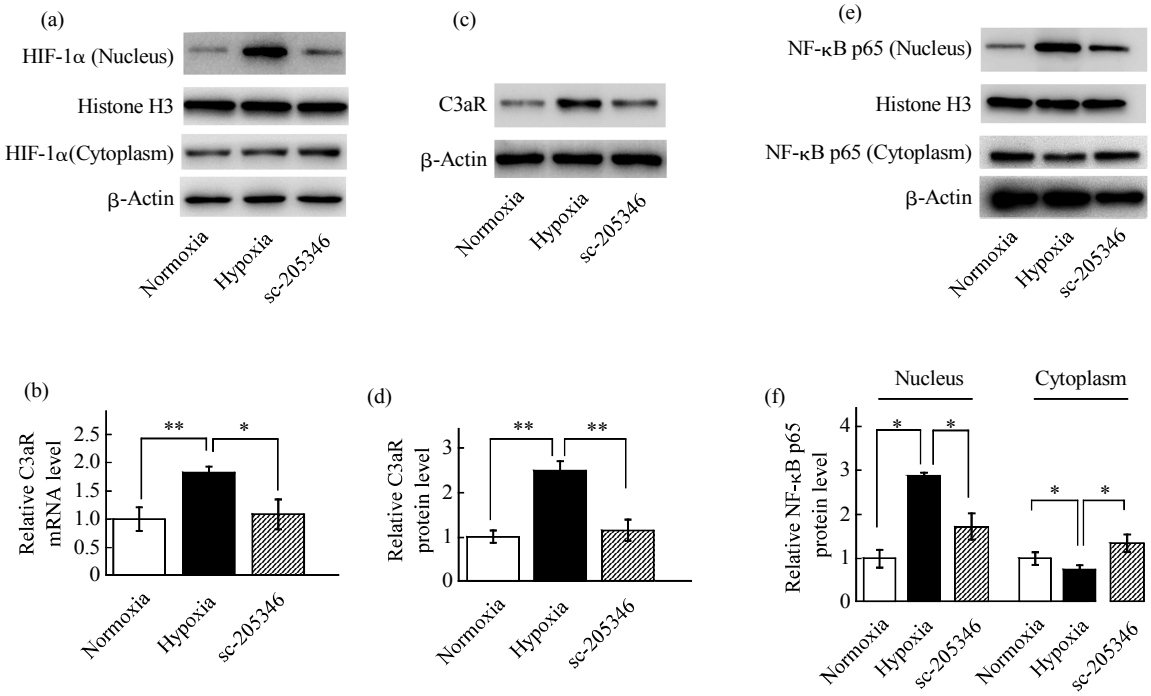

Fig. 3 HIF-1α inhibitor attenuated the induction of C3aR expression and NF-κB nuclear translocation by hypoxia

The hypoxia condition was mimicked by treatment of HK-2 cells with NaN_3. HK-2 cells were pretreated with HIF-1α inhibitor sc-205346 (30 μmol/L) for 30 min before exposure to 1 mmol/L NaN_3 for 4 h. (a) Representative picture of Western blotting demonstrating that HIF-1α inhibitor decreased HIF-1α in the nucleus of HK2 cells under hypoxia, in the meanwhile it increased the cytoplasmic HIF-1α level. (b) Results of quantitative PCR showing that pre-incubation with HIF-1α inhibitor significantly decreased the C3aR mRNA level induced by hypoxia in HK-2 cells. (c, d) Results of Western blotting showing that pre-incubation with HIF-1α inhibitor significantly decreased the C3aR protein level induced by hypoxia in HK-2 cells. (e, f) Results of Western blotting showing that HIF-1α inhibitor decreased the hypoxia-induced nuclear NF-κB level and increased the cytoplasmic NF-κB level (**P*<0.05, ***P*<0.01).

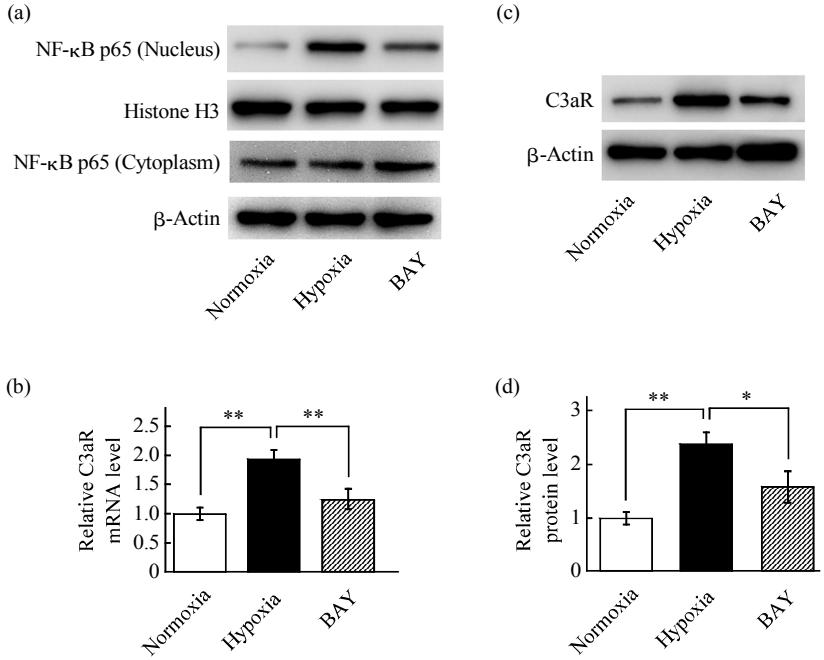

Fig. 4 NF-κB inhibitor attenuated the induction of C3aR by hypoxia

The hypoxia condition was mimicked by treatment of HK-2 cells with NaN_3. HK-2 cells were pretreated with NF-κB inhibitor BAY (10 μmol/L) for 30 min before exposure to 1 mmol/L NaN_3 for 4 h. (a) Representative picture of Western blotting demonstrating that NF-κB inhibitor (BAY) decreased nuclear NF-κB level of HK-2 cells under hypoxia, in the meanwhile it increased the cytoplasmic NF-κB level. (b) Results of quantitative PCR showing that pre-incubation with NF-κB inhibitor significantly decreased the C3aR mRNA level. (c, d) Results of Western blotting showing that pre-incubation with NF-κB inhibitor significantly decreased the C3aR protein level. (**P*<0.05, ***P*<0.01).

3 Discussion

In the present study, we investigated the influence of hypoxia on C3aR expression in tubular epithelial cells (HK-2 cells). C3aR expression was found to be upregulated significantly in response to hypoxia. In the meanwhile, hypoxia induced marked activation of HIF-1α (increased the translocation of HIF-1α to the cell nucleus) and inhibition of HIF-1α suppressed C3aR expression induced by hypoxia. Also, hypoxia significantly increased the activation level of NF-κB (as indicated by increased accumulation of NF-κB p65 in the nucleus of the cells), and inhibition of NF-κB attenuated the induction of C3aR expression by hypoxia. Furthermore, inhibition of HIF-1α suppressed the translocation of NF-κB into nucleus. Taken together, our results indicated that hypoxia induced C3aR expression through HIF-1α/NF-κB pathway.

Despite the kidney receives 20% of cardiac output, the oxygen tension in kidney is comparatively low. Special architecture of the vasculature makes countercurrent oxygen exchange between arteries and veins, resulting in the chronic oxygen deprivation in renal medulla. At the same time, tubular epithelial cells have a higher oxygen demand because of abundance of active reabsorption. As a result, the kidney is vulnerable to oxygen deprivation[15, 20–21]. This situation is even worse in the condition of kidney damage. Peritubular capillary loss decreased the oxygen supply, resulting in hypoxia. Subsequently, hypoxia leads to tubular cells injury and interstitial fibrosis. In turn, interstitial fibrosis extended distance between the capillaries and tubular epithelial cells and then reduced the oxygen diffusion to tubular cells[21–22]. In fact, hypoxia has been demonstrated as a common pathogenic factor in both acute kidney injury and chronic kidney disease [15–16, 20–21]. Several mechanisms have been reported to explain the hypoxia injury in kidney[23–25]. Complement activation is among them[26–29]. Since increased activation of complement could result in higher production of C3a, the nature ligand for C3aR, the upregulation of C3aR would mean excessive signaling through C3aR in hypoxia conditions. Previous studies have demonstrated that activation of C3aR pathway resulted in the tubular injury and epithelial-to-mesenchymal transition [26, 30–32]. Taken all these together, the present findings that C3aR was upregulated in hypoxia condition suggested that hypoxia could induce the renal injury through activating the C3aR signaling pathway.

As a critical mediator of inflammation, NF-κB plays important role in a lot of human diseases[33–35]. In the kidney, a wide range of stimuli relevant to kidney injury have been reported to be able to activate NF-κB, including cytokines, high glucose, immune mediators and proteinuria[36–40]. Through regulating the expression of inflammatory genes, such as the chemokines monocyte chemoattractant protein-1, interleukin-6 and interleukin-8[40–41], activation of NF-κB was demonstrated to contribute the progression of kidney damage, including renal tubular and interstitial damage [32, 42]. Previous studies have demonstrated the cross-talk between NF-κB and complement system [43–45], but the molecular mechanism was not completely understood. The present finding that NF-κB could regulate the expression of C3aR provide novel mechanism underlying the association of NF-κB with complement. In addition, this finding also implies that NF-κB may lead to renal injury through C3aR pathway. Thus, C3aR antagonist may be a potential target for inhibition of the NF-κB effects.

The present study had certain limitations. First, the present study only used the proximal tubular epithelial cell line. Whether these results can be extended to *in vivo* conditions remains to be determined. Second, chemical induction was used to mimic hypoxia conditions, which was a little different from the physiological conditions. Third, as NaN_3 is hypertoxic to the cells, in this hypoxia model, the cells can only be exposed to NaN_3 for a short period (less than 5 h, longer time of exposure to NaN_3 will cause severe cell death). The effect of longer period of hypoxia to C3aR expression is still to be evaluated by using other models.

In summary, the results of this study indicated that hypoxia increased the expression of C3aR in HK-2 cells *via* the HIF-1α/NF-κB pathway. These results provide important information for the largely unknown mechanism of the regulation of C3aR expression. The interplay in C3aR, HIF-1α and NF-κB may provide a new sight into how hypoxia causes renal injury. It is intriguing to speculate that targeting the HIF-1α or NF-κB pathway could be a therapeutic intervention in renal diseases associated with complement activation.

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缺氧通过 HIF-1α/NF-κB 通路诱导肾小管上皮细胞 C3aR 表达 *

赵文紧[1] 陈德君[2] 李丽娟[1] 郑敬民[1]**
([1] 南京军区南京总医院国家肾脏疾病临床医学研究中心，全军肾脏病研究所，南京 210002；
[2] 恩泽医疗中心，浙江省台州医院，临海 317000)

摘要 C3aR 是补体 C3a 的受体．在肾脏，已发现 C3aR 表达于包括肾小管上皮细胞在内的多种细胞．在特定的病理情况下，C3aR 表达上调并参与多种肾脏疾病的病理过程，但有关 C3aR 在肾脏细胞中的表达调控机制仍不清楚．小管间质缺氧是肾脏疾病中的一种常见现象，也是一种重要致病因素．为了探讨缺氧对 C3aR 的表达调控作用，本文利用体外缺氧模型，对模型条件下 C3aR 在肾小管上皮细胞中的表达变化情况进行了分析，同时检测了 HIF-1α 和 NF-κB 的表达变化及活化情况，以及 HIF-1α 和 NF-κB 抑制剂对 C3aR 的表达影响情况．结果发现缺氧可诱导 C3aR mRNA 及蛋白质水平的表达上调、HIF-1α 和 NF-κB 的核转移．HIF-1α 和 NF-κB 抑制剂可阻断缺氧对 C3aR 的诱导作用，且 HIF-1α 抑制剂可抑制 NF-κB 的核转移．这些结果说明缺氧可通过 HIF-1α/NF-κB 通路诱导肾小管上皮细胞 C3aR 的表达．考虑到 C3aR 活化可促进肾小管的损伤，我们推测 C3aR 通路可能参与了缺氧和 NF-κB 诱导的肾小管损伤过程，可能是防治缺氧和 NF-κB 诱导肾组织损伤的一个新靶标．

关键词 缺氧，C3aR，HIF-1α，NF-κB，表达
学科分类号 R394 **DOI**: 10.16476/j.pibb.2017.0110

* 国家自然科学基金资助项目(81370828).
** 通讯联系人.
Tel: 025-80863792, E-mail: zzjjmm7713@sina.com
收稿日期：2017-07-03，接受日期：2017-07-27