Pontifícia Universidade Católica do Rio Grande do Sul
Programa de Pós-Graduação em Medicina e Ciências da Saúde
Área de Concentração: Farmacologia Bioquímica e Molecular

Vanessa Sgnaolin

EXPRESSÃO E FUNÇÃO DOS RECEPTORES PARA CININAS EM CÉLULAS DE TUMOR DE BEXIGA: MECANISMOS RELACIONADOS

Porto Alegre
2012

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Dissertação apresentada como requisito parcial para obtenção do Título de Mestre pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde, Área de Concentração em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Profª. Dr. Maria Martha Campos
Co-Orientador: Profª. Dr. Fernanda Bueno Morrone

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Dedico este trabalho a minha família

e ao meu namorado André por sempre

terem me apoiando com muito

carinho e compreensão.

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RESUMO

O presente estudo teve por objetivo caracterizar, através de abordagens funcionais e moleculares, a relevância dos receptores B_1 e B_2 para as cininas no câncer de bexiga. Os dados obtidos mostraram que tanto o agonista dos receptores B_1, des-Arg^9-BK, quanto do receptor B_2, BK, foram capazes de estimular a proliferação das células de câncer de bexiga grau 3, denominadas T24. Além disso, a incubação dos antagonistas dos receptores B_1 e B_2, SSR240612 e HOE140, respectivamente, inibiram acentuadamente a proliferação das células T24. Por outro lado, apenas maiores concentrações de BK levaram à proliferação das células de câncer de bexiga grau 1 RT4; enquanto a incubação com des-Arg^9-BK não induziu sua proliferação. De maneira similar, os resultados revelaram que a expressão de mRNA dos receptores B_2 e, principalmente, dos receptores B_1 foi superior em células T24, em comparação com as células RT4, que apresentam baixo grau de malignidade. Além disso, os dados obtidos com biópsias de câncer de bexiga humano revelaram que a expressão do receptor B_1 foi claramente maior em todas as amostras tumorais ou, em uma biópsia obtida de um quadro de inflamação crônica de bexiga. Em relação às vias de sinalização relacionadas com os efeitos mitogênicos das cininas, os dados obtidos mostram que a inibição farmacológica da PI3Kγ com AS252424 reduziu de maneira concentração-dependente a proliferação das células T24, quando está foi induzida por BK ou des-Arg^9-BK. Finalmente, a incubação das células T24 com agonistas dos receptores de cininas produziu uma acentuada ativação das vias de sinalização PI3K/AKT e ERK 1/2, enquanto a via p38 MAP quinase permaneceu inalterada. Nossos resultados indicam que os receptores de cininas B_2 e, especialmente, os receptores B_1 parecem estar associados com a progressão do câncer de bexiga. Dessa forma, é possível sugerir

que antagonistas seletivos de receptores de cininas poderiam representar alternativas terapêuticas interessantes para o controle do câncer de bexiga.

Palavras-chave: câncer de bexiga, cininas, receptores, células T24 e RT4, biópsias.

ABSTRACT

This study was designed to characterize, by means of functional and molecular approaches, the relevance of kinin B_1 and B_2 receptors in bladder cancer. Our data clearly shows that both B_1 des-Arg^9-BK and B_2 BK receptor agonists were able to stimulate the proliferation of the grade 3-derived bladder cancer T24 cells. Furthermore, the incubation of B_1 and B_2 receptor antagonists, SSR240612 and HOE140, respectively, markedly inhibited the proliferation rate of T24 cells. In contrast, only higher concentrations of BK elicited the proliferation of the grade 1 bladder cancer cell line RT4, while des-Arg^9-BK incubation completely failed to induce its proliferation. Interestingly, real time PCR experiments revealed that mRNA expression of B_2, and mainly B_1 receptors was found superior in T24 cells, in comparison to the low malignity grade RT4 cells. Furthermore, data obtained using bladder cancer human biopsies revealed that B_1 receptor expression was visibly increased in all tumoral samples or under chronic inflammation of bladder. Concerning the signaling pathways related to the mitogenic effects of kinins, we bring novel evidence showing that pharmacological inhibition of PI3Kγ with AS252424 concentration-dependently reduced T24 cell proliferation induced by BK or des-Arg^9-BK. Finally, the incubation of T24 cells with kinin agonists led to a marked activation of PI3K/AKT and ERK 1/2 signaling pathways, whereas p38 MAP kinase remained unaffected. Our results indicate that kinin B_2, and especially B_1 receptors appear to be implicated in bladder cancer progression. It is tempting to suggest that selective kinin antagonists might represent potential therapeutic alternatives for bladder cancer control.

Keywords: bladder cancer, kinins, receptors, T24 and RT cells, biopsies

ABREVIAÇÕES

BK	Bradicinina
BCG	Bacilo de Calmette-Guérin
DNA	Ácido desoxirribonucléico
cDNA	DNA complementar
EDTA	Ácido Etilenodiamino Tetra-acético (do inglês, Ethylenediamine Tetraacetic Acid)
ELISA	Enzimaimunoensaio (do inglês, Enzyme-Linked Immunoabsorbent Assay)
ERK 1/2	(do inglês, Extracellular signal– Regulated Kinases 1 and 2)
FBS	Soro fetal bovino (do inglês, Fetal Bovine Serum)
HMWK	Cininogênio de alto peso molecular (do inglês, High Molecular Weight Kininogen)
LMWK	Cininogênio de baixo peso molecular (do inglês, Low Molecular Weight Kininogen)
Lys-BK	Calidina
MAPKs	Proteínas quinases ativadas por mitógeno (do inglês, Mitogen-Activated Protein Kinases)
M-MLV	(do inglês, Moloney Murine Leukemia Virus)
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina (do inglês, Thiazolyl blue Tetrazolium Bromide)
NF-κB	Fator de transcrição nuclear kappa B
PBS	(do inglês, buffer calcium-magnesium-free)
PCR	Reação em cadeia da polimerase

PI3K	(do inglês, phosphatidylinositol 3-kinase)
RNA	Ácido ribonucleico
RNAm	RNA mensageiro
RT-PCR	Reação em cadeia da polimerase precedida de transcrição reversa
TCC	Carcinoma de células transicionais (do inglês, Transitional Cell Carcinoma)

SUMÁRIO

1. INTRODUÇÃO

1.1. Tumor de Bexiga

O tumor de bexiga é o segundo tipo mais frequente de tumor maligno do trato geniturinário (Jemal *et al.*, 2005; Shabbir *et al.*, 2008; INCA, 2010). A incidência em homens é maior que em mulheres e os fatores de risco estão associados à industrialização e à ocupação. De fato, a causa mais comum de tumor de bexiga em todo o mundo é a infecção/inflamação que ocorre devido ao tabagismo (Jemal *et al.*, 2005).

A bexiga é anatomicamente constituída por quatro camadas: urotélio, lâmina própria, músculo e gordura perivesical. A neoplasia vesical pode-se originar no epitélio (urotélio) ou no mesênquima (demais camadas da bexiga). Os tumores de origem mesenquimal são raros, compreendendo apenas 1 a 5% dos tumores de bexiga, enquanto que as neoplasias oriundas das células transicionais, que constituem o urotélio, representam 90% dos casos (Przybylo *et al.*, 2005; Ma *et al.*, 2006; Shabbir & Burnstock, 2009).

Os tumores de bexiga superficiais de alto grau têm um grande risco de recorrência e progressão (Babjuk *et al.*, 2008). O tratamento utilizado para tumores avançados é a radioterapia e a cistectomia radical (Porter *et al.*, 2011). Porém, no momento do diagnóstico, o tumor de bexiga geralmente se apresenta como um tumor superficial, que é comumente tratado com ressecção transuretral. No entanto, esta abordagem terapêutica demonstra um alto risco de recorrência e progressão, o que constitui uma das principais dificuldades de cura dessa doença (Dozmorov *et al.*, 2006; Ma *et al.*, 2006; Shabbir *et al.*, 2008), uma vez que, os tumores invasivos muitas vezes surgem de neoplasias inicialmente superficiais (Shabbir *et al.*, 2008).

Estudos demonstram que o uso de agentes antitumorais é efetivo para prevenção da reincidência da doença (Swellam *et al.*, 2003; Shelley, 2004; Shabbir *et al.*, 2008). O tratamento que tem sido comumente realizado é a administração de Bacilo de Calmette-Guérin (BCG), após a ressecção transuretral, assim como o uso da quimioterapia adjuvante (Shabbir *et al.*, 2008). Contudo, sua eficácia é questionável devido aos efeitos adversos (dor vesical, hematúria e febre) e a progressão do tumor durante a terapia, ainda, pode ser observada (Ma *et al.*, 2006).

O estudo dos mecanismos relacionados com a progressão de tumores de bexiga pode ser realizado de diversas formas, dentre elas, destaca-se o emprego de linhagens de células tumorais em cultura. Com relação aos tumores de bexiga, duas linhagens celulares denominadas RT4 e T24 têm sido bastante utilizadas, por permitirem a realização de diferentes comparações, uma vez que a linhagem RT4 possui um padrão menos agressivo, enquanto que a linhagem T24 apresenta um perfil altamente invasivo (Stella *et al.*, 2010; Zhu *et al.*, 2011).

1.2. Câncer e Inflamação

Diversos estudos vêm ampliando o conceito da inflamação como um componente crítico na progressão do tumor e a caracterização dos mecanismos envolvidos nesta resposta tem sido alvo de recentes estudos (Weiss, 2008).

O processo inflamatório tem sido apontado como um fator etiológico de muitas doenças, incluindo algumas não comumente associadas à inflamação, como doença de Alzheimer, doenças cardiovasculares e tumores. Neste contexto, os mediadores da resposta inflamatória e seus receptores parecem representar importantes constituintes do ambiente tumoral, estando envolvidos em diversas etapas do desenvolvimento tumoral, como: transformação, proliferação, migração, invasão e angiogênese (Aggarwal *et al.*, 2006; Mantovani *et al.*, 2008).

A relação entre desenvolvimento tumoral e inflamação foi descrita, pela primeira vez, no final do século XIX, por Rudolf Virchow, cuja hipótese era que os tumores tinham origem em locais de inflamação crônica, onde a associação de fatores irritantes, com o processo inflamatório, determinaria um aumento da proliferação celular. No entanto, foi apenas durante a última década que o papel da inflamação na tumorigênese foi comprovado, e foram elucidados alguns dos mecanismos moleculares subjacentes (Karin, 2006).

A inflamação é caracterizada pelo aumento do fluxo sanguíneo no local afetado, aumento da permeabilidade vascular e migração de leucócitos para o sítio inflamatório. Esse influxo celular é regulado por mediadores inflamatórios produzidos por células endoteliais e inflamatórias. Os mediadores inflamatórios são moléculas solúveis e compreendem os produtos da degranulação dos mastócitos (histamina e serotonina), os peptídeos vasoativos (cininas, neurocininas e peptídeo relacionado ao gene da calcitonina), os componentes do sistema complemento, os mediadores lipídicos (prostaglandinas, leucotrienos e fator ativador de plaquetas), as citocinas, as quimiocinas e as enzimas proteolíticas, entre outros (Medzhitov, 2008).

As células tumorais também são capazes de produzir mediadores inflamatórios que agem direta ou indiretamente, ativando o endotélio vascular e recrutando leucócitos para o tumor, liberando fatores angiogênicos, mitogênicos, enzimas proteolíticas e fatores quimiotáticos. Esse processo promove o recrutamento de mais células inflamatórias e estimula a angiogênese, sustentando, dessa forma, o crescimento do tumor e facilitando metástases (Mantovani *et al.*, 2008).

O processo inflamatório é descrito como um componente essencial de todos os tumores, inclusive alguns em que uma relação causal direta com a inflamação ainda não está comprovada (Mantovani *et al.*, 2008). Apenas uma minoria dos tumores é causada por mutações germinativas, enquanto a grande maioria (90%) está ligada a mutações somáticas e a fatores ambientais (Grivennikov *et al.*, 2010). Cerca de 20% dos tumores estão associados a infecções crônicas; 30% podem ser atribuídos ao tabagismo e poluentes inalados (como sílica e amianto) e, 35% podem ser atribuídos a fatores dietéticos; entre esses, 20% estão ligados à obesidade (Aggarwal *et. al.*, 2009).

1.3. Cininas

Cininas são mediadores peptídicos biologicamente ativos que estão envolvidos em uma série de processos fisiopatológicos (Campos *et al.*, 2006). Apresentam como ações fisiológicas o controle da pressão arterial, o relaxamento e a contração da musculatura lisa e a natriurese (Campos *et al.*, 2006; Schulze-Thopoff *et al.*, 2008). Este grupo de peptídeos participa, também, da resposta inflamatória, promovendo vasodilatação, aumento da permeabilidade vascular, extravasamento plasmático e migração celular (Schulze-Thopoff *et al.*, 2008). As cininas também estão presentes em condições patológicas como sepse, dano pós-isquêmico, asma, pancreatite, cistite, alergia, diabetes, artrite reumatóide, colite, gastrite e tumores, além de causarem dor e hiperalgesia (Ni *et al.*, 2003; Calixto *et al.*, 2004; Fox *et al.*, 2005; Leeb-Lundberg *et al.*, 2005).

Em 1909, teve início o estudo do sistema das cininas, quando Abelous e Bardier demonstraram que a injeção de uma fração insolúvel da urina humana administrada por via endovenosa em cães era capaz de produzir uma queda

acentuada na pressão sanguínea. Frey e Kraut, em 1928, observaram o efeito hipotensor de um componente isolado da urina humana, que inicialmente foi chamado de substância F. Posteriormente, Kraut e colaboradores (1930) encontraram quantidades elevadas de substância F no pâncreas, sendo identificado como principal sítio de síntese dessa substância, e a substância F passou a ser chamada de calicreína (do grego: *kallikreas*, relacionado ao pâncreas). Em 1937, Werle e colaboradores demonstraram que a calicreína, quando incubada no plasma, era capaz de liberar uma potente substância contrátil a partir de um precursor inativo, denominada calidina. Da mesma forma, Rocha e Silva e colaboradores (1949) observaram que a incubação do veneno da serpente *Bothrops jararaca*, ou tripsina, com a fração pseudoglobulina do plasma era capaz de liberar um potente agente vasodilatador e contracturante, denominado bradicinina (BK; do grego: *bradi*, para lento; *kinesia*, para movimento). Lewis (1964) pela primeira vez demonstrou a capacidade da BK em desencadear os sinais clássicos da inflamação, tais como: aumento da permeabilidade vascular, formação de edema e dor. Posteriormente, Regoli e Barabé (1980) descreveram as ações fisiológicas da bradicinina e seus análogos em diferentes tecidos (Calixto *et al*., 2004; Costa-Neto *et al*., 2008).

Os mecanismos pelos quais ocorre a síntese das cininas são bem caracterizados. As cininas pertencem a um grupo de peptídeos com 9-11 aminoácidos, que incluem: BK, calidina, T-cinina e seus metabólitos ativos, des-Arg^9-cininas. São formadas a partir de G-globulinas chamadas de cininogênios, sendo conhecidos três tipos, os quais diferem em tamanho, função e estrutura. O cininogênio de alto peso molecular (High Molecular Weight Kininogen, HMWK) é uma proteína plasmática com massa molecular de 120 kDa e dá origem à BK. O cininogênio de baixo peso molecular (Low Molecular Weight Kininogen, LMWK) tem

massa molecular de 66 kDa e origina a calidina (Lys-BK), e estão amplamente distribuídos nos tecidos, em fibroblastos e em outras estruturas celulares do tecido conectivo. O terceiro, denominado tipo T, corresponde ao HMWK e é encontrado apenas em ratos (Mclean *et al*., 2000).

Os cininogênios são clivados por proteases chamadas calicreínas; até o momento foram identificadas a calicreína plasmática e a tecidual. A calicreína plasmática, de origem hepática, circula na forma inativada, chamada de pré-calicreína ou fator de Fletcher. Após clivada, origina a enzima ativa através de um processo dependente da ativação do fator de Hagemann (fator XII da coagulação sanguínea). A calicreína plasmática atua sobre o HMWK liberando BK, e este processo é exacerbado durante a resposta inflamatória (Mclean *et al*., 2000). A calicreína tecidual é distinta da calicreína plasmática na origem de síntese, nas funções biológicas, nas propriedades físico-químicas, e também na especificidade com os cininogênios. Elas atuam sobre os substratos sintéticos de maneira diferenciada e apresentam susceptibilidade diferenciada a vários inibidores naturais e sintéticos. As calicreínas teciduais estão amplamente distribuídas nos tecidos de mamíferos, principalmente rim, pâncreas, estômago, mucosa intestinal, glândulas salivares, e estão sendo identificadas e isoladas em outros órgãos (Gontijo, 2005).

As cininas sofrem uma rápida degradação metabólica por amino- carboxi- e endopeptidades encontradas em tecidos e fluidos biológicos (Couture *et al*., 2001). Deste grupo de enzimas denominado cininases, as mais relevantes fisiologicamente são a cininase I, conhecida como arginina carboxipeptidase, representada pela carboxipeptidase N (plasma) e carboxipeptidase M (membrana), que possui papel secundário na degradação da BK. Essa enzima é responsável pela remoção da arginina da porção C terminal da BK e da Lys-BK, gerando os metabólitos ativos

des-Arg^9-BK e Lys-des-Arg^9-BK, respectivamente. Já a cininase II, conhecida também como enzima conversora da angiotensina, possui maior afinidade pela BK e pela Lys-BK, gerando metabólitos inativos o que sugere que a formação dos metabólitos ativos não ocorre sob condições fisiológicas. Isto é evidenciado pela presença desses metabólitos em exsudatos inflamatórios, onde a formação de fibrina aumenta a atividade da cininase I em relação à cininase II (Campbell *et al*. 2000). A endopeptidase neutra e a aminopeptidase plasmática também exercem um papel importante no metabolismo das cininas. A primeira está presente nas células epiteliais e utiliza um mecanismo semelhante ao da cininase II para inativar a BK (Gafford *et al.* 1983; Bhoola *et al*. 1992). Já a aminopeptidase é capaz de converter a Lys-BK em BK, através da clivagem da porção N-terminal (Guimarães *et al*. 1973).

Depois de liberados, a BK e seus metabólitos podem ativar dois subtipos de receptores acoplados a proteína G, denominados B_1 e B_2. A classificação dos receptores cininérgicos foi realizada inicialmente através de estudos farmacológicos no final da década de 70 (Regoli *et al*., 1977; Regoli & Barabé, 1980; Calixto *et al*., 2000; Calixto *et al*., 2001; Campos *et al*., 2006). Posteriormente, a existência dos receptores B_1 e B_2 foi confirmada por estudos de clonagem e de deleção gênica (Calixto *et al*., 2000; Calixto *et al*., 2001; Pesquero & Bader, 2006). Em conjunto, os estudos farmacológicos e de biologia molecular permitiram determinar as principais características dos receptores para as cininas, bem como as diferenças entre os dois subtipos.

Os receptores B_1 são induzidos apenas em condições patológicas, tendo sido associados com a produção de mediadores inflamatórios, a ativação de células do sistema imune e, ainda, com a estimulação de diversas vias de sinalização intracelular e, com a amplificação e manutenção de alterações observadas nas

respostas inflamatórias crônicas (Calixto *et al*., 2004; Hara *et al*., 2008). O gene que codifica para o receptor B_1 apresenta várias sequências específicas para a ligação de fatores de transcrição. Uma série de evidências tem apontado o envolvimento do fator de transcrição NF-κB nos processos de regulação do receptor B_1 (Fernandes *et al*., 2005). O controle da expressão de receptores B_1 possivelmente ocorre em nível pós-transcripcional, através da modulação do RNAm (Zhou *et al*., 1999). Entretanto, esses mecanismos ainda são pouco elucidados, necessitando, assim, da realização de estudos adicionais. Sendo assim, os receptores B_1 representam alvos de grande importância para o desenvolvimento de drogas com potencial anti-inflamatório e podem ser úteis para o tratamento de doenças crônicas como asma, artrite e osteoartrite, neuropatias e doença periodontal, entre outras (Campos *et al*., 2006; Kuduk & Bock, 2008; Dornelles *et al.*, 2009).

Os receptores B_1 parecem representar um grupo especial de proteínas, que não são comumente expressos em condições normais, com exceção do sistema nervoso central, mas são rapidamente induzidos após estímulos como inflamação, infecção ou trauma, e apresentam afinidade pelos metabólitos ativos des-Arg^9-BK e Lys-des-Arg^9-BK. Em contrapartida, os receptores B_2 são expressos constitutivamente na maior parte dos tecidos e apresentam alta afinidade pela BK e pela Lys-BK. Tem sido sugerido que os receptores B_2 seriam responsáveis por mediar as respostas fisiológicas das cininas, mas também participariam da fase aguda dos processos inflamatórios (Calixto *et al*., 2004; Campos *et al.*, 2006).

1.4. Cininas e câncer

Um número crescente de evidências tem sugerido que as cininas e seus receptores parecem estar envolvidos no câncer (Mahabeer & Bhoola, 2000; Stewart,

2003). As conhecidas propriedades mitogênicas das cininas, além de sua capacidade de ativar as cascatas da tirosina e MAPKs (proteínas quinases ativadas por mitógeno) poderiam explicar, pelo menos em parte, os efeitos das cininas no crescimento tumoral e na migração celular (Mahabeer & Bhoola, 2000; Bhoola *et al*, 2001; Stewart, 2003). Além disso, como o crescimento tumoral e as metástases são extremamente dependentes da ativação de vias inflamatórias, é possível concluir que a regulação do receptor B_1 poderia desempenhar um papel importante neste cenário.

Diversos estudos de imunoistoquímica, imunofluorescência, hibridização *in situ* e autoradiografia têm apontado para a acentuada distribuição de receptores B_2 em várias linhagens de células tumorais humanas e de rato, ou ainda, em biópsias obtidas de pacientes (Wang *et al*., 2001; Wu *et al*., 2002; Chee *et al*., 2008). De forma interessante, um estudo recente conduzido por Jutras e colaboradores (2010) demonstrou efeitos anti-proliferativos e citotóxicos para o antagonista seletivo dos receptores B_2, BKM-570, sobre diferentes linhagens de células de tumor ovariano. Ademais, foi demonstrado que a BK, agindo através dos receptores B_2, leva ao aumento dos processos de migração e invasão de células de glioma em direção aos vasos sanguíneos (Montana & Sontheimer, 2011).

Com relação aos receptores B_1, há alguns dados controversos na literatura. Assim, alguns estudos realizados com células de osteosarcoma humano, MG63, e com células de sarcoma de camundongos 180, descartaram um possível envolvimento dos receptores B_1 no câncer (Wang *et al*., 2001;. Ishihara *et al*., 2001; 2002). A imunorreatividade do receptor B_1 foi significativamente aumentada em astrócitos e células endoteliais, bem como, nos vasos sanguíneos e em biópsias de astrocitomas humanos, tumores de pulmão e de mesotelioma pleural (Raidoo *et al*.,

1999; Chee *et al.*, 2007; 2008). A relevância dos receptores B_1 também foi demonstrada por um estudo funcional, utilizando células de câncer de mama, indicando que agonistas seletivos destes receptores são capazes de induzir proliferação celular, através da interação com o receptor do fator de crescimento epidermal, EGFR (Molina *et al.*, 2009). Além disso, a ativação dos receptores B_1 é capaz de induzir a proliferação de células de glioma, por meio da estimulação da ciclo-oxigenase-2 e da via PI3K/AKT (Lu *et al.*, 2010). Também foi demonstrado que a ativação dos receptores B_1 leva a um aumento da liberação de metaloproteinases em células de câncer de mama, responsivas ou não ao estrogênio (Ehrenfeld *et al.*, 2011a).

Barki-Harrington e colaboradores (2003) relataram que a ativação de receptores B_1 e B_2 parece estar relacionada com a sinalização mitogênica em células de câncer de próstata insensíveis a andrógeno, PC3. Esta evidência foi reforçada por outros dados demonstrando que os receptores B_1 estão presentes em neoplasias intra-epiteliais de próstata e nas lesões malignas, mas não em tumores benignos da próstata (Taub *et al.*, 2003). Um aumento da expressão dos receptores B_1 e B_2 também foi descrita em mastócitos e células gigantes de carcinoma de esôfago de células escamosas (Greco *et al.*, 2005). Finalmente, outros estudos têm apontado um papel crucial para o sistema cininérgico e seus receptores nos processos angiogênicos em diversos tipos tumorais, como, por exemplo, no câncer de mama e de próstata (Wright *et al.*, 2008).

Portanto, pode-se sugerir que antagonistas dos receptores B_1 e B_2 poderiam ser utilizados como adjuvantes na terapia de alguns tipos de câncer. Os mecanismos de sinalização celular subjacentes e o papel exato dos receptores para

cininas no câncer continuam a ser investigados e constituem uma área promissora e crescente de pesquisas.

2. OBJETIVOS

2.1. Objetivo Geral

Caracterizar a expressão e o papel funcional dos receptores para cininas em células de tumor de bexiga humano das linhagens RT4 e T24, bem como em biópsias tumorais, a fim de avaliar o possível envolvimento deste receptor na progressão dos tumores de bexiga.

2.2. Objetivos Específicos

a. Caracterizar as propriedades funcionais dos receptores cininérgicos, B_1 e B_2, em linhagens celulares de tumor de bexiga RT4 e T24, através de estudos *in vitro* de contagem celular e viabilidade, utilizando agonistas e antagonistas seletivos para os receptores de cininas.

b. Realizar estudos de expressão *in vitro* em linhagens celulares de tumor de bexiga RT4 e T24, por meio da técnica de reação em cadeia da polimerase precedida de transcrição reversa (RT-PCR), em tempo real, a fim de avaliar a presença dos receptores B_1 e B_2.

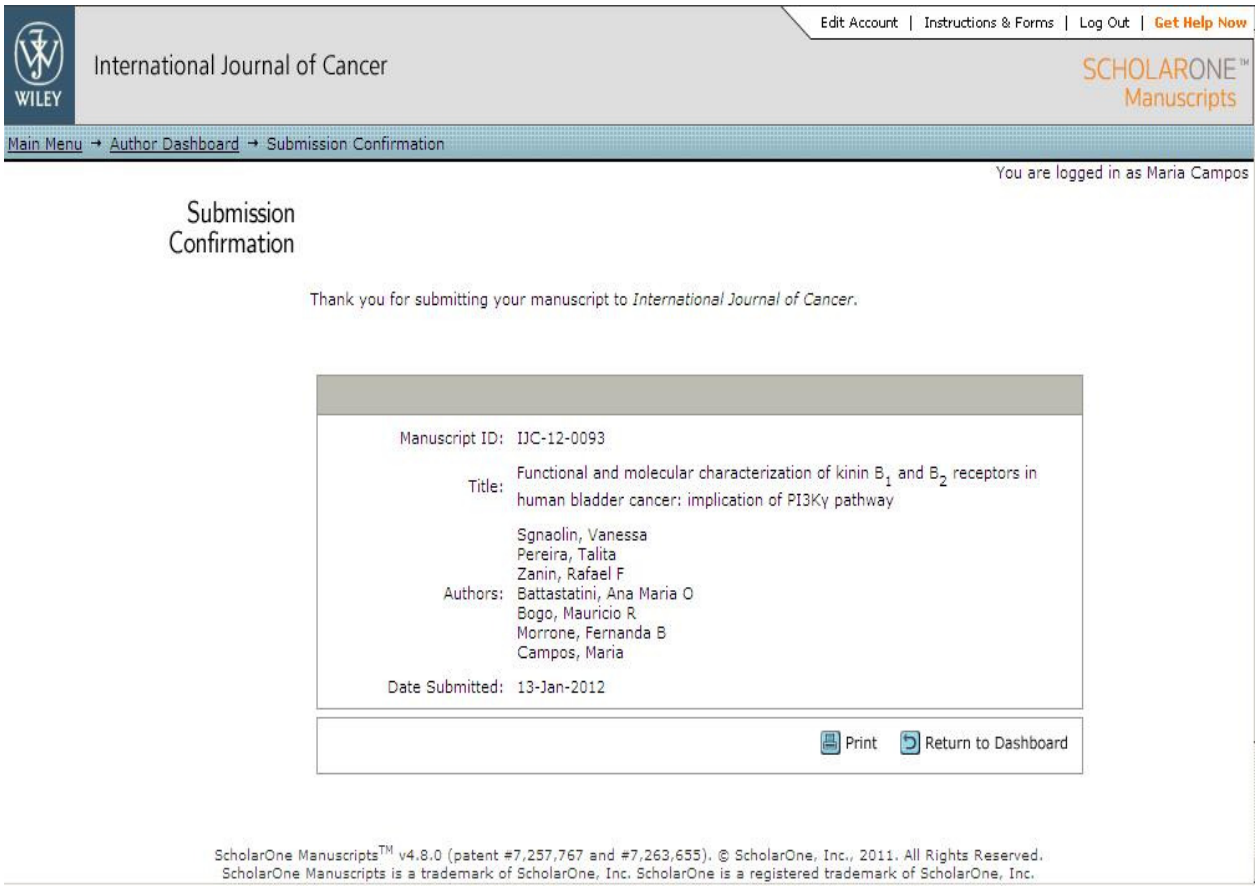
c. Avaliar a expressão dos receptores B_1 e B_2 para as cininas, em biópsias de tumor de bexiga, por meio de PCR em tempo real, a fim de verificar a presença dos receptores B_1 e B_2, em comparação com tecidos normais.

d. Avaliar a relevância da isoforma γ da PI3K nos efeitos proliferativos causados pelos agonistas cininérgicos na linhagem tumoral T24.

e. Verificar o perfil de ativação das MAP quinases, p38 e ERK 1/2 e da quisase relacionada à PI3K, AKT, após a incubação de agonistas cininérgicos.

3. ARTIGO CIENTÍFICO

Os resultados do presente trabalho foram submetidos ao periódico *International Journal of Cancer*. Fator de Impacto (ISI Web of Knowledge, 2010): 4,722.



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Functional and molecular characterization of kinin B_1 and B_2 receptors in human bladder cancer: implication of PI3Kγ pathway

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Running title: Kinin receptors and bladder cancer

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Abstract

This study was designed to characterize, by means of functional and molecular approaches, the relevance of kinin B_1 and B_2 receptors in bladder cancer. Our data clearly shows that both B_1 des-Arg^9-BK and B_2 BK receptor agonists were able to stimulate the proliferation of the grade 3-derived bladder cancer T24 cells. Furthermore, the incubation of B_1 and B_2 receptor antagonists, SSR240612 and HOE140, respectively, markedly inhibited the proliferation rate of T24 cells. In contrast, only higher concentrations of BK elicited the proliferation of the grade 1 bladder cancer cell line RT4, while des-Arg^9-BK incubation completely failed to induce its proliferation. Interestingly, real time PCR experiments revealed that mRNA expression of B_2, and mainly B_1 receptors was found superior in T24 cells, in comparison to the low malignity grade RT4 cells. Furthermore, data obtained using bladder cancer human biopsies revealed that B_1 receptor expression was visibly increased in all tumoral samples or under chronic inflammation of bladder. Concerning the signaling pathways related to the mitogenic effects of kinins, we bring novel evidence showing that pharmacological inhibition of PI3Kγ with AS252424, concentration-dependently reduced T24 cell proliferation induced by BK or des-Arg^9-BK. Finally, the incubation of T24 cells with kinin agonists led to a marked activation of PI3K/AKT and ERK 1/2 signaling pathways, whereas p38 MAP kinase remained unaffected. Our results indicate that kinin B_2, and especially B_1 receptors appear to be implicated in bladder cancer progression. It is tempting to suggest that selective kinin antagonists might represent potential therapeutic alternatives for bladder cancer control.

Introduction

Approximately 70,530 new cases of bladder cancer and 14,680 deaths from this tumor are estimated in the United States in 2010. Among men, this is the fourth most common cancer type and it is the ninth leading cause of death from cancer. The ratio of men to women that develop bladder cancer is approximately 3:1.[1] Of newly diagnosed bladder cancer cases, about 70% to 80% will present with nonmuscle-invasive disease, and despite endoscopic and intravesical treatments, 50 to 70% will recur and 10 to 30% will progress to muscle-invasive disease.[2,3] Most recurrences occur within 5 years,[4] and higher grade lesions are at a greater risk for tumor progression.[5]

Bladder tumors are predominantly presented as transitional cell (urothelial) carcinomas (TCCs). TCCs are often a mixture of heterogeneous cell populations with diverse morphological and clinical manifestations.[6] The three major risks for patients after initial treatment include tumor recurrence, progression to a higher grade or stage, and metastasis. Conventional clinical and pathologic parameters are widely used to grade and stage tumors and to predict clinical outcome of TCCs. Nevertheless, the predictive ability of these parameters is limited, and there is a lack of indices that could allow prospective assessment of risk for individual patients.[7]

Bladder cancer has several known risk factors, although many cases arise with no apparent exposure to carcinogens.[8] Age is a risk factor for developing this cancer type, which occurs more commonly in the elderly. The median ages of men and women presenting with bladder cancer are 72 and 74 years, respectively,[9] and cigarette smoking is the strongest risk factor.[10] Although the specific carcinogens in cigarette smoke responsible for the increased risk of bladder cancer are unknown, aromatic amines are thought to be the inciting factor.[11]

Kinins are a group of peptides involved in a series of pathophysiological processes. They are formed in plasma and tissues in response to infection, tissue trauma or inflammatory alterations.[12] Once formed and released, kinins exert most of their biological effects by the activation of two G-protein coupled receptors, denoted B_1 and B_2 receptors.[13,14] B_2 receptors are distributed in a constitutive manner throughout the central and peripheral tissues, and present higher affinity for bradykinin (BK) and Lys-BK peptides. On the other hand, B_1 receptors have a low expression under normal conditions and display high affinity for the metabolites des-Arg^9-BK and Lys-des-Arg^9-BK.[14,15] It is worth noting that B_1 receptor undergoes rapid up-regulation after inflammatory stimuli, cytokine stimulation or cell injury, all events highly relevant to neoplasia.[15,16]

An increasing body of evidence has emerged indicating that kinins and their receptors appear to be involved in cancer.[16-18] The known mitogenic properties of kinins and their ability to activate tyrosine and MAP (mitogen-activated-protein)-kinase cascades could explain, at least in part, the effects of kinins in tumor growth and migration.[17-19] In addition, cancer growth and metastasis are critically dependent on the activation of inflammatory pathways.[14] For instance, B_1 receptor seems to play an important role in human osteosarcoma cells and in mice bearing sarcoma 180 cells.[20-22] Additionally, increased immunopositivity for B_1 receptor has been detected in astrocytic tumors, as well as in lung and prostate cancer.[23-25] Of note, a series of previous studies has pointed out a marked distribution of B_2 receptor in several human and mouse tumor cells.[20] B_2 receptor was found overexpressed in human gliomas[26] and it was detected in gastric, duodenal, lung, and hepatic cancers.[27]

Following the aforesaid lines of evidence, the present study was designed to investigate the functional role, as well as the profile of expression of kinin receptors in human bladder cancer. We demonstrate, for the first time, that activation of both B_1 and B_2 receptors is likely related to cancer cell proliferation and viability, and the expression of kinin receptors is somewhat related to bladder cancer malignancy grade. We also provide novel data on the possible signaling pathways involved in the mitogenic actions of kinins in this tumor type. It is tempting to suggest that selective kinin receptor antagonists might be potentially useful as adjuvant therapies for bladder cancer.

Materials and Methods

Materials and reagents

RPMI1640, penicillin/streptomycin, trypsin/EDTA solution, and fetal bovine serum (FBS) were purchased from Gibco (Gibco BRL, Grand Island, NY). M-MLV reverse transcriptase (moloney murine leukemia vírus) (Promega, Madison, WI). Trizol LS, Taq-polymerase and oligonucleotides (Invitrogen Life Technologies, Carlsbad, CA). Des-Arg^9-BK, BK and HOE140 (Bachem, USA). SSR240612 (Sanofi Research, France). MTT (Thiazolyl Blue Tetrazolium Bromide) (Sigma, St. Louis, MO). Phosflow Buffer I, Phosflow Perm Buffer II, Alexa 488 anti–phosphor-p38, PE anti–phosphor-AKT and APC anti–phosphor-ERK 1/2 Ab (BD, Franklin Lakes, NJ). AS252424 (Tocris Bioscience, Missouri, USA).

Cell lineages

The human RT4 and T24 tumor bladder cell lines were obtained from American Type Culture Collection (Rockville, MD). RT4 and T24 tumor cells were grown in culture flasks in McCoy's or RPMI1640 culture medium, respectively, and supplemented with 10% (vol/vol) FBS, in the presence of the antibiotics penicillin/streptomycin 0.5 U/mL. Cell cultures were maintained in a 5% CO_2 incubator at 37°C and allowed to grow to confluence. RT4 and T24 are grade 1 and grade 3 bladder cancer-derived cell lines, respectively.[28]

Functional Studies

Cell counting

Human bladder cancer cells were seeded in 24-multiwell plates at densities of $1x10^4$ cells/well in a final volume of 500 µL of culture medium. Cells were blocked in G1 by initially reducing the concentration of FBS to 10.0%, and subsequently to 0.5%, for 24 h. The medium was changed 2 h prior to treatment, after which the cells were treated for 24 h with the selective B_1 or B_2 receptor ligands, as described below. At the end of this period, the medium was removed, cells were washed with calcium-magnesium-free PBS and 200 µL of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted in a hemocytometer.

MTT cell viability assay

This method provides a semi-quantitative measurement of the number of cells with metabolically active mitochondria and is based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay). Cells were plated in a 96-well plate at 2×10^3 cells/ well and treated with the selective B_1 or B_2 receptor ligands, as described in the next section. After 24 h of treatment, 10 µL of MTT (5 mg/mL solution) was added to culture wells and incubated for 3 h. The absorbance was read by an ELISA plate reader at 490 nm. The absorbance was linearly proportional to the number of live cells with active mitochondria.

Functional effects of kinins incubation

In order to functionally characterize kinin receptors in the human bladder cancer cells lines RT4 and T24, the cells were incubated with selective agonists or

antagonists of kinin receptors, alone or in combination, for a period of 24 h. The selective agonists for B_1 and B_2 receptors, des-Arg^9-BK and BK, respectively, were tested at the concentrations of 1, 3, 10, 30 and 100 nM, according to Molina *et al.*[29] The selective kinin B_1 or B_2 receptor antagonists, SSR240612 and HOE140, respectively, were tested at the concentrations of 1, 10, 30 and 100 µM, as previously described by Gougat *et al.* and Andoh *et al.*[30,31] In a separate series of experiments, the cells were treated with the combination of des-Arg^9-BK (10 nM) plus SSR240612 (10 µM), or BK (10 nM) plus HOE140 (10-30 µM).

Effects of selective PI3Kγ inhibition

The γ-isoform of PI3K has been demonstrated as a relevant signaling pathway for different G protein-coupled receptors, especially in the cancer context. Thus, we carried out an additional series of experiments in order to examine the effects of the pharmacological inhibition of PI3Kγ, on the proliferative effects of BK or des-Arg^9-BK in T24 bladder cancer cell line. Therefore, the cells were cultivated and plated as described before, and the proliferative effects of BK or des-Arg^9-BK (both at 10 nM) were assessed in the absence (control) or presence of the selective PI3Kγ blocker AS252424 (1, 3, 10 and 30 µM). The concentrations of this inhibitor were determined on the basis of previous literature data.[32]

Patients

To confirm the relevance of kinin receptors in bladder cancer, we have also assessed their expression in human biopsies. For this purpose, upon approval by the local ethics committee (protocol number 06/02970), specimens of bladder tumors and normal tissues were obtained from the same patients, who have been

pathologically diagnosed and have undergone surgical resection at the Hospital São Lucas/PUCRS. All samples were collected and rapidly frozen in -80ºC with RNA holder. Tissue specimens were ground and then sonicated in a TRIzol kit. The mRNA level was analyzed using RT-PCR analysis, as described below.

Molecular characterization of kinin receptors in human bladder cancer: quantitative PCR analysis

Total RNA from bladder tumors biopsies/control tissues, or T24 and RT4 cell-line cultures was isolated with Trizol LS reagent, in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg total RNA, in accordance with the manufacturer's instructions. Quantitative PCR was performed using SYBR Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA (1:50), containing a final concentration of 0.2 x SYBR Green I (Invitrogen), 100 µM dNTP, 1 x PCR Buffer, 3 mM MgCl2, 0.25 U Platinum Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers: 18S and GAPDH, used as reference genes;[33] B_1 receptor (forward 5′-TTCTTATTCCAGGTGCAAGCAG-3' and reverse 5′-CTTTCCTATGGGATGAAGATAT-3');[34] B_2 receptor (forward 5'-CAGCACCTTCCTGGATACGCTGCATC-3' and reverse 5'-CACCTCCCAAGACTTCTTTCGGAAGC-3') (designed using Oligos 9.6). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 94°C, 40 cycles of 15 s at 94ºC for denaturation, 10 s at 60°C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included

and fluorescence measured from 60 to 99°C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl). Relative RNA expression levels were determined using the 2-ΔΔCT method. The stability of the references genes 18S, β-actin, B2M, GAPDH (M-value) and the optimal number of reference genes according to the pairwise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/).

Flow Cytometry Analysis

To assess the possible signaling pathways implicated in the proliferative effects of kinin agonists in human bladder cancer cells, we performed a separate set of experiments to determine the activation of the MAP kinases ERK 1/2 and p38, or the PI3K-related serine/threonine kinase AKT. For this purpose, the expression of phospho-p38, AKT and ERK 1/2 in T24 cell line was measured by FACScantoII using BD Phosflow Protocol for Adherent Cells. T24 bladder cancer cells were stimulated with BK (10 nM), des-Arg^9-BK (10 nM) or FBS (10 %) for 0, 15 and 30 min in RPMI (supplemented or not with 0.5% FBS). In some cases, the cells were co-treated with the selective PI3Kγ inhibitor AS252424 (30 μM). Briefly, the cells were fixed in Phosflow Buffer I for 10 min at 37ºC. After washing, permeabilization was done with Phosflow Perm Buffer II for 30 min on ice. Then, T24 cells were washed and stained with Alexa 488 anti–phosphor-p38, PE anti–phosphor-AKT and APC anti–phosphor-ERK 1/2 Ab for 30 min at on ice.

Statistical analysis

Results are expressed as the mean ± standard error mean. The percentages of proliferation were determined for each individual experiment. For cell culture protocols, the results are expressed as the mean of four independent experiments, performed in triplicate. The experimental N for the expression experiments with human biopsies is detailed in Table 1. The statistical analysis was performed by one way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. *P* values smaller than 0.05 were considered as significant. All tests were performed using the GraphPad® 5 Software (USA).

Results

Stimulation of B_1 and B_2 receptors induces proliferation of bladder cancer cells

The induction of cell proliferation by BK has been reported in normal and tumor cells.[35,36] The Figure 1 shows the quantification of T24 cell counting, after incubation of the selective B_2 and B_1 receptor agonists and antagonists. The incubation of the selective B_2 BK (Fig. 1A), or B_1 des-Arg^9-BK (Fig. 1B) receptor agonists (1 to 100 nM) induced a marked increase of T24 cell proliferation, without a concentration-dependent profile. Indeed, cell counts showed a maximal increase of 51±4% (p<0.05) in T24 cell number when incubated with BK, and 64±3% (p<0.05) when treated with des-Arg^9-BK for 24 h, both at the 10 nM concentration, in comparison to the positive control FBS 10% (89±5%). As shown in Figure 1C and 1D, the incubation of the selective B_2 HOE 140 or B_1 SSR240612 receptor antagonists (1 to 100 µM) caused a concentration-related inhibition of T24 proliferation activity, which was maximal at 30 µM and 10 µM, respectively. A similar inhibitory effect was observed when HOE140 and SSR240612 were evaluated in the MTT assay (data not shown). In a separate series of experiments, the cells were treated with the combination of BK (10 nM) plus HOE140 (10-30 µM) or des-Arg^9-BK (10 nM) plus SSR240612 (10 µM). The use of selective antagonists was able to reverse the increased proliferation induced by kinin agonists, confirming the selectivity of kinin responses on cell proliferation (Fig. 1E and 1F, respectively).

Of high interest, the incubation of the RT4 cell line with the B_2 receptor agonist BK (1 to 100 nM) induced an increase of 23±4% in cell proliferation, only when stimulated with BK at 10 nM (Fig 2A). Otherwise, the selective B_1 receptor agonist des-Arg^9-BK (1 to 100 nM) completely failed to induce the proliferation of RT4 cells (Fig. 2B). As expected, the positive effects of BK (10 nM) on RT4 cell proliferation

were abolished by HOE140 (30 µM) (Fig. 2C), whereas SSR240612 (10 µM) did not significantly interfere with des-Arg^9-BK responses (Figure 2D).

Expression of kinin B_1 and B_2 receptors in bladder cancer cells

In this experimental set, we compared the mRNA expression of B_2 and B_1 receptors between T24 and RT4 cells, which represent grade 1 and grade 3 bladder cancer-derived cell lines, respectively. Of note, our data show that mRNA expression of B_2 (Fig. 3C and 3D), and mainly B_1 receptor (Fig. 3A and 3B) was significantly higher in T24 cells in comparison to RT4 cells, as assessed by real time PCR. The expression of B_1 receptor was around 7.3-fold superior to that seen in RT4 cell lineage, whereas for B_2 receptor this value corresponded to 5.7-fold, considering both reference constitutive genes 18S (Fig. 3A and C) and GAPDH (Fig. 3B and 3D).

Expression of kinin receptors in human bladder cancer biopsies

To gain further insights on the relevance of kinin receptors in bladder cancer, we have also evaluated the expression of both B_1 and B_2 receptors in human biopsies with different grades of malignancy (Table 1). From these experiments, it is feasible to observe that mRNA expression of B_1 receptor was generally augmented in tumoral in comparison to the normal tissues, independent on the cancer staging. Interestingly, the expression of B_1 receptor was markedly increased in a sample corresponding to chronic bladder inflammation. Otherwise, regarding the B_2 receptor, the pattern of expression was variable, depending on the sample analyzed, presenting no distinction between normal and tumoral tissues, or being slightly elevated (Table 1).

Participation of PI3Kγ in human bladder cancer cell proliferation induced by kinins

The enzyme PI3Kγ may be activated by a variety of growth factors or chemoattractants.[37] To examine the role of PI3Kγ in kinin-elicited bladder cancer proliferation, we evaluated the effects of the selective inhibitor of this isoform, named AS252424 (1 to 30 μM). As demonstrated before, the incubation of either BK or des-Arg^9-BK (10 nM) led to a marked proliferation of T24 human bladder cancer cells. Of note, the pre-treatment of cultures with AS252424 resulted in a significant and concentration-related decrease of cell proliferation induced by BK (Fig. 4B) or des-Arg^9-BK (Fig. 4C), as well as of that one caused by FBS 10% (Fig. 4A).

PI3K/AKT and ERK 1/2 are likely involved in kinin-induced bladder cancer cell proliferation

Next, we assessed the activation of distinct signaling pathways in response to FBS 10%, BK or des-Arg^9-BK incubation, by using flow cytometry. Stimulation of cells with BK and des-Arg^9-BK (both at 10 nM) induced an enhancement of AKT phosphorylation, which peaked at 15 min (Fig. 5A). Interestingly, the stimulation of T24 cells with BK and des-Arg^9-BK (10 nM) led to a striking increase of ERK 1/2 phosphorylation that peaked at 30 min (Fig. 5B). In both cases, the kinin effects were similar to that observed for FBS 10%. Noteworthy, the incubation of AS252424 (30 μM) largely prevented ERK 1/2 phosphorylation (data not shown). Lastly, both BK and des-Arg^9-BK incubation failed to induce any significant change of MAP kinase p38 phosphorylation, but a slight augmentation was seen with FBS 10% (Fig. 5C).

Discussion

Literature data clearly point out that kinin production is found increased in many kinds of cancer, and the expression of both B_1 and B_2 receptors is upregulated in cancer tissues from different origins.[16, 18, 38] Nevertheless, the importance of kinin receptors in various cancer types has not been fully explored, and the precise role of this group of peptides in development and promotion of cancer remains to be further investigated. On this account, the present work evaluated, for the first time, whether kinin B_1 and B_2 receptors might be relevant in bladder cancer.

To reach our main purposes, we have used T24 and RT4 lineages, two known bladder cancer cell lines with different grades of malignancy, to functionally characterize B_1 and B_2 receptors, and to investigate the possible downstream events that are connected to kinin receptors. Interestingly, our proliferation data on grade 3 T24 cells revealed that nanomolar concentrations of des-Arg^9-BK and BK produced a marked increase in cell counting, when compared to control cells. A similar outline of cell proliferation was observed in a previous work conducted by Molina *et al*., demonstrating that selective B_1 Lys-des-Arg^9-BK, or B_2 BK receptor agonists induced a marked proliferation of MCF-7 breast cancer cells at a nanomolar range.[29]

In order to extend the functional characterization of kinin receptors in bladder cancer cells, we have also assessed the effects of the selective B_1 and B_2 receptor antagonists, namely SSR240612 and HOE140, on the proliferation of T24 cells. Either antagonist was able to reduce in a concentration-dependent manner the proliferation of 10 % FBS-incubated T24 cells. Furthermore, both antagonists also reduced the cell viability, according to assessment in the MTT assay. Finally, the selectivity of kinin responses was demonstrated by the experiments using the combination of BK plus HOE 140, or des-Arg^9-BK plus SSR240612. This assembly

of results on bladder cancer cells confirms previous evidence showing that selective kinin antagonists, such as HOE140, display remarkable anticancer activities, both *in vitro* and *in vivo*.[18,39] Of note, the incubation of the potent B_2 receptor antagonist BKM-570 resulted in a marked inhibition the growth of epithelial ovarian cancer cells.[40] Likewise, Taub *et al*.[24] demonstrated that the peptide B_1 receptor antagonist des-Arg^9-[Leu^8]-BK was able to attenuate *in vitro* cell growth in androgen-insensitive prostate cancer PC3 cells. To our knowledge, we bring the first experimental evidence indicating that the non-peptide selective B_1 receptor antagonist SSR240612 displays anti-proliferative actions on cancer cells. It remains to be determined whether this antagonist might be active in animal models of cancer, when dosed orally.

We have also verified the effects of kinin receptor stimulation in the grade 1 bladder tumor-derived cell line RT4. Interestingly, BK incubation induced the proliferation of RT4 cells only when tested at the 10-nM concentration, whilst des-Arg^9-BK completely failed to induce cell proliferation in either tested concentration. The comparison of data on T24 and RT4 cells points out a relevant role of B_2, and mainly B_1 receptors on malignity grade and bladder cancer progression. Results presented by us are also reliable on the pattern of expression of the inducible kinin B_1 receptor. On this regard, we have decided to investigate the mRNA expression of kinin receptors in either cell lineages. Both kinin B_1 and B_2 receptors mRNAs were found expressed in T24 and RT4 human bladder cancer cells. However, extending functional data, the expression of B_2, and more intensely of B_1 receptors, was higher in the grade 3-derived cell line T24, in comparison to the grade 1 cell lineage RT4. Accordingly, it was previously demonstrated that low concentrations of BK induce an elevation of intracellular calcium levels in rat C6 glioma cells, whereas astrocytes

responded only to higher concentrations. Furthermore, the expression of B_2 receptors was found increased in C6 glioma cells, when compared with primary astrocytes or brain endothelial cells.[41]

Either functional or molecular data obtained in this study with T24 and RT4 bladder cancer cell lineages allow us to suggest that B_1 and B_2 receptors are likely implicated in bladder cancer malignance grade. Thus, to accomplish further insights on this hypothesis, we decided to perform real time PCR experiments using biopsies of human bladder cancer, categorized into distinct levels of progression, to determine the possible differences in kinin receptors expression. Remarkably, our data showed that B_1 receptor expression was found generally increased in most tumoral tissues, in relation to control samples, independent on the tumor classification. Similarly, the expression of B_1 receptors was also strikingly augmented in the transitional mucosa obtained from a patient with chronic bladder inflammation. In contrast, the expression of B_2 receptors was not changed, or it was even reduced in tumoral tissues. Worthily, a study conducted by Zelawski *et al*.[42] demonstrated a higher expression of B_1 receptors in biopsies of tubular adenomas, whereas an elevated expression of B_2 receptors has been found in hyperplastic polyps. Concerning the genitourinary system, an increased expression of both B_1 and B_2 receptors was observed in the parenchyma of renal carcinoma.[43] When allied to literature data, the experimental evidence presented herein is consistent with the inducible profile of B_1 receptors under stressful situations, such as cancer. Furthermore, the increased expression of B_1 receptors under bladder inflammation permits us to infer that this receptor subtype might be also implicated in the mechanisms of bladder tumor initiation. Concerning the B_2 receptors, we believe that they also display an important role in bladder cancer progression, but this is reliant

on the series of kinins produced in the tumor microenvironment. Nevertheless, it is tempting to propose that selective B_1, rather than B_2 receptor antagonists, might represent useful alternatives for the adjuvant treatment of bladder cancer.

It is well known that signal coupling of both B_1 and B_2 kinin receptors might lead to the activation of protein kinase C, MAP-kinase and tyrosine kinase pathways, coordinated with the stimulation of transcriptional factor NF-κB.[16] These receptors also stimulate phosphatidylinositol hydrolysis (PIP) leading to mobilization of intracellular Ca^{2+} levels, phospholipase C and phospholipase A_2.[14,36,44] In this study, we have made an effort to determine some of the transduction pathways linked to the mitogenic effects of kinins in bladder cancer cells. We provide novel and interesting evidence showing that proliferation of T24 cells induced by either BK or des-Arg^9-BK was markedly reduced, in a concentration-dependent manner, by the incubation of the selective inhibitor of the γ isoform of PI3K AS252424. Thus, we might suggest that PI3K/AKT pathway activation is probably related to kinin-induced bladder cancer cell proliferation. This proposition is reinforced by flow cytometry data indicating that stimulation of either B_1 or B_2 receptors by des-Arg^9-BK or BK led to a rapid and marked phosphorylation of AKT, as early as 15 min after incubation of agonists. The PI3K pathway plays a pivotal role in cell growth, proliferation and survival,[45] and signaling via this pathway is upregulated in many types of cancer.[46,47] The isoform PI3Kγ belongs to class IB, which is linked to the activation of G-protein-coupled receptors,[32] and it has been related to tumorigenesis mechanisms in neuroblastoma.[48] Moreover, recent studies have identified multiple molecular mechanisms by which PI3K pathway is activated in urothelial carcinoma.[49]

Previous results showed that BK led to increased activation of ERK 1/2 pathway in tumoral cells.[50] Furthermore, B_1 receptor-induced breast cancer cell

proliferation can be attenuated by selective PI3K or ERK 1/2 inhibitors.[29,36] To extend our knowledge on the intracellular signal pathways related to the positive effects of kinins on bladder cancer cell proliferation, we have also evaluated whether the incubation of BK or des-Arg^9-BK might lead to the activation of MAP-kinases ERK 1/2 or p38, by using T24 cells. Of note, our data show that both BK and des-Arg^9-BK induced a marked increase of ERK 1/2 phosphorylation, which was maximal at 30 min. On the other hand, either kinin agonists failed to significantly affect MAP-kinase p38 activation. In addition, we have also demonstrated that incubation of AS252424 was able to prevent the activation of ERK 1/2 MAP-kinase (data not shown). These results strongly suggest that proliferating effects of BK and des-Arg^9-BK depends on the activity of PI3Kγ, and subsequent ERK 1/2 phosphorylation.

In summary, this is the first study describing the expression and the functional role of B_1 and B_2 receptors in bladder cancer growth, and that B_1 receptor expression might well represent a marker of tumoral progression. Our results showed that the enhancement of T24 cell proliferation induced by BK and des-Arg^9-BK was mediated through an increase of PI3K/AKT and ERK 1/2 phosphorylation, confirming the importance of these intracellular signaling steps for cell proliferation. It is tempting to suggest that selective kinin antagonists might represent potential adjuvant therapeutic alternatives for bladder cancer control.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Expression of the B_1 and B_2 receptors in human bladder cancer tissues.

Case	Diagnosis	Age	Histological grade*	Fold-increase in receptor mRNA expression	
				B_1	B_2
1	Transitional mucosa with edema and acute and chronic inflammation	92	Absence of malignancy	5,1	1,8
2	Transitional cell carcinoma grade II of WHO, with invasion of the lamina propria	78	pT1	2,8	1,5
3	Transitional cell carcinoma grade II of WHO, with invasion of the lamina propria	76	pT1	1,1	0,8
4	Transitional cell carcinoma grade III of WHO, with invasion of the lamina propria	61	pT1	3,1	1,4
5	Transitional cell carcinoma grade II of WHO, with invasion of the lamina propria	71	pT1	1,6	1,5
6	Transitional cell carcinoma grade II of WHO, with invasion of the lamina propria	53	pT1	2,0	1,0
7	Transitional cell carcinoma grade II of WHO, with invasion of smooth muscle of the lamina propria	67	pT2	1,2	0,7

*Tumor classification based on World Health Organization (WHO).

Figure legends

Figure 1. Effects of kinin B_1 and B_2 receptor agonists and antagonists on the proliferation of T24 bladder cancer cells. The cells were incubated with selective agonists or antagonists of kinin receptors, for a period of 24 h. The selective agonists for B_2 and B_1 receptors, BK and des-Arg^9-BK, respectively, were tested at the concentrations of 1, 3, 10, 30 and 100 nM **(A and B)**. The selective kinin B_2 or B_1 receptor antagonists, HOE140 and SSR240612, respectively, were tested at the concentrations of 1, 10, 30 and 100 µM **(C and D)**. The cells were treated with the combination of BK (10 nM) plus HOE140 (10-30 µM) or des-Arg^9-BK (10 nM) plus SSR240612 (10 µM) **(E and F)**. Results are expressed as percentage of control cells. Graph bars represent the mean±SEM for four independent experiments. *p <0.05 **p<0.01 *versus* control. ANOVA followed by the post hoc test of Bonferroni.

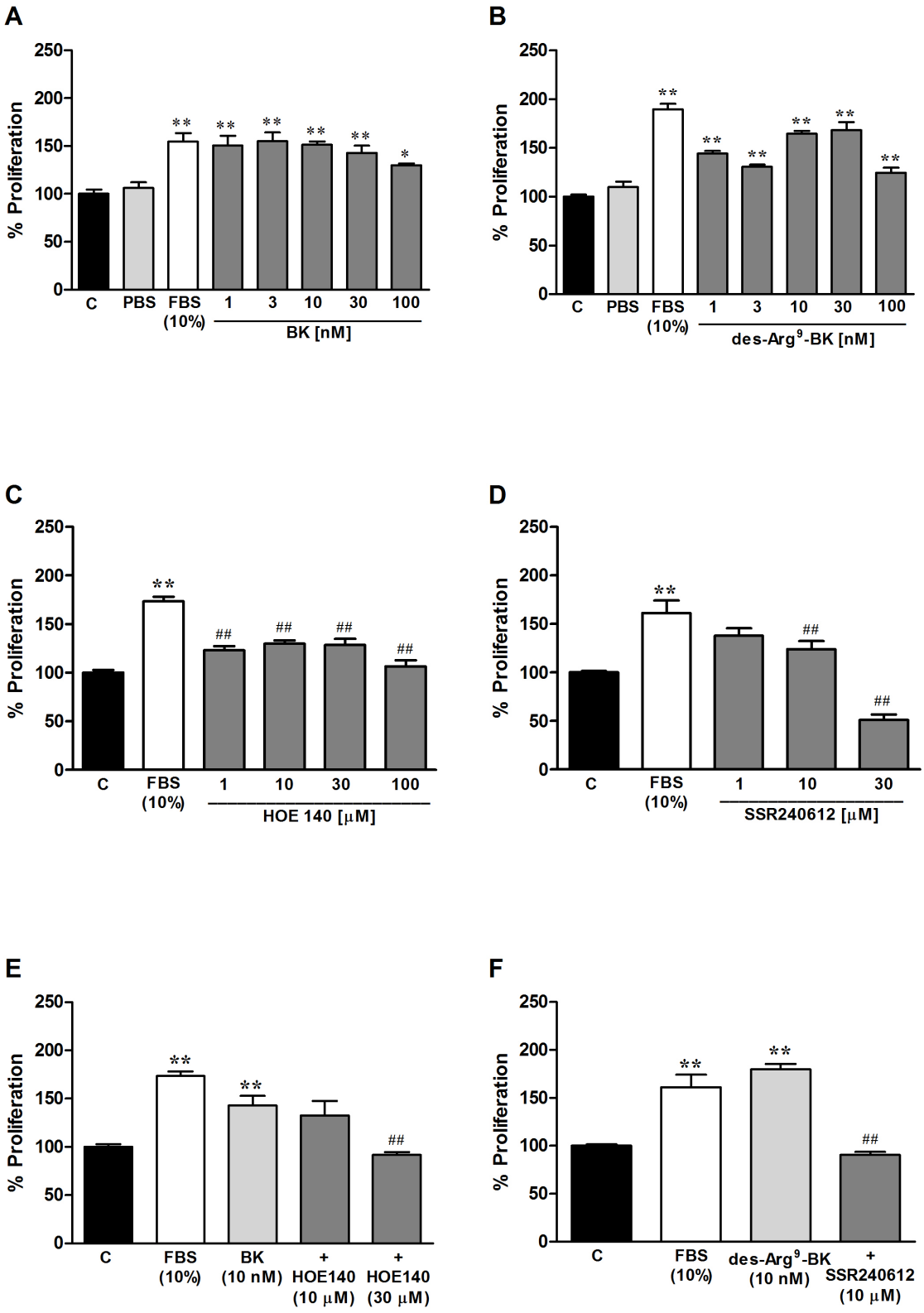
Figure 2. Effects of kinin B_1 and B_2 receptor agonists and antagonists on the proliferation of RT4 bladder cancer cells. The cells were incubated with selective agonists or antagonists of kinin receptors, for a period of 24 h. The selective agonists for B_2 and B_1 receptors, BK and des-Arg^9-BK, respectively, were tested at the concentrations of 1, 10 and 100 nM **(A and B)**. The selective kinin B_2 or B_1 receptor antagonists, HOE140 and SSR240612, respectively, the cells were treated with the combination of BK (10 nM) plus HOE140 (30 µM) or des-Arg^9-BK (10 nM) plus SSR240612 (10 µM) **(C and D)**. Results are expressed as percentage of control cells. Graph bars represent the mean±SEM of four independent experiments. *p <0.05 **p<0.01 *versus* control. ANOVA followed by the post hoc test of Bonferroni.

Figure 3. Expression of kinin B_2 and B_1 receptors in RT4 and T24 human bladder cancer cells lines. The expression level B_2 and B_1 receptors mRNA was determined by PCR real time and was normalized to that of GAPDH and 18S mRNA. Graph bars represent the mean±SEM of four independent experiments. *p <0.05 **p<0.01 RT4 *versus* T24 (t test).

Figure 4. Effects of incubation with the selective PI3Kγ inhibitor AS252424 (1, 3, 10, 30 µM; for 15 min), on the proliferation of T24 cells induced by FBS (10%) **(A)**, BK (10 nM) **(B)** and des-Arg^9-BK (10 nM) **(C)**. Results are expressed as percentage of control cells. Graph bars represent the mean±SEM of four independent experiments. *p <0.05 **p<0.01 *versus* control. ANOVA followed by the post hoc test of Bonferroni.

Figure 5. Effects of stimulation with FBS (10%), BK (10 nM) and des-Arg^9-BK (10 nM) (at 0, 15 and 30 min) on the phosphorylation of AKT **(A)**, ERK 1/2 **(B)** and p38 **(C)**. Graph bars represent the mean±SEM of four independent experiments. Representative images of the experiments are presented in the right column: FBS 10 % (a, d and g); BK 10 nM (b, e and h); des-Arg^9-BK 10 nM (c, f and i). *p <0.05 **p<0.01 *versus* control. ANOVA followed by the post hoc test of Dunnett's Test.

Figure 1.


A
% Proliferation
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0
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*
C
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FBS (10%)
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des-Arg9-BK [nM]
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##
C
FBS (10%)
BK (10 nM)
+ HOE140 (10 μM)
+ HOE140 (30 μM)
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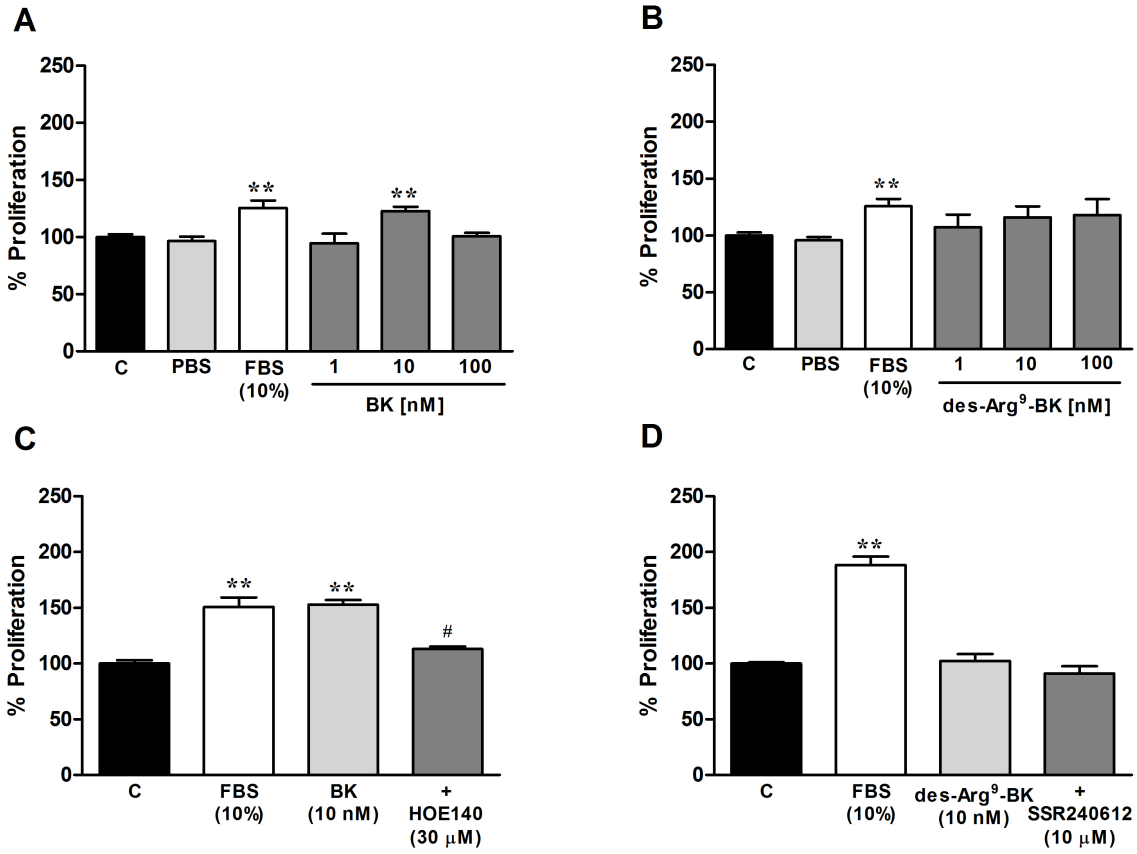
C
FBS (10%)
des-Arg9-BK (10 nM)
+ SSR240612 (10 μM)

Figure 2.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des-Arg9-BK [nM]
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C
FBS (10%)
BK (10 nM)
+ HOE140 (30 μM)
D
% Proliferation
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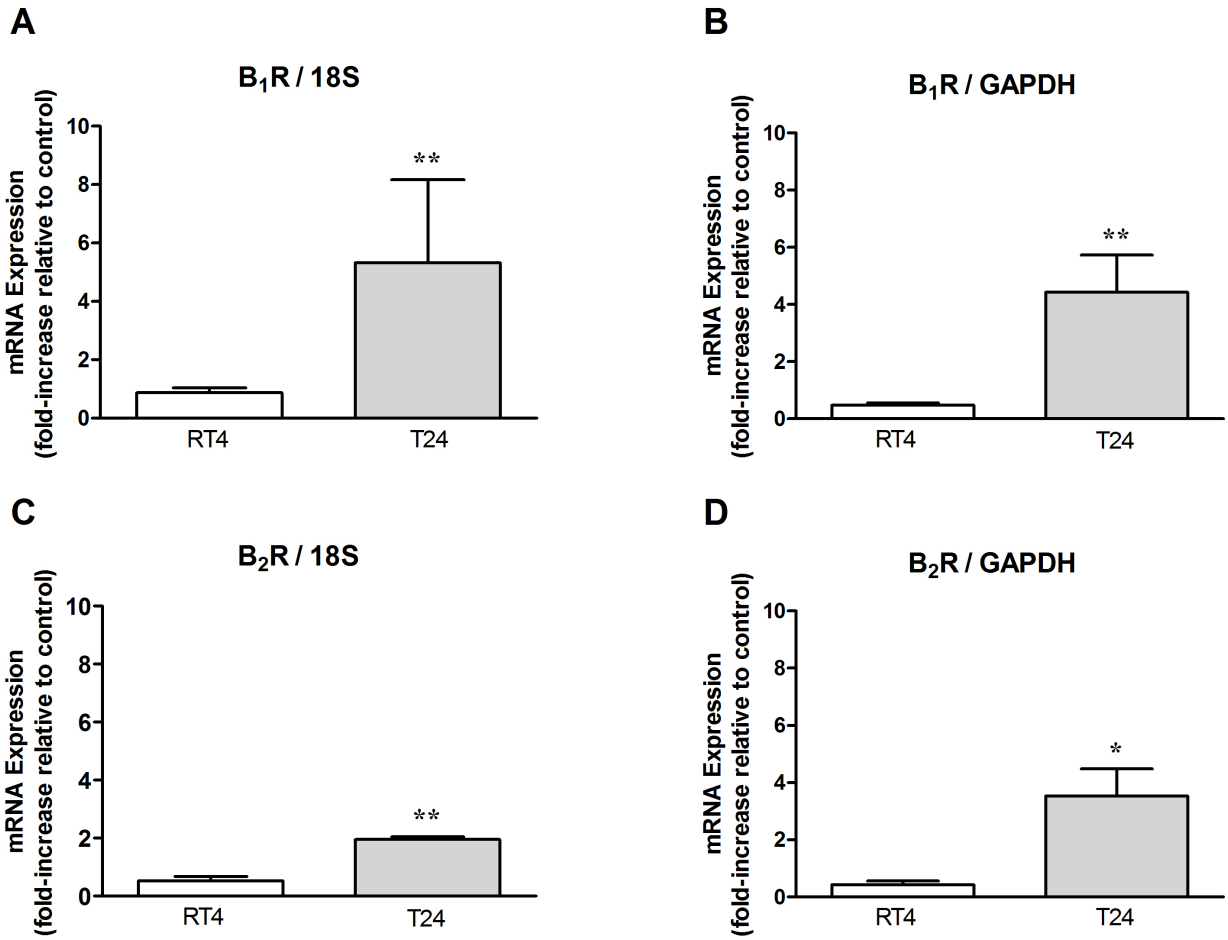
C
FBS (10%)
des-Arg9-BK (10 nM)
+ SSR240612 (10 μM)

Figure 3.


A
B1R / 18S
mRNA Expression
(fold-increase relative to control)
10
8
6
4
2
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**
RT4
T24
B
B1R / GAPDH
mRNA Expression
(fold-increase relative to control)
10
8
6
4
2
0
**
RT4
T24
C
B2R / 18S
mRNA Expression
(fold-increase relative to control)
10
8
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4
2
0
**
RT4
T24
D
B2R / GAPDH
mRNA Expression
(fold-increase relative to control)
10
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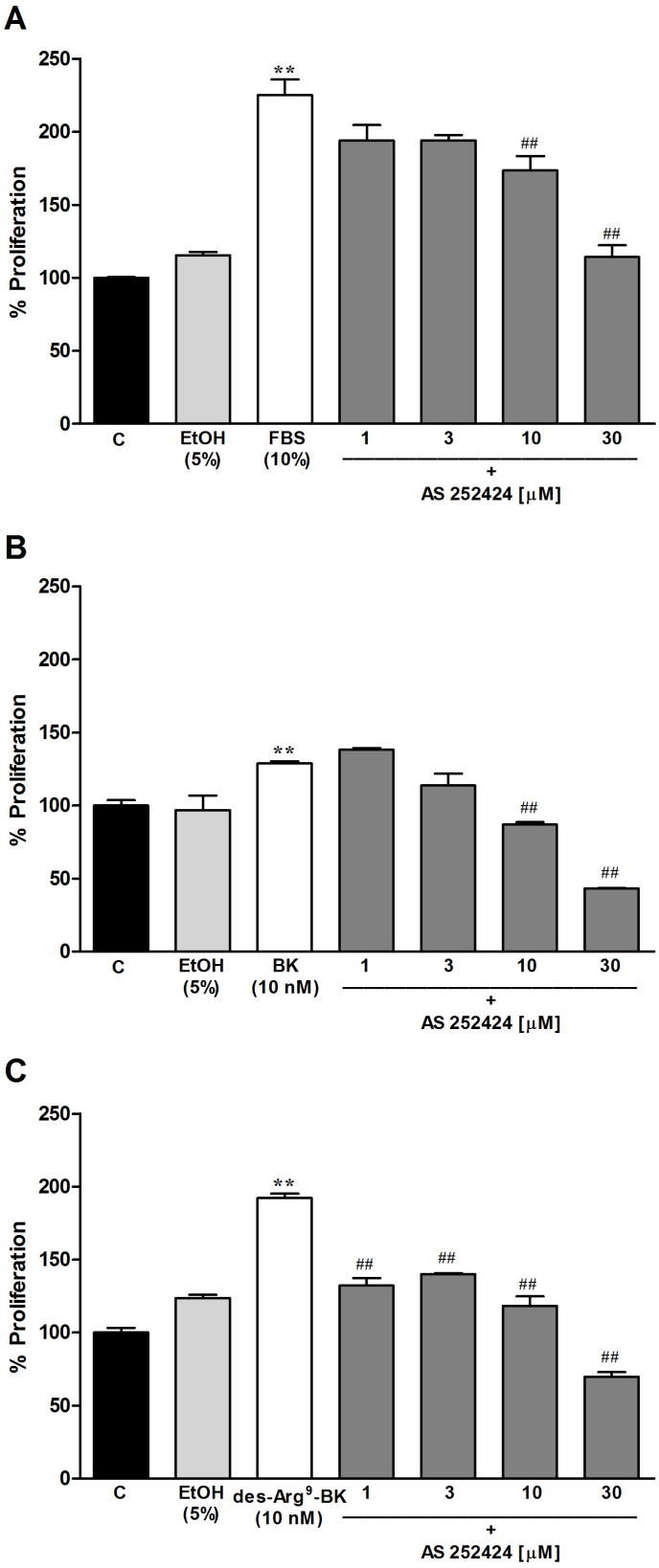
*
RT4
T24

Figure 4.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##
C
EtOH (5%)
FBS (10%)
1
3
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+
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B
% Proliferation
250
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##
C
EtOH (5%)
BK (10 nM)
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AS 252424 [μM]
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% Proliferation
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C
EtOH (5%)
des-Arg9-BK (10 nM)
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3
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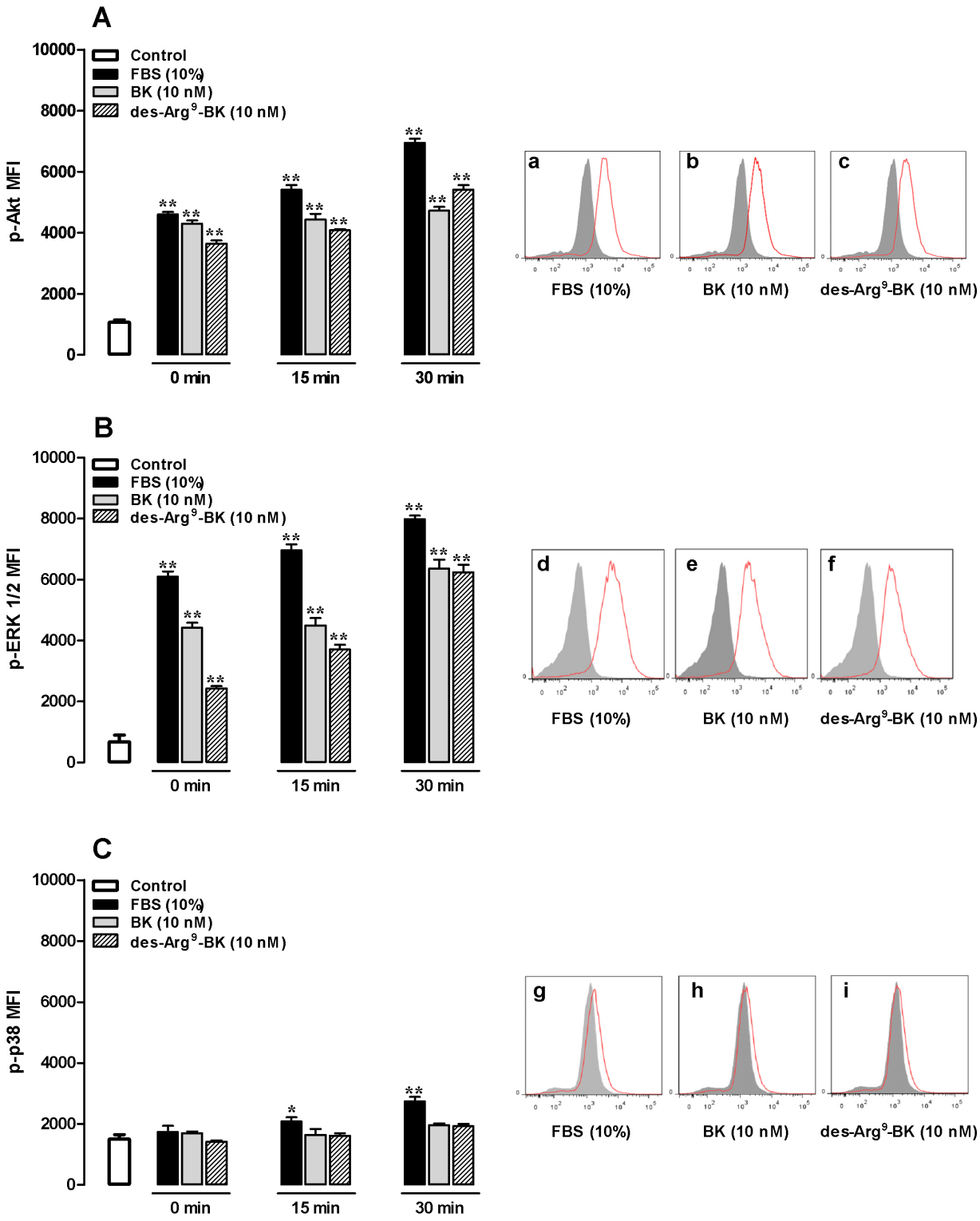
30
+
AS 252424 [μM]

Figure 5.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p-Akt MFI
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8000
6000
4000
2000
0
Control
FBS (10%)
BK (10 nM)
des-Arg9-BK (10 nM)
0 min
15 min
30 min
a
b
c
FBS (10%)
BK (10 nM)
des-Arg9-BK (10 nM)
B
p-ERK 1/2 MFI
d
e
f
C
p-p38 MFI
g
h
i

4. CONSIDERAÇÕES FINAIS

As cininas são mediadores peptídicos biologicamente ativos, envolvidos em uma série de processos fisiopatológicos (Campos *et al*., 2006). Além de seu papel fisiológico, estes peptídeos participam da resposta inflamatória, promovendo vasodilatação, aumento da permeabilidade vascular, extravasamento plasmático e migração celular (Schulze-Thopoff *et al*., 2008). Uma vez formadas e liberadas, as cininas podem ativar dois subtipos de receptores acoplados à proteína G, denominados B_1 e B_2. Os receptores do tipo B_1 não são comumente expressos em condições normais, com exceção do sistema nervoso central, mas são rapidamente induzidos após estímulos como inflamação, infecção ou trauma, e apresentam afinidade pelos metabólitos ativos des-Arg^9-BK e Lys-des-Arg^9-BK. Em contrapartida, os receptores B_2 são expressos constitutivamente na maior parte dos tecidos e apresentam alta afinidade pela BK e pela Lys-BK, sendo responsáveis por mediar as respostas fisiológicas das cininas e, também por participar da fase aguda dos processos inflamatórios (Calixto *et al*., 2004; Campos *et al*., 2006).

Dados da literatura apontam que a produção de cininas está aumentada em diversos tipos de câncer e a expressão de ambos os receptores é regulada nesses tecidos (Stewart, 2003; Bhoola *et al*, 2007; Ehrenfeld *et al*. , 2011b). No entanto, a importância dos receptores de cininas no câncer ainda não foi totalmente explorada. Dessa forma, o presente trabalho avaliou, pela primeira vez, o papel dos receptores B_1 e B_2 para cininas no câncer de bexiga.

Os resultados obtidos demonstraram que a estimulação dos receptores para cininas, através da incubação de agonistas seletivos, induziu a proliferação celular em linhagens de câncer de bexiga. Quando comparados diferentes tipos celulares, observou-se que na linhagem T24 a proliferação celular ocorreu diante da

estimulação de ambos os receptores e foi mais acentuada. Em contrapartida, na linhagem RT4, a proliferação ocorreu apenas quando o receptor B_2 foi estimulado em maiores concentrações. A fim de confirmar esses resultados funcionais, foi realizada a incubação das células T24 com antagonistas dos receptores B_1 e B_2, SSR240612 e HOE140, respectivamente, os quais inibiram acentuadamente sua proliferação. De forma inovadora, o presente trabalho apresentou pela primeira vez a atividade anti-proliferativa do antagonista seletivo SSR240612 sobre as células cancerosas. Complementando os dados, a avaliação através do ensaio MTT também demonstrou a redução da viabilidade celular com o uso de ambos os antagonistas. Dessa forma, os resultados reforçam o efeito inibitório dos antagonistas na proliferação e confirmam o envolvimento dos receptores B_1 e B_2 na progressão do câncer de bexiga.

O presente trabalho também mostrou que a expressão dos receptores B_2, e principalmente B_1 está aumentada nas células T24, que apresenta um perfil mais invasivo, em comparação às RT, de padrão menos agressivo. Da mesma forma, quando avaliadas biópsias de pacientes com diagnóstico de câncer de bexiga foi evidenciado um aumento visível da expressão dos receptores B_1. Assim, estes dados apontam para uma provável associação entre o grau de malignidade tumoral e a expressão dos receptores para cininas, confirmando a presença de receptores B_1 apenas nos tumores de maior grau de malignidade, ou seja, em ambientes mais nocivos.

Aprofundando os estudos funcionais e moleculares, foram analisadas algumas vias de sinalização intracelular, que poderiam estar relacionadas com os efeitos mitogênicos das cininas. Esta análise demonstrou que a inibição farmacológica da PI3Kγ com AS252424 reduziu de maneira concentração-

dependente a proliferação das células T24, quando estimuladas pela BK ou des-Arg^9-BK. Além disso, dados de citometria de fluxo evidenciaram que o uso de agonistas cininérgicos ocasionou uma acentuada ativação das vias de sinalização PI3K/AKT e ERK 1/2, enquanto a via p38 MAP quinase permaneceu inalterada. Sendo assim, podemos sugerir que a ativação da via PI3K/AKT está provavelmente relacionada à proliferação celular induzida pelas cininas no câncer de bexiga. Ademais, resultados não apresentados revelam que a inibição seletiva da PI3Kγ impede a ativação da ERK 1/2, demonstrando a dependência de ativação entre as vias.

Com base nos dados aqui apresentados é possível inferir que: (I) os receptores B_1 são induzidos em situações de estresse como no câncer; (II) a estimulação dos receptores B_1 atua na progressão do câncer de bexiga; (III) os receptores B_2, também apresentam um papel importante na progressão do câncer de bexiga, mas isso depende da produção de cininas no microambiente tumoral; (IV) a expressão dos receptores B_1 pode estar associada ao grau de malignidade tumoral; (V) as vias PI3K/AKT e ERK 1/2 estão relacionadas com proliferação celular induzida pelas cininas; (VI) os antagonistas seletivos de receptores de cininas podem representar potenciais alternativas terapêuticas para o controle do câncer de bexiga.

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