K⁺ regulates DNA binding of transcription factors to control gene expression related to neuronal apoptosis

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Sponsorship: Supported by grants from the Major State Basic Research Program of China (G200077800) and projects 30321002, 30225025 from the National Natural Science Foundation of China.

Received 28 April 2006; accepted 9 May 2006

The loss of intracellular K⁺ promotes neuronal apoptosis. The mechanism by which K⁺ acts on apoptosis, however, remains largely unknown. Here we showed that K⁺ selectively affects DNA binding activity of transcriptional factors in vitro. Low K⁺ concentration ([K⁺]) promoted the DNA binding activity of p53 and Forkhead, proapoptotic transcriptional factors, whereas it inhibited that of cAMP-responsive element-binding protein, an anti-apoptotic transcriptional factor. In contrast, K⁺ did not affect the DNA binding activity of Ying Yang 1, CCAAT/enhancer binding protein and early growth response protein-1. The expression of bax and bim, proapoptotic genes known to be regulated by p53 and Forkhead, respectively, was enhanced in cortical neurons deprived of serum, a condition known to cause K⁺ loss, whereas the expression of c-fos, a cAMP-responsive element-binding protein target gene, was inhibited. Furthermore, blocking K⁺ channels suppressed the enhancement of bim mRNA level and the reduction of c-fos mRNA level induced by K⁺ loss, whereas it had no effect on the stimulation of Forkhead or cAMP-responsive element-binding protein induced by K⁺ loss. These results suggest that low intracellular [K⁺] selectively affects DNA binding activity of transcriptional factors to regulate gene expression related to neuronal apoptosis.

NeuroReport 17:1199–1204 © 2006 Lippincott Williams & Wilkins.

Keywords: cAMP-responsive element-binding protein, DNA binding, Forkhead, neuron apoptosis, p53, potassium

Introduction

Potassium homeostasis plays a critical role in apoptosis of many cell types [1]. A drastic reduction in intracellular K⁺ concentration ([K⁺]ᵢ) has been found in the early phase of apoptosis [2–4]. It is believed that [K⁺]ᵢ reduction is not merely a consequence of apoptosis but a permissive step of apoptosis [1]. In cultured cortical neurons, apoptosis induced by serum deprivation (SD) was mediated by loss of total intracellular K⁺ [5]. Blocking K⁺ loss by tetraethylammonium (TEA), a broad-spectrum K⁺ channel blocker, or elevating extracellular K⁺ prevents the apoptosis [4–7]. The mechanism by which low [K⁺]ᵢ promotes apoptosis, however, remains largely unclear. It has been suggested that the physiological [K⁺]ᵢ suppresses the endonuclease activity and prevents the activation of caspase-3; however, low [K⁺]ᵢ does not activate caspase-3 directly [2,4,8].

As protein synthesis is required for neuronal apoptosis induced by low [K⁺]ᵢ [5], it is possible that gene transcription regulated by low [K⁺]ᵢ is involved in the apoptosis. We thus hypothesized that low [K⁺]ᵢ, regulates the expression of genes related to apoptosis through its direct effect on DNA binding activity of transcription factors (TFs) to affect neuronal survival. Here, we showed that in-vitro low [K⁺] (~60 mM), similar to the [K⁺]ᵢ found during apoptosis [1], augmented the DNA binding activity of p53 and Forkhead, known to be pro-apoptotic [8,9], but suppressed that of cAMP-responsive element-binding protein (CREB), known to be anti-apoptotic [10]. Similarly, we also found the mRNA levels of their target genes were enhanced or suppressed by intracellular K⁺ loss. Taken together, these results suggest that [K⁺]ᵢ regulates DNA binding activity of TFs to control neuronal survival.

Materials and methods

Cell culture and treatment

The cortical neurons isolated from 18-day-old fetal rats were cultured in Eagle’s minimal essential medium (MEM, Gibco, Grand Island, New York, USA) with 10% fetal bovine serum and 10% horse serum [11]. Three days after plating in vitro, 15 mg/ml 5-fluoro-2’-deoxyuridine and 35 mg/ml uridine
were added to inhibit non-neuronal cell growth. Cultures were used for experiments 11–13 days after plating in vitro. For SD treatment, medium was replaced by MEM without serum after rinsing twice with MEM. Tetraethylammonium (TEA) (5 mM), valinomycin (20 nM) and nigericin (30 nM) were used and were obtained from Sigma (St Louis, Missouri, USA).

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) was conducted with 20 ng nuclear extracts of cortical neurons or 0.5 ng recombinant proteins as described previously [12]. Briefly, the nuclear proteins extracted by the buffer [in mM, 20 N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9, 25% glycerol, 1.5 MgCl₂, 0.2 ethylene-diamine tetraacetic acid (EDTA), 0.2 phenylmethylsulfonyl fluoride (PMSF) and 0.5 dithiothreitol] from neuronal nuclei, were incubated in the binding buffer (in mM, 20 HEPES pH 7.9, 25% glycerol, 1.5 MgCl₂, 0.2 EDTA, 0.2 PMSF, 0.5 dithiothreitol, 0.1 μg/μl poly-dI-dC, 0.4 μg/μl bovine serum albumin) containing indicated K⁺ concentrations and 15 mM Na⁺ for 15 min at room temperature before incubation with 32P-labeled oligonucleotides. Unlabeled oligonucleotides were used as cold probes in competition experiments. The DNA–protein complex was separated on a non-denaturating 6% polyacrylamide gel electrophoresis and visualized by autoradiography. Signal densities were quantified by Image Quant software (Amerham). Primers used in PCR were: F-AAT GCC TCC AGG ATG GTG GCA GAG GAG ACC TAC-3; bax, 5’-TGG TAT CTT GAT CAG GCC GAA GAC-3; p53, 5’-GGA ATA GAT GCC TGG TGC TAC-3; bim, 5’-GCT AAT CCC GAC GGC GAA GAG AC-3 and 5’-GAT AAT CCC GAC GGC GAA GAG AC-3; c-fos, 5’-GGA GCT GAG GGC GAG GAG CCG CAG TCA GAT-3 and 5’-GTA AAT CCC GAC GGC GAA GAG ACC-3; c-fos, 5’-GGA GTG AGC CCT-3; nur77, 5’-TCT GCT GAC TGG TGC TAC-3 and 5’-GCC ACC ACC TCC TCC AGC TGC-3; nor-1, 5’-GAA AGA GCC GAA GAG CAG GCT GAT-3 and 5’-GGA GTA GGA AAG GAG GCC ATC-3; bim, 5’-GCT AAT CCC GAC GGC GAA GAG AC-3 and 5’-GAT AAT CCC GAC GGC GAA GAG ACC-3; c-fos, 5’-GGA GCT GAC TCA GAT-3 and 5’-ATT CTA GGC TGC CCA-3; c-fos, 5’-GGA GCT GAG GGC GAG GAG CCG CAG TCA GAT-3 and 5’-GTA AAT CCC GAC GGC GAA GAG ACC-3; c-fos, 5’-GGA GTG AGC CCT-3.

**Western blotting**

Briefly, whole cell extracts were separated on 8–10% sodium dodecyl sulfate electrophoresis polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Amersham) and probed with the primary antibody (Bax, c-fos and β-actin, from Santa Cruz Biotechnology, Santa Cruz, California, USA) at 4°C overnight and probed with secondary antibodies (Amersham) at room temperature for 1 h. The bands were visualized by an Amersham ECL system.

**Generation of glutathione-S-transferase-p53d370 and GST-FKDBD**

Glutathione-S-transferase (GST)-p53d370 was generated by PCR amplification of the amino acids 1–370 of hp53 using primers 5’TCC CCG GGG ATG GAG GAG CAG TCA GAT-3 and 5’-TAG AAT TCC TCA CAG GTG GCT GTA GTG AGC CCT-3 followed by cloning into Smal and EcoRI-restricted pGEX-KG [13]. GST-p53d370 and GST-FKDBD were expressed in BL21(DE-3) induced by 0.5 mM isopropyl-thio-BD-galactopyranoside at 25°C overnight. GST-fusion protein was purified by the GST Purification Kit (Clontech, Palo Alto, California, USA) and GST tag was removed by thrombin (Sigma) digestion [14]. The purity was assessed by Coomassie blue staining.

**Results**

**Low [K⁺] enhanced DNA binding activity of p53, Forkhead, HIF and Oct-1**

To study whether [K⁺] affects DNA binding activity of TFs to control neuron survival, we performed EMSA using nuclear extracts of cortical neurons in the binding buffer containing different K⁺ concentrations.

As shown in Fig. 1a, the DNA binding activity of p53, Forkhead, HIF and Oct-1 was increased when [K⁺] was decreased from 150 mM, similar to the normal [K⁺] (~140 mM) [1], to 0 mM. Statistical analysis indicated that the threshold [K⁺] to significantly alter DNA binding activity of these TFs was about 90 mM (Fig. 1b), which was higher than [K⁺] finally attained during apoptosis (~50 mM) [14]. DNA binding activity of p53 and Forkhead at 60 mM [K⁺], was enhanced to 206±20 and 353±17% of that found at 150 mM [K⁺], respectively (P<0.01, n=3) (Fig. 1b). Similarly, DNA binding activity of HIF and Oct-1 was increased to 185±20 and 178±5%, respectively (P<0.01, n=3) (Fig. 1b). Furthermore, DNA binding activity of these TFs could be completely blocked by cold specific probes but not by cold non-specific probes, indicating the specificity of EMSA probes.

To demonstrate that low [K⁺] directly enhances DNA binding activity of the TFs, we examined K⁺ effect on the purified truncated forms of p53 and Forkhead. Consistent with the EMSA results of neuron extract, DNA binding activity of purified p53d370, a truncated form of p53 in
which its C-terminal 24 amino acids were deleted to promote p53 DNA binding activity [13], was also markedly increased at 60 mM [K⁺], compared with that found at 150 mM [K⁺] (Fig. 1c). Similarly, DNA binding activity of FKDBD, which only contained the DNA binding domain of the Forkhead transcription factor FKHRL1, was also greatly enhanced at 60 mM [K⁺] (Fig. 1c). In addition, DNA binding activity of FKDBD exhibited a similar sensitivity to K⁺ as that of wild-type Forkhead, suggesting that the K⁺-sensitive site is within the DNA binding domain. Together, these results indicated that low [K⁺] directly enhanced DNA binding activity of some pro-apoptotic TFs in vitro. As p53, Forkhead and HIF are reported as pro-apoptotic TFs known to be regulated by p53 and Forkhead, respectively [8,13,15–17], these results suggested that low [K⁺] might increase DNA binding activity of several pro-apoptotic TFs to promote apoptosis.

**Intracellular K⁺ loss enhanced expression of bax and bim**

To provide evidence that low [K⁺], up-regulated DNA binding activity of pro-apoptotic TFs to promote apoptosis, we used cortical neurons deprived of serum as an apoptotic model induced by intracellular K⁺ loss [5], and examined the expression of bax and bim, two pro-apoptotic genes known to be regulated by p53 and Forkhead, respectively [8,13,15]. Consistent with the previous work, the cortical neurons underwent apoptosis 24–48 h after SD and addition of TEA, which blocks intracellular K⁺ loss, prevented the cell death (data not shown). Reverse transcription PCR analysis illustrated that mRNA levels of bax and bim were increased 4–8 h after SD and preventing [K⁺] loss by TEA blocked their increases (Fig. 2a). These results indicated that low [K⁺], increased bax and bim transcription while high [K⁺], inhibited their transcriptions. Furthermore, we found that bax protein level was also increased 2–10 h after SD and TEA treatment partially blocked the increase (Fig. 2b). These results suggested that bax and bim might be involved in apoptosis of neurons deprived of serum and their transcriptions increased by low [K⁺], might be due to K⁺ direct effect on p53 and Forkhead DNA binding activity.

This notion was further supported by the findings that exposure of the neurons to valinomycin, a K⁺ ionophore known to induce intracellular K⁺ loss and apoptosis in these neurons [5], greatly increased bax and bim mRNA levels 2 to 8 h after the treatment (Fig. 2c). Similarly, nigericin, another K⁺ ionophore also markedly augmented bax and bim mRNA levels (Fig. 2c). Together, these results suggested that low [K⁺], enhances DNA binding activity of p53 and Forkhead to up-regulate bax and bim, leading to neuronal apoptosis.

**Low [K⁺] decreased DNA binding activity of CREB and expression of CREB target genes**

As described above, DNA binding activity of some TFs was increased in low [K⁺]. In contrast, we found that DNA binding activity of CREB, known to be important for cell survival [18,19], was decreased when [K⁺] was reduced from 150 to 0 mM (Fig. 3a and b). Statistical analysis showed that DNA binding activity of CREB at 60 mM [K⁺] was decreased to 38 ± 9% of that found at 150 mM [K⁺] (P < 0.01, n = 3) (Fig. 3b). These results suggested that low [K⁺], might decrease DNA binding activity of anti-apoptotic TFs to promote cell apoptosis.

We then studied whether the expression of c-fos, nur77 and nor-1, three genes known to be regulated by CREB [20], was affected by low [K⁺]. As shown in Fig. 3c, mRNA levels of these genes were reduced 4–8 h after SD. Blocking K⁺ loss by TEA prevented the reduction induced by SD. Moreover, the mRNA levels of c-fos, nur77 and nor-1 were also decreased in neurons treated with valinomycin and nigericin for 2–8 h (Fig. 3d). These results indicated that low [K⁺], decreased the expression of CREB target genes and preventing intracellular K⁺ loss blocked their reductions. Furthermore, the c-fos protein was also decreased 2–10 h after SD and TEA treatment blocked its reduction (Fig. 3e). Altogether, these results supported the idea that low [K⁺], decreased CREB transcription activity through its direct effect on CREB DNA binding activity to promote neuron apoptosis.

We also found the DNA binding activity of AP-1 and Npas2 was decreased by lowering [K⁺] (Fig. 3a and b), although their roles in apoptosis remain unclear. We further found that the DNA binding activity of C/EBP, YY1 and Egr-1 was not affected by different [K⁺] (Fig. 3f), suggesting that K⁺ selectively affects TF DNA binding activity.
Activation of Forkhead and inactivation of CREB in neurons deprived of serum were insensitive to TEA

Activation of transcription factors involves sequential reactions, including phosphorylation, degradation and nuclear translocation [9,21]. Therefore, it is possible that $[\text{K}^+]_i$ affects gene expression through its action on the stimulation pathways to activate or inactivate TFs. To test this possibility, we examined the effect of TEA treatment on the activation of Forkhead and CREB by EMSA, which is also an approach to detect the activation and nuclear translocation of TFs.

![Fig. 2](image1.png)

**Fig. 2** Reduction of intracellular $\text{K}^+$ concentration ($[\text{K}^+]_i$) increased bax and bim expression. Effects of serum deprivation (SD) with or without tetraethylammonium (TEA) (5 mM) (a) or valinomycin (20 nM) and nigericin (30 nM) (c) on mRNA expression of bax and bim were analyzed by reverse-transcription polymerase chain reaction. (b) Western blot analysis of effects of SD with or without TEA on Bax protein expression.

![Fig. 3](image2.png)

**Fig. 3** Low $\text{K}^+$ concentration ($[\text{K}^+]_i$) decreased DNA binding activity of cAMP-responsive element-binding protein (CREB) and its downstream gene expression. (a) Effects of $\text{K}^+$ on the DNA binding activity of CREB, activator protein-1 (AP-1) and neuronal PAS domain protein-2 (NPAS2) were analyzed by electrophoretic mobility shift assay (EMSA). (b) Quantitative data of (a) were represented as mean ± SEM of three independent experiments. The asterisks (*) denoted data significantly different from that at 150 mM $\text{K}^+$: *$P < 0.05$, **$P < 0.01$. Effects of serum deprivation (SD) with or without tetraethylammonium (TEA) (c) or valinomycin and nigericin (d) on mRNA levels of c-fos, nur77 and nor-1 were analyzed by reverse-transcription polymerase chain reaction. (e) Western blot analysis of effects of SD with or without TEA on c-Fos protein expression. (f) Effects of $\text{K}^+$ on DNA binding activity of CCAAT enhancer binding protein (C/EBP), YinYang1 (YY1) and early growth response protein-1 (Egr-1) were analyzed by EMSA. Unlabeled specific [a] or non-specific [b] oligonucleotides were used as cold probes.
K⁺ REGULATES DNA BINDING OF TRANSCRIPTION FACTORS

Translocation of TFs. EMSA was conducted using the same amount of nuclear extracts in the binding buffer containing 90 mM K⁺. As shown in Fig. 4a and b, DNA binding ability of Forkhead was increased after SD for 3–9 h; however, TEA treatment did not affect its binding ability augmented by SD. Additionally, DNA binding ability of CREB was decreased after SD for 3–6 h (Fig. 4a and b) and this SD-induced reduction in its DNA binding ability was insensitive to TEA. Taken together, these results suggested that preventing K⁺ loss did not affect the signaling pathways regulating Forkhead and CREB.

Discussion

The present study revealed that K⁺ affects DNA binding activity of TFs in three different manners in vitro. Low [K⁺] promoted DNA binding activity of p53, Forkhead and HIF, whereas inhibited that of CREB. In contrast, DNA binding activity of YY1, C/EBP and Egr-1 was not affected by K⁺. Using rat cortical neurons deprived of serum or treated by K⁺ ionophores as models known for K⁺ loss-induced apoptosis [1], we provided evidence that reduction in [K⁺], increased the expression of the downstream genes of p53 and Forkhead, whereas decreased that of CREB target genes. Furthermore, preventing K⁺ loss had no effect on the stimulation of Forkhead or CREB. All these results supported a notion that K⁺ directly and selectively affects DNA binding of TFs and consequently regulates the expression of their downstream genes to control neuronal survival. It is consistent with the idea that physiologic [K⁺], acts as a repressor of apoptotic effectors and low [K⁺] promotes apoptosis [1]. It will be interesting to study whether in vitro K⁺ can also directly regulate DNA binding activity of transcription factors and control the transcription of their target genes.

Although the mRNA abundance changes might be due to changes in the mRNA stability, EMSA results (Figs 1A and 3A) indicated that mRNA transcription of bim, bax and c-fos was regulated by [K⁺].

It has been known that on the basis of the protein structures TFs can be categorized into four types of basic domain: (1) leucine zipper: such as CREB, C/EBP; (2) helix-turn-helix: such as Forkhead, E2F, HIF, Oct-1, NPAS2; (3) b-scaffold factors with minor groove contacts: such as p53, AP-1, STAT, Egr-1 and (4) zinc finger: such as YY1. It was evident that K⁺ effect on TF DNA binding activity was not dependent on these different groups. The mutant probes in which mutated nucleotides were outside the binding core region, did not alter the K⁺ sensitivity of CREB binding to DNA (data not show). It is not clear at present how K⁺ affects DNA binding ability. As the pore region of K⁺ channels is selective to K⁺ [22,23], it is possible that some domains in TFs are sensitive to K⁺.

As physiologic K⁺ homeostasis and transcription factor activity are important to a variety of cellular activity, the effect of K⁺ on TF DNA binding activity may represent a novel mechanism by which gene expression is controlled.

Conclusion

In-vitro EMSAs showed that low [K⁺] promoted DNA binding activity of p53 and Forkhead, pro-apoptotic transcription factors, whereas inhibited that of CREB, an anti-apoptotic transcription factor. It is possible that low [K⁺], regulated the expression of apoptosis-related genes through its direct and selective effect on DNA binding activity of various transcription factors to promote cell death.

Acknowledgements

We thank Dr Samuel Benchimol for kindly providing pcDNA3/hwtptp53 plasmid, Dr Jonathan Ham for pGEX-6P-2-FKHRL (DBD) plasmid and Ms Z-j Fan for cortical cell preparations.

References


