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Efeito do NFAT (Fator nuclear de células T ativadas) sobre o controle da expressão de WNK4 (With no Lysine Kinase 4) em células de néfron distal.

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RESUMO

Este trabalho objetivou avaliar como a expressão de WNK4 (*With No Lysine Kinase 4*) é modulada por NFAT através da modulação por AngII (Angiotensina II) e Ciclosporina A (CsA). WNK4 é uma cinase que tem papel fundamental na regulação do transporte iônico ao longo do néfron distal, pois fosforila outras cinases (SPAK – *Proline Alanine-Rich Kinase* e OSR1 – *Oxidative Stress Responsive 1*) que fosforilam, e assim ativam, o co-transportador sódio-cloreto sensível aos tiazídicos (NCC), o que acarreta no aumento da reabsorção de sódio, cloreto e água, transporte esse sabidamente regulado pelo hormônio Angiotensina II. O estudo genético que revelou que mutações no gene de WNK geravam um quadro de hipertensão, hipercalemia e hipercalcemia, denominado de "pseudohipoaldosteronismo tipo II" (PHAII) ou Sídrome de Gordon, foi crucial para a descoberta da importância das WNKs. Este quadro é revertido quando se utiliza diuréticos tiazídicos, mostrando a relação com o NCC. Interessantemente, um quadro similar ocorre quando pacientes transplantados recebem tratamento com CsA. Estudos comprovam que AngII interfere agudamente no conteúdo da proteína de WNK4, por ativação da PKC e fosforilação da Kelchl3 (*Kelh-like 3*), culminando com inibição do complexo Ubiquitina-Ligase-E3, importante no processo de degradação da WNK4. Ainda não se sabe qual o papel do NFAT sobre a regulação de WNK4 e como AngII e CsA modulação a transcrição gênica de WNK4. Através dos métodos de westernblotting verificamos que CsA aumenta o conteúdo de WNK4. Por RT-PCR observamos também que AngII e CsA são capazes de aumentar significativamente o mRNA-WNK4 após 24h de incubação. Por ensaio da atividade da luciferase utilizando um vetor que apresenta o gene da luciferase sob o controle de promotor contendo elementos para ligação com NFAT, observamos que AngII parece aumentar atividade de NFAT. Observamos que CsA inibe significativamente a atividade da calcineurina através de ensaio enzimático e AngII não teve efeito significativo. Além disso, amplificamos o promotor do gene de WNK4 por PCR do DNA genômico e seguimos com a clonagem deste fragmento no vetor pGL4.10 (que apresenta o gene de luciferase como gene repórter). Neste constructo, vimos que tanto AngII como CsA são capazes de estimular o promotor do gene de WNK4. Após mutações pontuais para elementos NFAT no promotor de WNK4, observamos que o NFAT pode se ligar em diferentes elementos e essa diversidade gera efeitos estimulatórios e inibitórios na síntese proteica de WNK4, pois os elementos apresentam comportamentos diferentes na regulação da transcrição do gene repórter regulado pelo promotor do gene de WNK4. Assim, concluímos que AngII é capaz de estimular a síntese proteica de WNK4 por via dependente de NFAT.

ABSTRACT

This work aimed to understand how WNK4 expression is modulated by NFAT through AngII and CsA. Wnk4 is a kinase which plays a significant role in ionic transport regulation in distal nephron by inducing phosphorylation of other kinases such SPAK and OSR1, which ultimately lead to NCC phosphorylation. WNK4 activation increases sodium, water and chloride reabsorption in this segment and it´s already know that AngII can modulate this pathway. Genetic mapping studies showed that mutations in WNK gene lead to a hypertension, hyperkalemia and hypercalcemia condition, called Pseudohypoaldosteronism type II (PAH II) or Gordon's Syndrome. This finding was crucial for WNK's discovery. Interestingly, this disease is controlled with thiazide diuretics treatment, showing that NCC participates in its pathogenesis. Curiously, a similar condition occurs when transplant recipient are treated with cyclosporine A. AngII changes the WNK4 protein content, thought PKC activation and KLHL3 phosphorylation, leading to an inhibition of ubiquitin-ligase E3 complex, which is important to WNK4 degradation. It is still unclear how NFAT may modulate WNK4 gene expression. We show, by western blotting technique, that CsA increased WNK4 expression, but AngII had not significant effect. After 24h, AngII and CsA increased mRNA-WNK4 by RT-PCR. Using luciferase assay, we observed that AngII increases NFAT activity and CsA decreases NFAT activity, both significantly. AngII did not show effect on calcineurin activity but CsA was able to decrease it, after incubation during 4h. WNK4 gene promoter was amplified by PCR using genomic DNA as a template, and the sequence obtained was cloned in a recombinant vector which has a luciferase gene reporter. Using this recombinant vector cloned with WNK4 gene promoter, we observed that both AngII and CsA increase WNK4 expression. We made a mapping of genome sites for NFAT binding at WNK4 promoter and we identified four elements for NFAT binding. Point mutations in these sites were engineered in order to evaluate the NFAT action in WNK4 promoter activity. We could see that NFAT had an ambiguous behavior and this effect is dependent on which element NFAT is bounded. In summary, we conclude that AngII may increase WNK4 expression through activation of NFAT.

INTRODUÇÃO

No ano 2000, Melanie Cobb e seus colaboradores[1], buscando novas proteínas da família MEK (MAP/ERK) cinases no sistema nervoso central, descobriram uma nova família de cinases que não apresentam o aminoácido lisina invariante no subdomínio II, mas sim no subdomínio I. Essa característica é bastante peculiar, tendo em vista que a lisina no subdomínio catalítico aparece em todas as proteínas cinases serina/treonina já descritas e é crucial para a ligação com o ATP e consequente atividade da enzima. Dessa forma, a equipe nomeou essa nova família de quinases de WNKs – *With no lysine(K) kinases* – em tradução livre "cinases sem lisina" (Xu et al., 2000).

Em mamíferos, são descritas 5 isoformas de WNKs (WNK1, WNK2, WNK3, WNK 4 e KS-WNK1). Em humanos, os 4 genes que codificam as WNKs (1 – 4) foram localizados nos cromossomos 12p13.33 (WNK1), 9q22.31 (WNK2), Xp11.22 (WNK3) e 17q21.31 (WNK4) (NCBI database). Vale ressaltar que a KS-WNK1 é uma isoforma truncada da WNK1, que parece ser expressa especificamente do túbulo convoluto distal do rim, dai o nome "KS" de "*kidney specific*" (Delaloy et al., 2003).

Devido ao fato das WNKs serem expressas em diversos tecidos, elas estão ligadas à regulação de várias funções fisiológicas, muitas delas ainda desconhecidas. WNK1 é amplamente expressa, sendo descrita nos testículos, coração, rim, musculatura esquelética, vasos sanguíneos, cólon, fígado, pâncreas e cérebro (O'Reilly et al., 2003); WNK2 é expressa principalmente no coração, cérebro e cólon (Verissimo & Jordan, 2001); WNK3 é expressa em baixos níveis no cérebro, pulmão, rim, fígado, pâncreas e em tecidos fetais incluindo placenta, cérebro, pulmão, rim, coração, timo e baço fetais (Holden; Cox; Raymond, 2004); WNK4 é expressa em tecidos que contém epitélios secretores como rim, pâncreas, ductos biliares, cólon, barreira hematoencefálica, epidídimo e pele (Kahle et al., 2004).

WNK1 e WNK4 vêm apresentando maior destaque por estarem relacionadas com doenças genéticas. Após 1 ano da descobertas das WNKs, Richard P. Lifton e seus colaboradores[2] demonstraram que mutações nos genes que codificam WNK1 e WNK4 estão associadas ao desenvolvimento de um tipo de hipertensão sensível a sal associada à hipercalemia, condição denominada de "Pseudohipoaldosteronismo Tipo II" (PAHII), "Hipertensão Hipercalemica Familiar" (FHHt) ou ainda "Síndrome de Gordon" (Wilson et al., 2001). A partir daí, inúmeros grupos de pesquisa vêm se dedicando ao estudo da regulação, função e demais mecanismos envolvidos com estas proteínas. A fim de otimizar a compreensão deste trabalho, focaremos na WNK1 e na WNK4.

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Conclusão

Nossos experimentos mostram que a angiotensina é capaz de ativar significativamente a via de sinalização por NFAT, mesmo não apresentando efeito sobre a atividade da calcineurina. Além disso, AngII foi capaz de aumentar o conteúdo de mRNA de WNK4 e de estimular a síntese proteica no promotor de WNK4. Esses resultados mostram que AngII é capaz de estimular a síntese de WNK4 através da modulação da via de sinalização por NFAT.

Observamos também que a CsA aumenta o conteúdo de mRNA e de proteína WNK4. CsA também estimula significativamente o promotor de WNK4. Entretanto, contrariamente aos efeitos da AngII, CsA inibe a sinalização por NFAT e a atividade da calcineurina. Assim, podemos concluir que o efeito estimulatório de CsA sobre a síntese proteica de WNK4 parece ser independente de NFAT.

Os achados deste trabalho trazem perspectivas interessantes de como o NFAT pode se comportar na regulação da transcrição gênica de WNK4. Observamos que os elementos para ligação de fatores de transcrição NFAT no núcleo apresentam comportamentos diversos, tendo sido identificados elementos para ligação com fator de transcrição NFAT tanto estimuladores como repressores da síntese proteica. Além disso, observamos que esses elementos apresentam grau de afinidade de ligação ao NFAT, além de capacidade estimulatória e repressora diferentes, tornando assim, o estudo da regulação gênica de WNK4 por NFAT bastante complexo.

Em resumo, Ang é capaz de estimular a síntese de WNK4 através de ativação da via de sinalização por NFAT e que a CsA induz síntese de WNK4 provavelmente por uma via de sinalização independente de NFAT.

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