UNIVERSIDADE ESTADUAL DE CAMPINAS

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"REGULAÇÃO DA EXPRESSÃO E LOCALIZAÇÃO DO RECEPTOR DE ANDRÓGENO EM CÉLULAS MUSCULARES LISAS PROSTÁTICAS *IN VITRO*"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientador: Prof. Dr. Hernandes Faustino de Carvalho

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RESUMO

O crescimento e função prostática dependem da estimulação androgênica e interação epitélio-estroma. Além dos andrógenos, outros fatores interagem com a próstata e são igualmente importantes para sua fisiologia. Sabe-se que os estrógenos exercem um importante papel no desenvolvimento prostático e que, combinados com andrógenos, podem contribuir para o aparecimento de patologias. A insulina é outro hormônio que afeta a atividade destes hormônios sexuais nos tecidos, inclusive na próstata. Os níveis séricos de esteróides sexuais estão intimamente relacionados com a sensibilidade a insulina, embora esta relação ainda seja pouco esclarecida. No estroma prostático, as células musculares lisas são o tipo celular predominante, influenciando a atividade do epitélio por mecanismos parácrinos e modificando a matriz extracelular em situações de remodelação, como no crescimento, na regressão e na invasão tumoral. Sabe-se que estas células apresentam receptores de andrógeno (AR) e que respondem à privação androgênica, alterando sua morfologia. O presente estudo buscou verificar a influência da testosterona, estradiol e insulina sobre a expressão e localização do AR em células musculares lisas da próstata ventral de ratos Wistar cultivadas in vitro. Os resultados mostraram que o estradiol causou alterações nos níveis protéicos, os níveis de RNAm do AR foram pouco afetados e a localização do AR foi predominantemente nuclear independente da dose de estradiol. Os tratamentos feitos com insulina mostraram que a sua presença causou uma queda da expressão da proteína e uma localização predominante nuclear do AR na situação em que os dois hormônios, insulina e testosterona, foram administrados juntamente. Os resultados permitem sugerir que a expressão do AR pode ser modulada por outros fatores como estrógeno e insulina.

ABSTRACT

The prostate function and growth depends on the androgenic stimulation and epithelium-stroma interaction. Besides androgens, other factors interact with the prostate and are also important for its physiology. It is known that estrogens exert an important role in prostate development, and combined with androgens they can contribute for the appearance of pathologies. The insulin is another hormone that interacts with these sexual hormones in tissues, and also in the prostate. The serum levels of sexual steroids are closely related with the sensitivity to the insulin, even though this relation is not yet clear. In prostatic stroma, smooth muscle cells are the predominant cell type. They influence the activity of the epithelium through paracrine mechanisms and modify the extracellular matrix in remodeling situations, such as gland growth and regression, and during tumor invasion. It is known that these cells express androgen receptor (AR) and respond to androgen deprivation by modifying its phenotype. The present study was undertaken to verify the influence of testosterone, estradiol and insulin on the expression and localization of the AR in the smooth muscle cells from the Wistar rat ventral prostate cultured *in vitro*. The results showed that estradiol caused alterations in the AR protein levels, the mRNA level was less affected and AR localization was predominantly nuclear irrespective of the estradiol dose. Insulin treatments caused a decrease in the expression of the protein and a predominant nuclear localization of AR in the presence of testosterone. The results suggest that AR expression and regulation might be modulated by others factors such as estrogen and insulin.

1. INTRODUÇÃO

A próstata é uma glândula exócrina do aparelho reprodutor masculino que contribui com uma fração importante do líquido seminal e com a capacitação e sobrevivência dos espermatozóides, fornecendo íons, lipídios estruturais de membrana, substâncias imunossupressoras e antiinflamatórias. A próstata é composta por um conjunto de estruturas epiteliais túbulos-alveolares, nas quais as estruturas epiteliais encontram-se envolvidas por um denso estroma fibromuscular (Hayward & Cunha, 2000, Aümuller *et al*.,1979).

Em roedores, a próstata é uma glândula multilobulada localizada em torno da uretra postero-inferior a bexiga. Na próstata ventral de rato, cada lobo prostático consiste de oito conjuntos de dutos que se originam a partir da uretra como uma estrutura tubular simples que se ramifica distalmente. Esse conjunto de dutos é dividido em três regiões morfológica e funcionalmente distintas denominadas distal, intermediária e proximal, de acordo com sua posição em relação à uretra (Lee *et a*l., 1990; Shabsigh *et al*., 1999). Cada lobo tem características histológicas distintas e também expressam diferentes grupos de proteínas secretoras (Cunha *et al*., 2004, Hayashi e*t al*., 1991). Na região distal, são encontradas células epiteliais colunares altas com atividade proliferativa, circundada por células musculares lisas que formam uma camada esparsa e descontínua, associada a um grande número de fibroblastos (Nemeth & Lee, 1996). Na região intermediária, as células epiteliais também são colunares altas, apresentando características de células secretoras, sem atividade proliferativa. Nesta região, a camada de células musculares lisas é fina e contínua. Na região proximal, as células epiteliais são cúbicas e baixas, sendo freqüentes células apoptóticas e as células musculares lisas formam uma camada espessa. Tanto na região intermediária como na proximal, os componentes fibrosos estão presentes no espaço entre os dutos e, ocasionalmente, intercalando a camada de células musculares lisas.

Em contraste com a próstata de roedores **(Fig.1)**, a próstata humana é uma glândula compacta sem lobos distintos (Cunha 2004), embora esteja subdividida em três zonas: central,

periférica e transicional (McNeal, 1980, 1983). Observações comparativas do desenvolvimento prostáticos de roedores e humanos demonstram que a morfogênese prostática ocorre de maneira análoga em ambos.

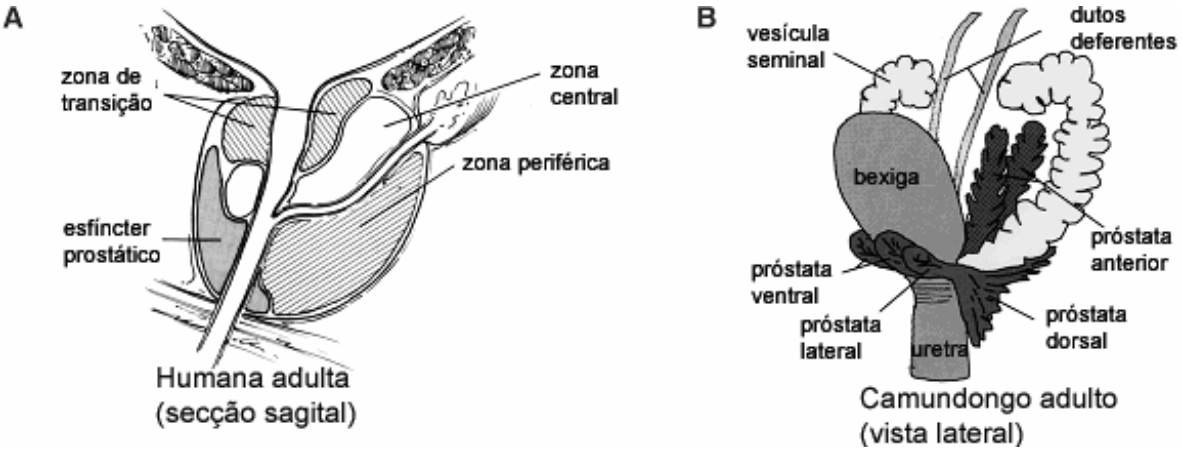

Figura 1. Ilustração esquemática da anatomia da próstata humana (A) e de camundongo (B) (adaptados de McNeal, 1969 e de Cunha *et al*. 1987, respectivamente).

A próstata do rato adulto é composta por células epiteliais luminais, basais e neuroendócrinas, células musculares lisas, fibroblastos, macrófagos e células endoteliais. Cada tipo de célula desempenha um papel importante e específico na função secretora e viabilidade do tecido (Prins *et al*., 1991). O desenvolvimento prostático ocorre como resultado de interações recíprocas mesenquimal-epitelial, em que o mesênquima induz a diferenciação epitelial e o epitélio induz diferenciação mesenquimal (Hayward & Cunha, 2000; Cunha *et al*, 2004). A interação epitélio-estroma desempenha um papel fundamental na regulação e manutenção da atividade funcional da próstata (Lee, 1996).

Os principais tipos de célula encontrados no estroma são as células musculares lisas (CML) e fibroblastos. As células musculares lisas representam 22% da área total da próstata humana (Shapiro *et al*, 1992), predominando ao redor dos ductos, onde se encontram em íntimo contato com a lâmina basal das células epiteliais. Já na próstata ventral de ratos, as CML ocupam 5% do volume total da glândula e cerca de 14% do estroma (Antonioli *et al*., 2004). As CML têm

um papel preponderante no controle da organogênese prostática (Chrisman & Thomson, 2004) e nos mecanismos de estimulação parácrina, especialmente sobre o epitélio (Farnsworth, 1999).

Os andrógenos são essenciais para o desenvolvimento, crescimento, diferenciação e manutenção da morfologia e função secretória da próstata (Davies & Eaton, 1991) e também para manter a funcionalidade da glândula durante a vida adulta (Thomson, 2001). Andrógenos masculinizam o trato reprodutivo durante o estágio ambissexual do desenvolvimento e conduz a formação da próstata. O desenvolvimento urogenital do macho, dependente de andrógeno, é mediado por receptores de andrógeno (AR) (Cunha, 2004) via interações mesenquimal-epitelial, em que o mesênquima induz a morfogênese epitelial, regula a proliferação e evoca a expressão de proteínas secretoras tecido específico (Cunha,1992). Estudos têm mostrado que o AR é primeiro observado nas células mesenquimais, precedendo o aparecimento nos brotos epiteliais prostáticos (Takeda *et al*., 1985). Da mesma forma que a sinalização parácrina do mesênquima regula o desenvolvimento e proliferação epitelial, a sinalização também ocorre em outra direção: do epitélio para o mesênquima. A sinalização epitelial regula a diferenciação do mesênquima em estroma, composto de células musculares lisas e fibroblastos (Hayward et al., 1996), e é possível que andrógenos regulem estes sinais ou interaja com este caminho para controlar a diferenciação (Thomson, 2001). A organogênese prostática culmina no desenvolvimento de uma glândula madura composta de células epiteliais secretoras e células musculares lisas contráteis altamente diferenciadas (Cunha *et al*.,2004).

As células prostáticas respondem a uma variedade de fatores esteróides e não esteróides e produzem uma grande variedade de fatores de crescimento, que combinados criam um ambiente glandular ótimo assegurando o equílibrio (Daves & Eaton, 1991). Multiplas vias de transdução de sinal influenciam na ação esteróide, ou pelo aumento da resposta ao andrógeno ou pela ativação do receptor independente do ligante (Gerdes *et al*., 1998).

Na próstata, fatores de crescimento foram relacionados com o crescimento normal e anormal, e foram sugeridos como possíveis mediadores parácrinos de interações epitelial-estromal (Story, 1991). Estes fatores incluem mitógenos como fator de crescimento de fibroblasto FGF-1, FGF-7 e FGF-10; morfógenos como fator de crescimento de hepatócitos; e fatores de

crescimento transformante e PS-20, que podem impedir a proliferação (Hayward & Cunha, 2000 ; Davies & Eaton, 1991).

A insulina é um fator de crescimento (Lehrer *et al*., 2002) e foi visto ser importante na manutenção de explantes prostáticos e, quando usada juntamente com testosterona, sua ação é potencializada (Lostroh,1971). Webber (1981), baseado em seus estudos com epitélio prostático *in vitro,* sugeriu que a insulina é estritamente necessária para a manutenção da glândula, alterando a permeabilidade celular, aumentando a atividade pinocítica, a captação de aminoácidos e estimulando a síntese de RNA, DNA e proteínas. Estudos epidemiológicos associam altos níveis de insulina no sangue com o aumento do risco de câncer de próstata (Lehrer *et al*., 2002 ; Barnard *et al*., 2002). Tendo em vista que a homeostase prostática depende de uma variedade de fatores esteróides e não esteróides, e o fato de que a próstata humana é alvo de uma série de doenças como hiperplasia prostática benigna e câncer, nos quais a idade e hormônios esteróides são fatores de risco (King *et al*,.2005), o estudo dos mecanismos de ação hormonais e de fatores de crescimento são importantes para um melhor entendimento do funcionamento prostático.

1.1. Eixo hipotálamo-hipófise-testículo e a regulação por hormônios esteróides sexuais

O controle neuroendócrino do metabolismo, do crescimento e de certos aspectos da reprodução é mediado por uma combinação de sistemas neurais e endócrinos centrados no eixo hipotálamo-hipófise. A secreção de gonadotrofinas (hormônios luteinizante – LH e folículo-estimulante – FSH) pela adeno-hipófise é regulada pelo hipotálamo, através do hormônio liberador de gonadotrofinas (GnRH) (Klonoff & Karam, 1995). O LH é uma glicoproteína que estimula a ovogênese nas fêmeas e a espermatogênese nos machos. Neste último, a sua principal ação ocorre através do estímulo da produção de testosterona pelas células de Leydig (Klonoff & Karam, 1995).

Os andrógenos são requeridos para o crescimento normal e atividades funcionais da próstata. No homem, os principais andrógenos circulantes são a 5α-diidrotestosterona (DHT) e a testosterona (T). Esta última é produzida, na sua maior parte (cerca de 95%), pelos testículos. As glândulas adrenais contribuem com menos de 5% da produção dos esteróides sexuais (Coffey,

1992; Cheng *et al.,* 1993; Geller *et al.,* 1984), sendo regulada pelo hormônio adrenocorticotrófico (ACTH).

Estima-se que apenas 2% a 3% da T encontra-se disponível em sua forma livre, sendo que o restante encontra-se ligado a proteínas séricas como a SHBG (*sex hormone-binding globulin*), albumina e globulina de ligação com corticoesteróide (Vermeulen, 1973). Entre as três proteínas, a SHBG possui a maior afinidade pela testosterona.

Ambos os efeitos reprodutivos e anabólicos dos andrógenos são mediados por sua interação com o receptor de andrógeno (AR), que funciona como um fator de transcrição ativado por ligante (Roy *et al*., 1999). A conversão de T em DHT é realizada pela enzima 5α-redutase. Embora os dois hormônios atuem através do mesmo receptor de andrógenos (AR), parece que exercem funções distintas. Assim, a função da 5α-redutase é essencial para a morfogênese prostática, sendo que sua deficiência resulta na quase total inexistência do órgão. Na vida adulta, a concentração tecidual de DHT é mantida elevada, quando comparada com os níveis plasmáticos. Ambos os andrógenos são capazes de manter a atividade prostática, porém a DHT apresenta 1,6 a 1,9 maior potência que a testosterona (Rittmaster *et al*., 1991; Wright *et al*., 1999), amplificando consideravelmente o sinal androgênico e funcionando como um superandrógeno (Roy *et al*.,1999).

Adicionalmente, está bem estabelecido que alguns dos andrógenos circulantes são convertidos a estrógenos em vários tecidos periféricos, através da enzima aromatase (Simpson *et al*., 1999). Interessantemente, a aromatase foi identificada na próstata humana, sugerindo um local de aromatização e uma possível fonte local de estrógeno (Tsugaya *et al*., 1996).

Ainda que a próstata seja um tecido andrógeno dependente, os estrógenos influenciam as funções normais e mudanças patológicas. Este efeito pode ser indireto, inibindo o eixo hipotálamo-hipófise, ou direto, atuando diretamente no órgão. As evidências de uma ação direta dos estrógenos na próstata surgem da observação da existência de receptores para estrógenos (ERα e ERβ) nas células prostáticas estromais e epiteliais, sendo que ERα é encontrado predominantemente no estroma e o ERβ é encontrado predominantemente no epitélio prostático (Makela *et al*.,2000). Um efeito direto do estrógeno, estimulando o crescimento prostático, foi demonstrando em camundongo hipogonadais (*hpg*), que não possuem andrógenos circulantes. O

crescimento induzido foi bem menor que os controles e estava associado a diferentes neoplasias (Bianco *et al*., 2002). Com base na cinética de apoptose das células epiteliais prostáticas frente à castração e ao tratamento com estrógeno, foi sugerido que alta dose de estrógeno possui um efeito inicial direto sobre a próstata, antecipando a apoptose das células epiteliais, quando comparado com a castração (Garcia-Flórez & Carvalho, 2005).

Além disto, há também um mecanismo denominado *imprinting* estrogênico, que se caracteriza pela exposição perinatal ao estrógeno e a manifestação de efeitos na adolescência e na vida adulta. Os efeitos do estrógeno têm ação não normotônica, ou seja, efeitos opostos quando diferentes dosagens são empregadas. Dosagens baixas resultam em estímulo do crescimento, enquanto dosagens mais elevadas resultam em redução do crescimento. Os efeitos do *imprinting* estrogênico são múltiplos e melhores conhecidos através de seus efeitos feminilizantes por ação direta no hipotálamo, além de atuar antecipando a puberdade em ratos (Putz *et al*., 2001b). Altas doses de estrógeno neonatal induzem também uma quase completa eliminação da expressão do receptor de andrógeno, como determinado por imunocitoquímica (Putz *et al*., 2001a). Foram observados ainda rearranjos das junções comunicantes (Habermann *et al*., 2001) e alterações na expressão dos receptores para ácido retinóico (Prins *et al*., 2002). O estrógeno parece exercer suas ações através do receptor de estrógeno do tipo β, presente no estroma prostático. Trabalhos mostram que o estradiol causa uma redução do AR, por destinar o produto proteico ao sistema de degradação por proteassomos (Woodham *et al*., 2003). Foi também observado que a administração de estrógeno leva a uma reorganização dos sítios de *splicing* em vários tecidos, especialmente no útero (George-Tellez *et al*., 2002). Apesar destas evidências, os mecanismos moleculares envolvidos no *imprinting* estrogênico são desconhecidos.

1.2. Célula muscular lisa e a próstata

A célula muscular lisa (CML) é o principal componente celular do estroma prostático, e marca uma importante proporção do estroma do trato urogenital (UGT) sendo derivada do mesênquima por diferenciação (Chrisman & Thomson, 2006) foi sugerido que as CMLs participam do desenvolvimento prostático pela regulação da interação do mesênquima com o epitélio durante o processo de indução prostática (Thomson *et al*., 2002). A porção ventral do

mesênquima do seio urogenital (UGM) é uma área condensada separada do epitélio uretral por uma camada de músculo liso (Thomson *et al*., 2002). Esta camada de músculo liso é menos desenvolvida em machos, permitindo que os brotamentos prostáticos passem através da camada descontínua de musculo liso para entrar no UGM, onde subseqüente morfogênese do crescimento e ramificação ocorrem (Thomson *et al*., 2002). Nas fêmeas, como a camada de musculo liso é contínua, o UGM fica isolado da uretra tornando raro o aparecimento de brotamentos epiteliais prostáticos. Sendo assim, o músculo liso pode agir como um regulador da elongação dutal prostática e morfogênese da ramificação. Andrógenos parcialmente inibem o desenvolvimento da camada muscular lisa peri-uretral, modula a diferenciação e leva ao dimorfismo sexual desta camada muscular lisa (Thomson *et al*., 2002; Cunha *et al*.,2004).

Os andrógenos regulam a diferenciação da CML e, então, controlam a sinalização envolvida na indução prostática. O mecanismo preciso pelo qual os andrógenos regulam a organogênese prostática não é conhecida até o presente. É possível que os andrógenos regulem os sinais de indução prostática ou interajam com estas vias para controlar a diferenciação (Thomson, 2001; Thomson & Cunha,1999).

Na glândula prostática madura, a CML é o tipo celular estromal responsável pelo efeito andrógeno-induzido que possuem receptores de andrógeno ativos (AR), o que suporta que os andrógenos podem afetar diretamente esta células (Prins et al.,1991). Antonioli et al (2004), mostraram que as CML tiveram seu volume total diminuido e fenótipo alterado frente à deprivação androgênica. A mudança fenotípica das CML está ligada a alterações gerais do estroma e, parece, que estas células são responsáveis por algumas das modificações, incluindo a reorganização das fibras de colágeno, com as quais as células musculares lisas estão intimamente associadas após a castração (Vilamaior et al.,2000).

As CML respondem a andrógenos e também a estrógenos (Chrisman & Thomson, 2006), sendo alvos diretos da ação do estrógeno no desenvolvimento de próstatas estrogenizadas (Prins et al., 1998). Experimentos feitos in vitro mostram que o estrógeno interfere na arquitetura das células musculares do UGT levando a um aumento no conteúdo celular assim como nas junções comunicantes das células musculares (Chrisman & Thomson, 2006). Tais alterações são diretamente mediadas pela presença de receptores para estrógeno no estroma. Frente ao

tratamento com testosterona, as células tiveram um comportamento oposto, diminuindo o número de células musculares e inibindo a junção entre células (Chrisman & Thomson, 2004). Estes resultados reafirmam que os andrógenos são responsáveis pelo dimorfismo sexual durante o crescimento do órgão em machos e fêmeas. Haja visto que o tratamento com testosterona leva ao aumento do crescimento e aumento da atividade da próstata em fêmeas (Santos et al.,2006).

Dado o envolvimento das células musculares lisas nas funções prostáticas e sua participação na estruturação do estroma e reorganização frente à castração (Vilamaior et al., 2000) e seu provável papel nas interações epitélio-estroma (Thomson et al., 2002; Cunha et al., 1996), o estudo dos mecanismos de regulação da função destas células parece ser fundamental para um melhor entendimento das funções do estroma em diversos processos relacionados com a modulação do crescimento e da função prostática.

1.3. O receptor de andrógeno e o funcionamento da próstata

O receptor de andrógeno (AR) é um membro da família dos receptores nucleares, funcionando como um fator de transcrição ativado por ligante. O AR é responsável pela mediação dos efeitos fisiológicos dos andrógenos através da sua ligação a seqüencias de DNA específicas, influenciando a transcrição de genes responsivos a andrógenos. Este receptor possui diferentes envolvimentos ao longo da vida contribuindo para o desenvolvimento morfológico de machos, para a gênese da hiperplasia prostática benígna e desenvolvimento do câncer de próstata com a idade (Gelmann, 2002).

O AR clássico tem 110kDa e, como outro membro da família dos receptores nucleares, possui diversos domínios funcionais **(Fig. 2)**. Estes são o domínio de ligação com o DNA (DBD), constituído de dois complexos, cada um composto por quatro resíduos de cisteína e um átomo de zinco. O primeiro dedo de zinco liga-se com o elemento responsivo ao andrógeno (ARE) e o segundo dedo estabiliza a ligação do complexo através de interações hidrofóbicas com o primeiro e contribui para a especificidade da ligação do receptor com o DNA. A região em dobradiça (hinge), que se localiza na C-terminal do domínio DBD, compreende a sequëncia de localização nuclear e é, portanto, responsável pela translocação do receptor do citoplasma para o núcleo assim que este se liga no hormônio. O domínio de ligação com o ligante (LBD) corresponde à

região de ligação com o hormônio esteróide e, juntamente com a região N-terminal, é o local de interação com cofatores transcricionais (coativadores e co-repressores) (Roy *et al*., 1999; Gelman, 2002).

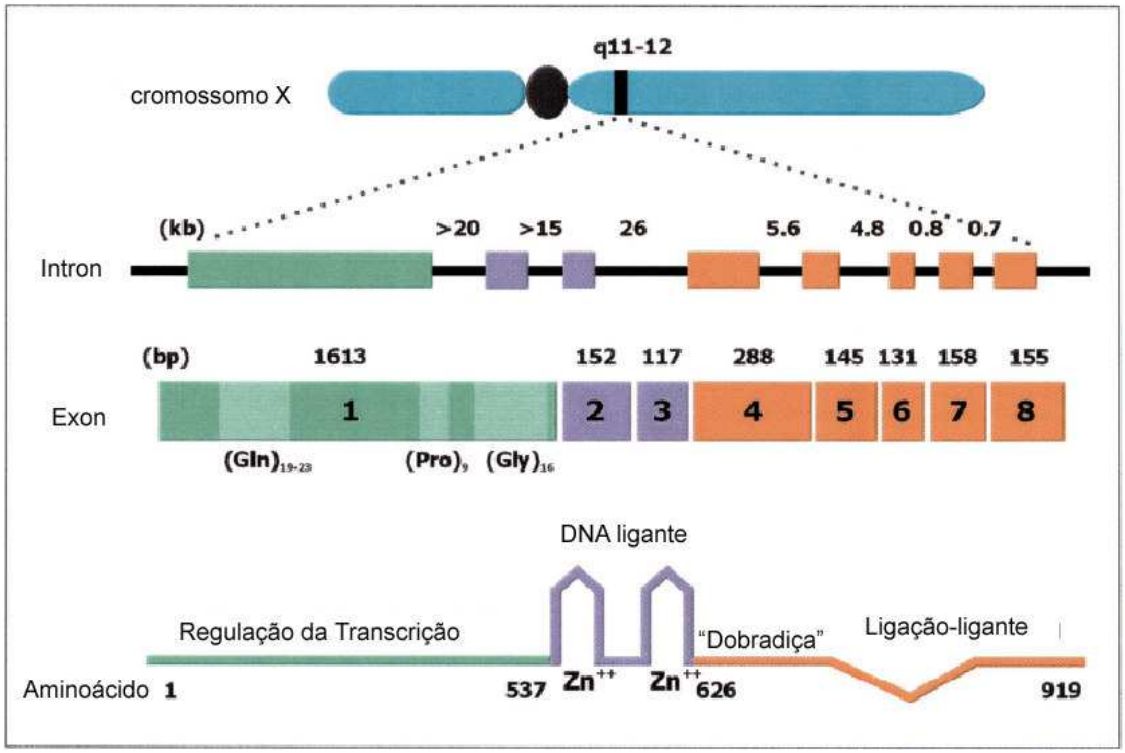

Figura 2. Organização genômica do gene do AR. O diagrama da estrutura da proteína mostra as regiões funcionais do receptor de androgeno (Quigley *et al.*,1995).

O AR sintetizado **(Fig. 3)** fica localizado no citoplasma e, com a ligação da testosterona (T) ou da 5α-diidrotestosterona (DHT), dissocia-se de uma proteína HSP (heat shock protein), dimeriza-se e é tranlocado para o núcleo onde, em conjunto com uma série de coativadores e co-repressores, ativa ou inativa diferentes conjuntos de genes (Jia *et al*., 2003). Hoje são conhecidas ações não genômicas do andrógeno, que envolveriam a ativação de várias vias de sinalização e diferentes efeitos sobre o comportamento celular (Heilein & Chang, 2002). As características principais destas ações são a sua velocidade de ocorrência, antecipando qualquer efeito de ativação e transcrição gênica (Castoria *et al*., 2003).

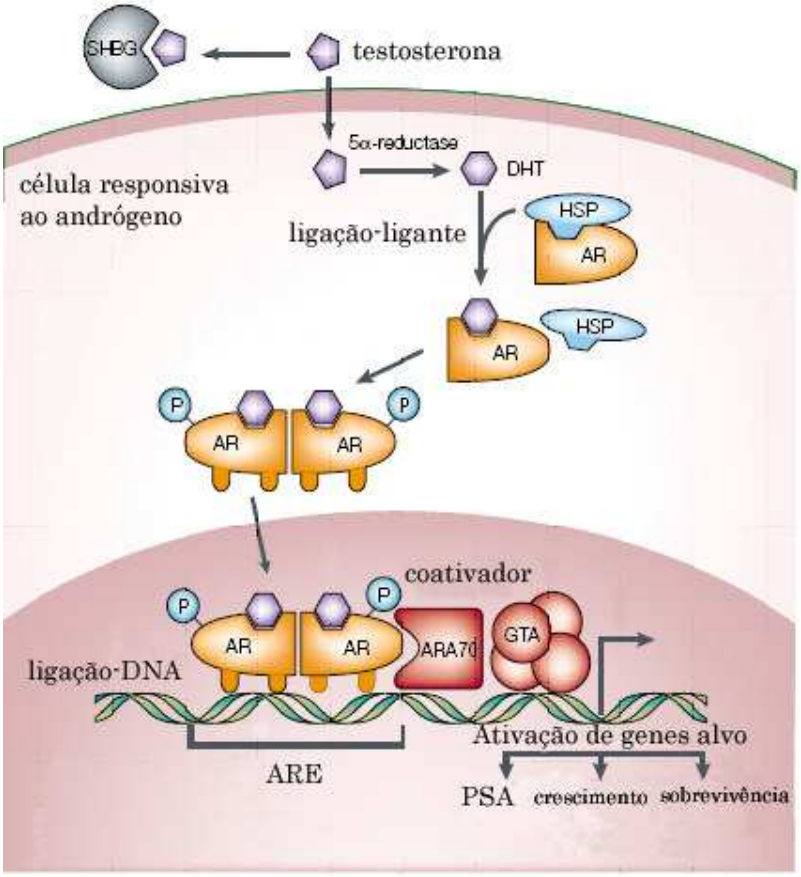

Figura 3. Ação do Andrógeno (adaptado de Feldman & Feldman, 2001).

Na próstata, AR está entre os fatores de transcrição cuja atividade é influenciada pela cascata de transdução de sinal, ou através da fosforilação direta do AR ou através da fosforilação de correguladores do AR e, a falha da interação normal entre a transdução de sinal e a transativação do AR podem contribuir para a progressão do câncer de próstata (Heilen & Chang, 2004).

O AR também pode ser funcional na ausência de andrógenos. Crescimento tumoral pode inicialmente responder bem à terapia de ablação hormonal, causando a regressão do tumor dependente de andrógeno. No entanto, a terapia pode resultar no crescimento do câncer independente de andrógeno. A ativação do receptor de andrógeno pode ocorrer através de vários

mecanismos como aumento da sensibilidade do AR a baixos níveis de andrógeno, ativação do AR por fatores de crescimento ou promiscuidade do receptor (Feldman & Feldman, 2001).

No caso de tumores, a expressão do AR é bastante heterogênea. As células LNCaP são células que expressam um AR com uma mutação T876A (Heilen & Chang, 2004). Como a mutação ocorre no domínio de ligação com o ligante, o receptor pode ser ativado por esteróides não androgênicos. As PC3 e DU145 são outras duas linhagens tumorais que não expressam AR. O padrão de metilação de duas regiões correspondentes a ilhas CpG na região promotora regula a expressão do AR. A ausência de expressão do AR na PC3 e na DU145 pode ser atribuída a um elevado grau de metilação nestas regiões. O mesmo estudo demonstrou que o padrão de metilação do gene do AR é heterogêneo nos tumores prostáticos (Nakayama *et al*.,2002).

Tendo em vista que o AR é o mediador chave da sinalização do andrógeno, responsável pelo desenvolvimento normal e também pelo desenvolvimento de doenças prostáticas, torna-se necessário um estudo detalhado do seu funcionamento assim como sua relação com outros fatores que podem interferir no funcionamento normal do receptor.

2. OBJETIVO

Os receptores de andrógeno são importantes mediadores do efeito androgênico no desenvolvimento e manutenção prostáticos. Sabe-se que parte destes efeitos se dá via células estromais em mecanismos parácrinos e que as células musculares lisas expressam AR. Baseado neste contexto, as ações de outros fatores como o estrógeno e a insulina, além dos andrógenos, conhecidos por interferir na atividade normal e anormal do receptor, foram estudadas a fim de definir aspectos da regulação da expressão da proteína e modificações na distribuição celular do AR, causadas pelo tratamento de células musculares lisas prostáticas com estradiol e insulina.

3. ARTIGOS

Este trabalho foi organizado na forma de dois artigos escritos em língua inglesa.

3.1 Estradiol Affects the Androgen Receptor Levels in Prostatic Smooth Muscle Cells. Sheila C. S. Victório, Hernandes F. Carvalho.

3.2 Insulin Affects AR-Ligand Location and Stability in Prostatic Smooth Muscle Cells. Sheila C. S. Victório, Hernandes F. Carvalho.

3.1. Estradiol Affects the Androgen Receptor Levels in Prostatic Smooth Muscle Cells

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Key words: prostate, smooth muscle cell, estrogen, androgen receptor

Abbreviations: E_2, estradiol; T, testosterone; AR, androgen receptor

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Abstract

Androgen receptors (AR) play crucial roles in prostate physiology and pathology. Estrogens are also important for growth and development of the gland. The aims of this study were to investigate the effects of estradiol (E_2) on AR expression and localization in rat ventral prostate smooth muscle cells (SMC). SMC were cultured in medium supplemented with estradiol (1nM, 10nM, 1μM) with or without testosterone, and AR expression was measured at the protein level by Western blotting and at the mRNA level using RT-PCR. Immunocytochemistry was used to determine AR subcellular distribution. AR expression was affected by E_2 hormonal treatment. The non-monotonic effect of E_2 was observed with the lowest and the highest estrogen concentrations causing marked reduction, while the intermediate concentration did not affect AR protein levels. AR mRNA percentual levels were less affected, suggesting that E_2 effects on AR protein are caused by increased proteolysis, without manifested effect on AR expression or activation. Immunocytochemistry showed that estradiol, irrespective of the dose, did not change the predominantly nuclear localization of AR.

1. Introduction

Androgens are important in the maintenance of both structure and functions of mature prostate (Trapman & Brinkmann, 1996).

The androgens stimulation on prostatic development is mediated via androgen receptors (AR) in the context of mesenchymal-epithelial interactions (Cunha et al., 1992). Initial expression of AR protein occurs exclusively in the mesenchyme. Subsequent expression of AR occurs via stromal induction of basal epithelial cells, concurrent with differentiation of the mesenchyme cells into smooth muscle and mature fibroblasts (Gerdes et al., 1996).

The adult prostatic stroma is composed of AR-expressing smooth muscle cells (100% positive) as well as interacinar fibroblasts cell (~30% positive) (Gerdes et al., 1996; Hayward & Cunha, 2000).

AR is a member of the nuclear receptor superfamily. It regulates the expression of several genes, important for prostatic growth and differentiation (Black & Paschal, 2004). Furthermore, it acts as a transcription factor mediating not only androgen dependent growth, but also the development of cancer cells and importantly the androgen independent growth of prostate tumors (Häag et al., 2005). The newly synthesized AR resides in the cytoplasm associated with a multi-protein chaperone complex. After specific interaction with its ligands, a conformational perturbation takes place, which leads the multiprotein receptor complex to dissociate, exposes phosphorylation sites and the nuclear localization, and allows interactions with transregulatory proteins (Beekman et al., 1993). The ligand-induced conformational changes facilitates the formation of AR homodimer complex that can then recognize and bind to androgen response elements (ARE) in the promoter region of target genes.

Androgens are known to exert their effects through the activation of intracellular receptors that regulate the transcription of target genes. In addition to transcriptional or genomic mode of action by androgens, non-genomic mechanisms have been described, including cross-talk with proteins from signaling pathways such as the mitogen-activated kinase (MAPK), protein kinase A

(PKA) and protein kinase C (PKC) (Häag et al., 2005; Gatson, Kaur & Singh, 2006; Heinlein & Chang, 2002).

Estrogens are also important in both normal and abnormal growth of the prostate. Neonatal exposure to high concentrations of exogenous estrogen leads to hormonal imprinting, compromising prostate growth and response to androgens. On the other hand, low concentrations increase prostate weight (Putz et al., 2001). Zhu et al (2005), studying different cell types, showed that estrogen inhibit or enhance effects of androgens on prostate cells depending of the ER isoform expressed by the cell, as well as dose and ligand type.

In this work we have investigated the AR behavior in cultured prostatic SMC treated with different concentrations of estradiol, both in the presence or absence of testosterone. We have studied the effects of such treatment at the protein and mRNA levels, besides checking receptor localization by immunocytochemistry.

2. Materials and methods

2.1. Cell culture

Primary cultures of Wistar rat ventral prostate smooth muscle cell were established by organ explant cultures. Smooth muscle cells were prepared using a modification of the protocol described by Gerdes et al. (1996). The ventral prostate of young adult Wistar rats (between 60 and 90 days) was minced into 1-2mm^3 fragments and placed in fenol red free DMEM (Nutricell, Campinas, SP, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution) supplemented with 0.5µg/mL testosterone ciprionate (Novaquímica- Sigma Pharma, Hortolândia, SP, Brazil) and 5µg/mL insulin (BioBrás, Belo Horizonte, MG, Brazil) in 24-well culture plates. Medium was replaced every 48h.

After reaching confluence, SMC were replated using trypsin/EDTA (Nutricell) and cultured in 25cm^2 culture flasks at 37°C in a humidified atmosphere with 5% CO_2. All the experiments used SMC cultures at passages 4 and 5.

2.2. Western Blotting Analysis of Androgen Receptor

For steroid hormone effect studies, SMC cells were cultured until 95% confluence. Medium was then replaced for the serum free medium, with insulin and supplemented with the indicated concentration of estrogen with or without testosterone for 48h.. Passed the time, the cells were scraped using Trypsin-EDTA and centrifuged for 10 min at 590g. The cell pellet was diluted in protein buffer extraction (1mM aminohexanoic acid, 5mM benzamidine, 1mM PMSF, 5mM NEM, 10mM EDTA, 600mM Tris and 0.1% Triton X-100). Total protein concentration was measured using Bio-Rad Bradfords protein assay.

Western blotting was performed after electrophoresing 75µg protein of each lysate on 10% polyacrylamide gel under reducing conditions and electric transfer for nitrocellulose membranes (Hybond-ECL, Amersham Biosciences). Membranes were blocked for 1h with 5% non fat dry in TBS-T (Tris buffered saline plus 0.2% Tween 20) at room temperature with agitation. Anti-AR polyclonal antibody (N-20; cat. sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:500 in 1% non fat dry in TBS-T and incubated for 2h at room temperature with agitation. After three TBS-T washes, HRP conjugated goat anti-rabbit IgG (cat. 61-8100; Zymed Laboratories, South San Francisco, CA, USA) were added 1:2500 dilution, in TBS-T for 1h at room temperature with agitation. After another set of washes, detection of bound antigen was achieved by chemiluminescence (Santa Cruz Biotechnology). The same membrane was stripped (100mM β-mercaptoetanol, 2% SDS, 62.5mM Tris-HCl pH 6.7) and after was blocked for 1h with 1% non fat dry in TBS-T at room temperature with agitation. Then, goat polyclonal antibody anti-human actin (cat. I-19; Santa Cruz Biotechnology) diluted 1:1000 in TBS-T containing 0.1% non fat dry milk and incubated for 2h at room temperature with agitation. Following three TBS-T washes, HRP-conjugated rabbit anti-goat IgG (H+L) (cat. 81-1620 Zymed Laboratories) were added after 1:500 dilution in TBS-T for 1h at room temperature with agitation. After another set of washes, detection of bound antigen was achieved by chemiluminescence (Santa Cruz Biotechnology). Band intensity was determined by densitometry using Kodak 1D software (Scientific Imaging System).

2.3. Immunocytochemistry

The SMC were plated on glass coverslips in 24 well plates, and maintained in serum containing medium until semi-confluence. They were changed to serum free medium for 48h, supplemented with the indicated concentration steroid hormones as specified. Coverslips were washed three times in PBS (phosphate buffered saline, pH 7.4) and fixed for 15 min in 4% paraformoldehyde.

The cells were permeabilized by exposure to TBS-T (Tris-buffered saline plus 0.2% Tween 20) three times at room, and blocked in TBS-T with 3% BSA (bovine serum albumin) for 1h. The same anti-AR primary antibody was diluted 1:200 in solution of 1% BSA in TBS-T, and applied to the cells for 2h at room temperature. Coverslips were washed three times with TBS-T and incubated with a fluorescein conjugated goat anti-rabbit IGg (cód. F-0382, Sigma Chemical Co, Saint Louis, Mo, USA) diluted 1:200 in 1% BSA in TBS-T for 1h at room temperature. The cells were then washed in TBS-T and counterstained with propidium iodide (10µg/mL) for 2 min.

Observations were made using a Bio-Rad MRC 1024 UV confocal microscope.

2.4. RNA Extraction and Reverse Transcription - Polymerase Chain Reaction

The SMC were treated with hormones as described. Total RNA extraction was isolated using TRizol reagent (Invitrogen Life Technologies, São Paulo, SP, Brazil). Samples were homogenized in 1 mL and the subsequent procedures were carried out according to manufacturer. The concentration was quantified by spectrophotometry using Ultraspec 2100 pro equipment (Amersham Biosciences, São Paulo, SP, Brazil). For cDNA synthesis, 5µg of total RNA samples was reverse transcribed with 200U SuperScript III (Invitrogen Life Technologies) and Oligo $(dT)_{12\text{-}18}$ Primer (Invitrogen Life Technologies) according to the supplier's instructions. The cDNA was quantified by spectrophotometry as above.

AR and β-actin synthetic oligonucleotide primers were determined and designed using Gene Runner 3.05 (Hastings Software. Inc). AR, forward 5'CGCTTCTACCAGCTCACCAAG3'; reverse 5'TGGGCACTTGCACAGAGATG3'; β-actin forward 5'-CTGGCCTCACTGTCCACCTT-3', reverse 5'-AGTACGATGAGTCCGGCCC-3'. All primers were synthesized by Invitrogen Life Technologies.

For AR, PCR thermal cycling conditions were as follows: an initial step at 94ºC for 5 min; 30 cycles of: 94ºC – 30 sec, 62ºC – 30 sec, 74ºC – 45 sec and a final cycle of 5 min at 74ºC. It was performed in a 13µL final volume containing 150ng cDNA, 0.39U Taq DNA Polymerase (Invitrogen Life Technologies) and 2mM $MgCl_2$. For β-actin, PCR thermal cycling conditions were: an initial step at 94ºC for 5 min; 30 cycles of: 94ºC – 40 sec, 64ºC – 10 sec, 72ºC – 15 sec and a final cycle of 5 min at 72ºC, in a 13µL final volume containing 150ng cDNA, 0.39U Taq DNA Polymerase (Invitrogen Life Technologies) and 2.5mM MgCl2.

PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. Images were captured and analyzed with a Kodak EDAS 290 Camera using the Kodak Digital Science 1D 3.6 software (Eastman Kodak Company).

2.5. Statistical Analysis

Data were expressed as the mean of band intensity for all experiments. Differences between groups were determined by one way analysis of variance (ANOVA). When the differences were determined, paired comparison of mean values was assessed by Tukey's test (MINITAB 14 software). The differences were considered statistically significant when $p \leq 0.05$. Values were expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Inhibition of AR expression by steroid hormones

Figure 1A shows a representative Western blotting for AR. It was apparent that AR content in SMC was much lower than that for LNCaP cells and that with E_2 affected the relative amount of AR in SMC. Quantification of the bands **(Fig. 1C)** showed that the effects of E_2 cause significant variation in AR protein. E_2 treatment in absence of testosterone caused a non-monotonic effect with a bell shaped curve. The effect observed with 1nM E_2 on reducing AR content was reverted at 10nM. The highest concentration employed (1µM) again reduced

significantly AR content. In the presence of testosterone all E_2 concentrations caused marked reduction in AR content.

Statistical analysis was performed using four repeated experiments.

3.2. Hormonal effects of AR mRNA expression

Figure 2A shows that AR mRNA was detected both in the presence and in the absence of testosterone. Estradiol alone increased the expression of AR mRNA and, in the situations which testosterone was included the levels of AR mRNA levels were lower. Band intensity was determined for each experiment and their variation assessed with respect to β-actin **(Fig. 2B)**, which was used as an internal control. **Figure 2C** shows the quantitative results which showed no significant variation between the groups.

3.3. Effect of hormonal treatments on AR expression and distribution

SMC cultures in serum-free medium, in the presence of insulin, supplemented with estradiol (1nM, 10nM, 1μM) and testosterone exhibited a predominantly nuclear localization pattern of AR irrespective of the E_2 dose **(Fig. 3)**. A non-uniformy labeling was identified for the different experimental situations. In addition, some cells showed cytoplasmic aggregation of the AR.

4. Discussion

The androgen receptor (AR) is involved in the normal prostate development and cancer progression, being sensitive to androgen until its independence, which corresponds to mutations or high sensitivity to low androgens levels after hormonal ablation. (Häag et al., 2005; Feldman & Feldman, 2001).

Estrogens are also involved in the growth and development of the prostate and can interfere in the progression of pathologies by inhibiting or promoting androgen action in the

prostate (Zhu et al., 2005; Yeh et al., 1998; King, Nicholson & Assinder, 2005). Experimental work in rodent prostates demonstrated that the treatment with high doses of estrogen caused reduction of prostatic size and alterations in the expression of AR and estrogen receptor (ER), whereas the treatment with low doses caused an increase of the gland size (Putz et al., 2001; Hayward & Cunha, 2000).

In the present work, we have studied the effect in vitro of the estrogen on the expression and localization of AR in smooth muscle cells (SMC) of rat prostates. The results obtained for Western blotting showed that the treatments of the SMC with steroid hormones, estrogen and testosterone caused significant alterations in the protein levels of AR. The presence increasing concentrations of estradiol promoted a decrease in the AR protein level, which was more conspicuous in presence of testosterone.

It is known that androgens are powerful stimulators of the growth and differentiation of the prostate and that they act mainly through androgen receptors. However, functional activity of the steroid hormones receptors could also be affected by other factors, including peptide growth factors and through cross talking with other intracellular signaling pathways such as MAPK (mitogen-activated kinase) (Häag et al., 2005; Heinlein & Chang, 2002; Gnanapragasam et al., 2000). Direct actions of estrogens in the prostate are mediated by its binding to the ER (Hayward & Cunha, 2000), and the modulation of the androgen action by estrogen is certainly via either ERα or Erβ (Zhu et al., 2005). Estrogens interfere in the transcription of genes responsive to androgens and could be involved in the development of the benign prostate hiperplasia and prostate cancer (Fugimoto et al., 2004). It is known that AR action in the activation of genes is modulated by other coativators and co-repressor proteins (Jia et al., 2003; Hong et al., 2005). The coativators recruit histone acetiltransferases and metiltransferases, while co-repressors, desacetilates core histones for the transcription complex resulting in the decondensation or condensation of the chromatin (Jia et al., 2003).

After its translation, AR remains in the cytoplasm associated with a multiple protein complex in a conformation that prevents its nuclear translocation and binding to the DNA (Feldman & Feldman, 2001). After association with the ligand, a conformational change takes place, and the complex is translocated to the nucleus where it will act in the transcription of

several genes. The results obtained by immunocytochemistry demonstrated, as an ordinary characteristic, the predominance of the nuclear localization of the AR in the experiments with estradiol and testosterone, and showed that the estrogen did not intervene in the translocation of the protein to the nucleus. It could be noted the fact that not all cells express AR. AR exerts an important role in the proliferation of the human prostate cancer and confers a great prognostic in the breast cancer. The stability of the AR mRNA is important for the regulation of the expression of the protein in the cells of prostate and breast cancer (Yeap, Wilce & Leedman, 2004). We have observed for RT-PCR that the content of AR mRNA, of the cells that received different treatments, have oscillated, but did not suffer significantly alterations. Considering the alterations in the protein levels it could be suggested that the main effect of the E_2 is in the stimulation of the proteolytic degradation of AR.

Similar effect was demonstrated by Woodham et al (2003) in prostates of neonates, with great direction of AR for the degradation in proteassomo. Moreover, the marked fact is that E_2 showed a non monotonic effect on the AR concentration in the absence of testosterone but not on its presence. As the estradiol, the testosterone was present in the medium and, in consequence, the AR was translocated to the nucleus and a proeminent nuclear localization was visualized.

5. Acknowledgments

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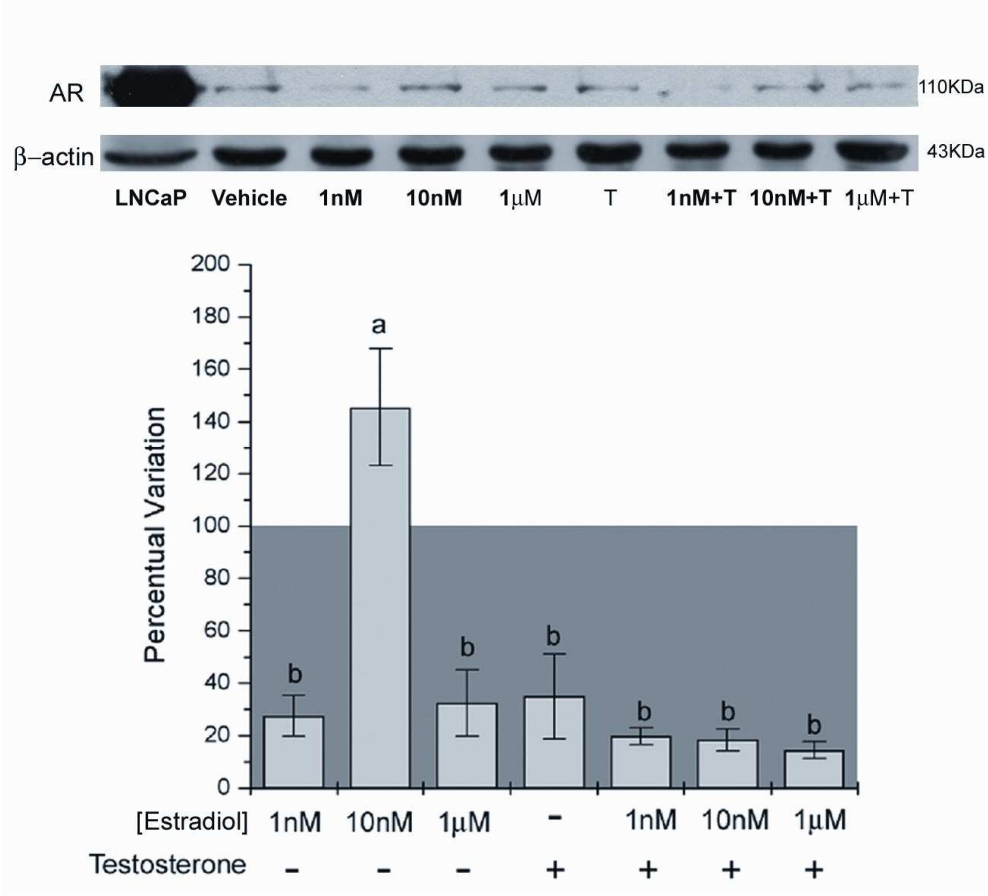
FIGURE LEGENDS

Fig 1. **(A)** Representative Western blotting showing AR. Protein levels of AR in SMC following estrogen treatment with or without testosterone. LNCaP cells were used as reaction control. **(B)** β-actin was used as loading control. Protein levels were evaluated for 48h period of exposure to the different E_2 concentrations. **(C)** Percentual variation of band density AR for SMC treated with medium supplemented with estrogen (1nM, 10nM, 1μM) and testosterone. The vehicle only treated group was used as reference (100%). Data are expressed as means ± standard error of the means. Different letters indicate significant differences ($p \leq 0.05$) as assessed by the Tukey's paired test. All the groups were significantly different of the control group, except the treatment with 10nM E_2.

Fig 2. (A) AR mRNA levels in SMC treated with estrogen and testosterone. **(B)** β-actin was used as an internal control. **(C)** AR mRNA percentual variation as determined by semi-quantitative RT-PCR. The vehicle only treated group was used as reference (100%). No significant variation was observed.

Fig 3. Confocal images showing effects of hormonal treatments with estrogen (1nM, 10nM, 1μM) plus testosterone on AR distribution. **(A)** Treatment only with testosterone (T). All the treatments show the nuclear localization pattern of AR, independent of the dose. (A) The arrow indicate cell with cytoplasmic AR location. (C, D) The arrowheads indicate cells with no labeling for the AR. AR labeling was not uniform. Magnification, 400X.

Figure 1


(A)
AR
110KDa
(B)
β–actin
43KDa
LNCaP
Vehicle
1nM
10nM
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(C)
Percentual Variation
200
180
160
140
120
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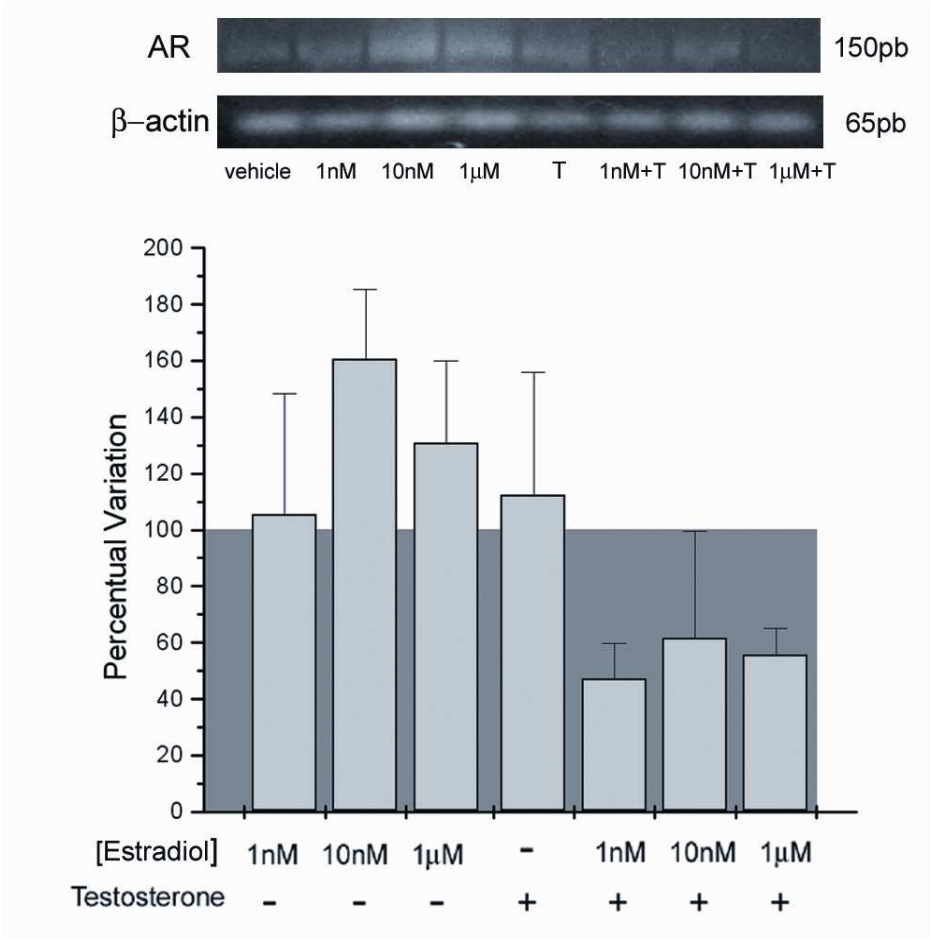
Figure 2


(A)
AR
150pb
(B)
β–actin
65pb
vehicle 1nM 10nM 1μM T 1nM+T 10nM+T 1μM+T
(C)
Percentual Variation
200
180
160
140
120
100
80
60
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20
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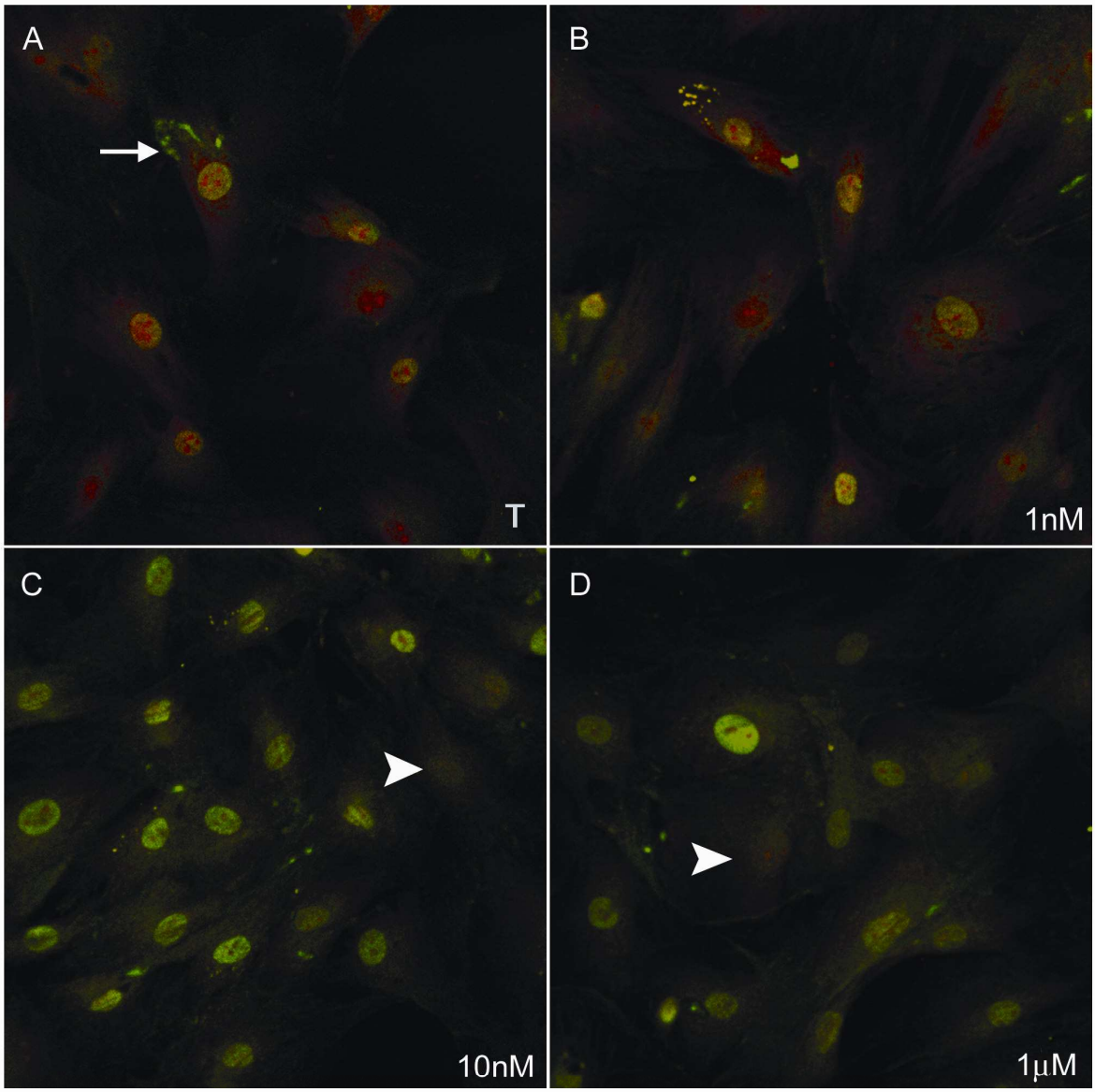
Testosterone - - - + + + +

Figure 3



3.2. Insulin Affects AR-Ligand Location and Stability in Prostatic Smooth Muscle Cells

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Key words: Prostate, Smooth Muscle Cell, Insulin, Androgen Receptor

Abbreviations: I, insulin; T, testosterone; AR, androgen receptor

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Abstract

The androgen receptor (AR) is present in all phases the prostate cancer and is also important in the establishment of benign prostatic hyperplasia (BPH). Besides androgens, which bind to and activate AR, other signal transduction pathways affect AR function. Insulin has also been implicated in prostate cancer development. The aim of this work was to investigate the influence of insulin in AR protein and mRNA levels, and cellular location in prostatic smooth muscle cells (SMC). SMC were culture in medium supplemented with insulin and/or testosterone. AR protein and mRNA were measured by Western bloting and semi-quantitative RT-PCR, respectively. AR localization was determined by immunocytochemistry. It was demonstrated that AR expression was affected by insulin. AR protein levels were markedly reduced by simultaneous administration of testosterone and insulin, while the levels of AR mRNA were not affected. Immunocytochemistry showed that insulin promoted nuclear translocation in the presence of testosterone. Insulin alone caused no evident effects on AR. Insulin promotes efficient nuclear translocation of AR. In the presence of insulin, androgen-AR complexes seem to be less stable and, as so, more prone to degradation, causing diminished levels of protein at constant AR mRNA levels, so evidencing that insulin might reinforce androgen stimulation and contribute to normal response of the gland and also to BPH.

1. Introduction

Androgens are important for the development, growth and maintenance of the prostate. Androgen function is mediated by the androgen receptor (AR). Besides its important role in normal physiology of the prostatic gland, AR is expressed in all stages of prostate cancer development. In reality, tumors become androgen-independent in response to mutations in the androgen receptor gene that results in higher sensibility to androgens allowing the tumor to grow even after castration (Feldman & Feldman, 2001).

In addition to the conventional hormone-dependent regulation, non-genomic mechanisms for AR function have been proposed to occur through interactions with the SRC-Raf-Ras-MAP kinase in the cytosol.. This non-genomic mechanism also requires the presence of androgen ligand and does not explain the progression of the disease in a ligand-independent manner (Heinlein & Chang, 2004; Kousteni et al., 2001; Pandini et al., 2005). Overexpression of co-activators and/or decreased expression of co-represso have been suggested as mechanisms for ligand-independent AR signaling (Feldman & Feldman, 2001). Certain peptide growth factors can act directly at the androgen-binding domain of the AR or indirectly by modifying co-regulators to initiate AR signaling (Sadar, 1999; Lin et al., 2001).

Although the prostate depends primarily on androgens, other hormones modify androgen's action in the prostate and accessory sex organs (Webber, 1981). Insulin is an important hormone that stimulates cell metabolism (through the phosphatidylinisitol 3-kinase pathway) and cellular proliferation (through the mitogen activated protein kinase) pathway (Begum et al., 1998; Montagnani et al., 2002). Insulin causes the reduction of serum level of sex hormone binding globulin (SHBG) and stimulates the production of IGF-1 while inhibiting the production of insulin-like growth factor binding protein 1 and 2 (IGFBP) (Barnard et al., 2002). The changes of serum levels of IGF-1 and IGFBP are associated with increased risk for several cancers, including the prostate (Yu & Rohan, 2000).

It has been evidenced (Shirai et al., 2004) that insulin replacement in rats with induced diabetes restores the protein and mRNA levels of AR in induced diabetic rat model. Another

report (Lin et al., 2005) has shown that AR knockout rat developed insulin resistance and, moreover, the administration of DHT did not reverse this effect, showing that insulin resistance is mediated by AR.

Since SMC play pivotal role in epithelial regulation (Hayward, Rosen & Cunha, 1997), show specific response to androgen deprivation (Antonioli, Della-Coleta & Carvalho, 2004, Vilamaior, Taboga & Carvalho, 2005) and are involved with the installation of benign prostatic hyperplasia, in this work we have examined the effect of insulin in AR expression and localization in prostatic SMC, using Western blotting and immunocytochemistry. The results show that insulin caused a reduction in AR protein levels and, in the presence of testosterone, a predominant AR nuclear localization.

2. Materials and methods

2.1. Cell culture

Primary cultures of rat ventral prostate smooth muscle cell were established by organ explant cultures. Smooth muscle cells were prepared using a modification of the protocol described by Gerdes et al. (1996). The ventral prostate of young adult Wistar rats (between 60 and 90 days) was minced into 1-2mm^3 fragments and placed in fenol red free DMEM (Nutricell, Campinas, SP, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution) supplemented with 0.5µg/mL testosterone cypionate (Novaquímica- Sigma Pharma, Hortolândia, SP, Brazil) and 5µg/mL insulin (BioBrás, Belo Horizonte, MG, Brazil) in 24-well culture plates. Medium was replaced every 48h.

After reaching confluence, SMC were replated using trypsin/EDTA (Nutricell) and cultured in 25cm^2 culture flasks at 37°C in a humidified atmosphere with 5% CO_2. All the experiments used SMC cultures at passages 4 and 5.

2.2. Western Blotting Analysis of Androgen Receptor

For insulin effect studies, SMC cells were cultured until reached 95% confluence. Medium was then replaced with serum free medium and without hormones for 24h before the treatments. The medium was then replaced and supplemented with 1.0g/L insulin, 0.55g/L transferrin, and 0.67g/L sodium selenite (ITS) and/or testosterone for 48h. Cells were scraped using Trypsin-EDTA and centrifuged for 10 min at 590g. The cell pellet was diluted in protein extraction buffer (1mM aminohexanoic acid, 5mM benzamidine, 1mM PMSF, 5mM NEM, 10mM EDTA, 600mM Tris e 0.1% Triton X-100). Total protein concentration was measured using Bio-Rad Bradford´s protein assay.

Western blotting was performed after electrophoresing 75µg protein of each lysate on 10% polyacrylamide gel under reducing conditions and electroctransferred to nitrocellulose membranes. Membranes were blocked for 1h with 5% non fat dry milk in TBS-T (Tris-buffered saline plus 0.2% Tween 20), and applied to the cells for 1h at room temperature with agitation. Anti-AR polyclonal antibody (N-20; cat. sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:500 and incubated for 2h at room temperature with agitation. After three TBS-T washes, HRP conjugated goat anti-rabbit Ig (cat. 61-8100, Zymed Laboratories, South San Francisco, CA, USA) diluted 1:2500 in TBS-T was added for 1h at room temperature with agitation. After another set of washes, detection of bound antigen was achieved with a chemiluminescence substrate (Santa Cruz Biotechnology). β-actin was used as an internal loading control The same membrane was stripped (100mM β-mercaptoetanol, 2% SDS, 62.5mM Tris-HCl pH 6.7), blocked for 1h with 1% non fat dry milk in TBS-T at room temperature and incubated with goat polyclonal antibody anti-human actin (cat. I-19; Santa Cruz Biotechnology) diluted 1:1000 in TBS-T containing 0.1% non fat dry milk and incubated for 2h at room temperature with agitation. Following three TBS-T washes, HRP-conjugated rabbit anti-goat IgG (H+L) (cat. 81-1620 Zymed Laboratories) was added after 1:500 dilution in TBS-T for 1h at room temperature with agitation. After another set of washes, detection of bound antigen was achieved by chemiluminescence (Santa Cruz Biotechnology). Band intensity was determined using Kodak 1D software (Scientific Imaging System). The experiments were repeated three times.

2.3. Immunocytochemistry

The SMC were plated on glass coverslips in 24 well plates, and maintained in serum containing medium until semi-confluence and treated as above. Coverslips were washed three times in PBS (phosphate buffered saline, pH 7.4) and fixed for 15 min in 4% paraformaldehyde. Cells were permeabilized by exposure to TBS-T (Tris-buffered saline plus 0.2% Tween 20) three times at room, and blocked with 3% bovine serum albumin (BSA) in TBS-T for 1h. The same anti-AR primary antibody was diluted 1:200 in 1% BSA in TBS-T, and applied to the cells for 2h at room temperature. Coverslips were washed three times with TBS-T and incubated with a fluorescein conjugated goat anti rabbit IgG (cat. F-0382, Sigma Chemical Co., Saint Louis, MO, USA) diluted 1:200 in 1% BSA in TBS-T for 1h at room temperature. The cells were then washed in TBS-T and mounted in DABCO (Sigma Chemical Co.). Observations were made using an Olympus BX51 microscope and images were acquired using the Image-Pro 4.0 software.

2.4. RNA Extraction and Reverse Transcription - Polymerase Chain Reaction

Total RNA extraction was isolated using TRizol reagent (Invitrogen Life Technologies, São Paulo, SP, Brazil). Samples were homogenized in 1mL and the subsequent procedures were carried out according to the manufacturer. The concentration was quantified by spectrophotometry using Ultraspec 2100 pro equipment (Amersham Biosciences, São Paulo,SP, Brazil). For cDNA synthesis, 5µg of total RNA samples was reverse transcribed with 200U SuperScript III (Invitrogen Life Technologies) and Oligo (dT)12-18 Primer (Invitrogen Life Technologies) according to the supplier's instructions. The cDNA was quantified by spectrophotometry as above. AR and β-actin synthetic oligonucleotide primers were designed using Gene Runner 3.05 (Hastings Software. Inc). AR, forward 5'-CGCTTCTACCAGCTCACCAAG-3'; reverse 5'-TGGGCACTTGCACAGAGATG-3'; β-actin forward 5'-CTGGCCTCACTGTCCACCTT-3', reverse 5'-AGTACGATGAGTCCGGCCC-3'. All primers were synthesized by Invitrogen Life Technologies.

For AR, PCR thermal cycling conditions were as follows: an initial step at 94ºC for 5 min; 30 cycles of: 94ºC – 30 sec, 62ºC – 30 sec, 74ºC – 45 sec and a final cycle of 5 min at 74ºC. The reaction was performed in a 13µL final volume containing 150ng cDNA, 0.39U Taq DNA Polymerase (Invitrogen Life Technologies) and 2mM $MgCl_2$. For β-actin, PCR thermal cycling conditions were: an initial step at 94ºC for 5 min; 30 cycles of: 94ºC – 40 sec, 64ºC – 10 sec, 72ºC – 15 sec and a final cycle of 5 min at 72ºC, in a 13µL final volume containing 150ng cDNA, 0.39U Taq DNA Polymerase (Invitrogen Life Technologies) and 2.5mM $MgCl_2$. The experiments were done in triplicates.

2.5. Statistical Analysis

Data were expressed as the mean of bands intensity for all experiments. Differences between treated groups were determined by one way analysis of variance (ANOVA). Post comparison of mean values by Tukey's determined where differences occurred (MINITAB 14 program). The differences were accepted as statistically significant when $p \leq 0.05$. The values were expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. AR protein content is diminished in the presence of insulin

Figure 1A shows a representative Western blotting for AR. It was apparent that insulin affected the relative amount of AR in SMC. Quantification of the bands (**Fig. 1C**) showed that insulin presence caused a significative drop in AR content in the presence of testosterone. SMC cultured only in medium, insulin or testosterone produced similar amounts of AR.

3.2. Insulin effects in mRNA of AR

Figure 2A shows a representative PCR result. Band intensity was determined for each experiment and their variation assessed with respect to β-actin (**Fig. 2B**), which was used as an

internal control. The variation of the endogenous control was less than 20% among treatments. **Figure 2C** shows the semi-quantitative results for AR mRNA. No significative variation in AR mRNA was detected using β-actin and the internal control.

3.3. Hormonal effects of AR distribution

The SMC cultured in medium with or without testosterone showed a nuclear and cytoplasmic localization pattern of the AR (**Figs. 3** and **4**). Nuclear staining was ligand independent irrespective of the presence of insulin. SMC cultured with non-supplemented medium, and in the presence of insulin or testosterone exhibited the same proportion of cells with nuclear, cytoplasmic or nuclear plus cytoplasmic distribution of AR (**Fig. 4**). The medium supplemented with insulin and testosterone caused more cells to have a predominant nuclear localization as compared to the other situations (**Fig. 4**). These data indicate that androgens are not required for AR localize to the nucleus of cultured SMC.

4. Discussion

The androgen receptor (AR) mediates androgen stimulation to regulate normal and abnormal prostatic function. Nevertheless, other signal transduction pathways have been shown to influence steroid action leading to enhanced response to androgen responses and, in some instances, ligand-independent receptor activation (Gerdes et al., 1998). Certain growth factors play important role in prostate cancer progression and possibly in the progression to the androgen independent stage of the disease, interfering with signal transduction cascades and AR transactivation (Heinlein & Chang, 2004).

Epidemiologic studies have assessed the relationship of serum levels of insulin and the risk of prostatic cancer development (Lehrer et al., 2002). Insulin resistance and compensatory hyperinsulinaemia have direct effect on the liver. It produces potent promoters of prostate cancer such as IGF-1 and reduces the serum level of sex hormone binding globulin (SHBG) and insulin-like growth factor biding protein 1 and 2 (IGFBP-1,-2) (Barnard et al., 2002).

In contrast with the limited molecular knowledge on the effects of insulin on the prostate gland, studies showed that the proliferative effect of insulin on vascular SMC is mediated by activation of the MAPK cascade, which in turn phosphorylates and activates other kinases and a variety of transcription factors, leading to increased DNA and protein synthesis (Begum et al., 1998).

Our results showed that insulin reduced the content of AR protein in the presence of testosterone. Insulin alone did not change significantly the protein expression compared to the other situations. It is possible that insulin decreases the stability of AR, thus promoting its faster degradation. This idea seems probable since the expression of AR at the mRNA was proven not to differ from the control situation.

At the physiological level, sex steroids are thought to be involved in maintaining normal insulin sensitivity on tissues. Excessive androgen production and insulin resistance are associated to the polycystic ovarian syndrome (Livingstone & Collison, 2002). However, it has been shown that the knocking down of androgen receptor in mice caused insulin resistance even at constant levels of testosterone (Lin et al., 2005). Another report showed that insulin treatment restored AR mRNA and protein levels in diabetic rats, with a suggestion that the observed effect might be mediated through IGF-1 (Shirai et al., 2004).

Other factors can be responsible for regulating androgen independent AR signaling, since under some conditions, some androgen regulated genes continue to be expressed (Barnard et al., 2002; Iwamura et al., 1993). IGF-1 signaling is one such factor that seems to be tightly related to the androgen-independent activation of AR (Barnard et al., 2002; Iwamura et al., 1993).

Classically, in the absence of ligand, AR remains in the cytosol in an inactive form (Wu et al., 2006). Our results demonstrated that in the presence of insulin, AR was present in the cell nucleus in most of the cells, that testosterone stimulation alone was not enough to translocate the AR pool to the cell nucleus, what was more evident when insulin and testosterone were administered simultaneously. It is in agreement with the results published by others (Gerdes et al., 1998), who showed residual cytoplasmic AR staining in SMC treated with DHT.

From the present result, it might be suggested that under activation of the insulin signaling, AR activation by testosterone is more efficient, as measured by its preferential nuclear localization, though resulting in diminished stability of the complex, thus reflecting in lesser amount of protein at the same levels of AR mRNA.

5. Acknowledgments

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FIGURE LEGENDS

Figure 1. (A) Western blot for AR (110kDa) in SMC treated with insulin and/or testosterone. **(B)** β-actin was used as loading control. **(C)** Percentual variation of band density AR for SMC treated with medium supplemented with insulin and testosterone. The SMC untreated with hormones was used as reference (100%). Different letters indicate significant differences ($p \leq 0.05$) as assessed by the Tukey's paired test.

Figure 2. (A) AR mRNA levels in SMC treated with insulin and/or testosterone. **(B)** β-actin was used as an internal control. **(C)** AR mRNA percentual variation as determined by semi-quantitative RT-PCR. The cells treated with non-supplemented medium were used as reference (100%). No significant variation was observed.

Figure 3. Immunolocalization of AR in SMC treated with insulin and/or testosterone. **(A)** Culture medium, **(B)** insulin, **(C)** testosterone, **(E)** insulin plus testosterone. **(A)** The arrow indicates a cell with no nuclear AR labeling. **(C)** The arrowhead shows nuclear AR. Magnification, 400X.

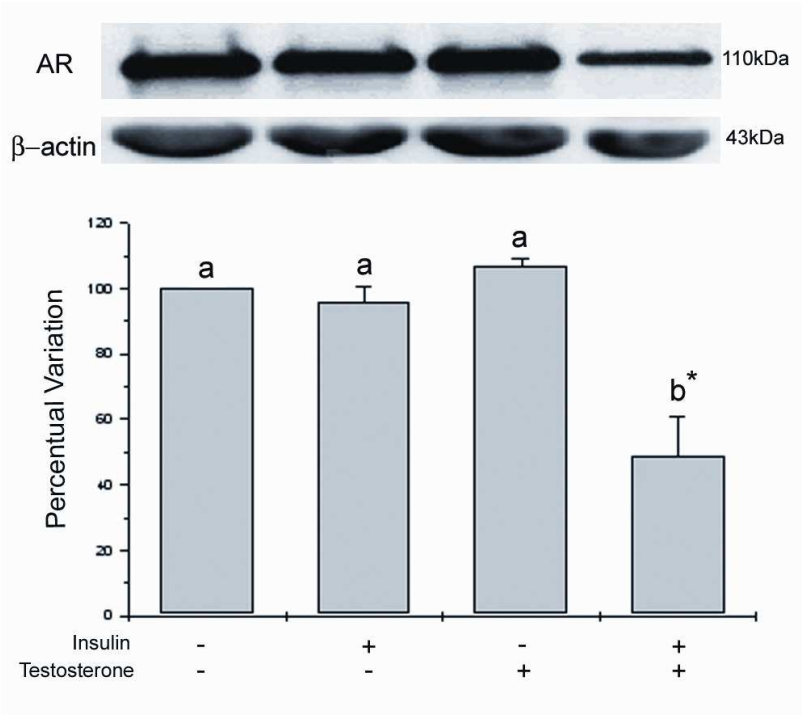
Figure 4. AR localization in SMC. Culture medium (n=141), insulin (n=109), testosterone (n=187), insulin/testosterone (n=61). Data are expressed as means ± SEM. * indicates differences with respect to the controls, ** indicate differences with respect to all other groups ($p<0.05$).

Figure 1

(A)

(B)

(C)


AR
110kDa
β–actin
43kDa
Percentual Variation
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40
60
80
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120
a
a
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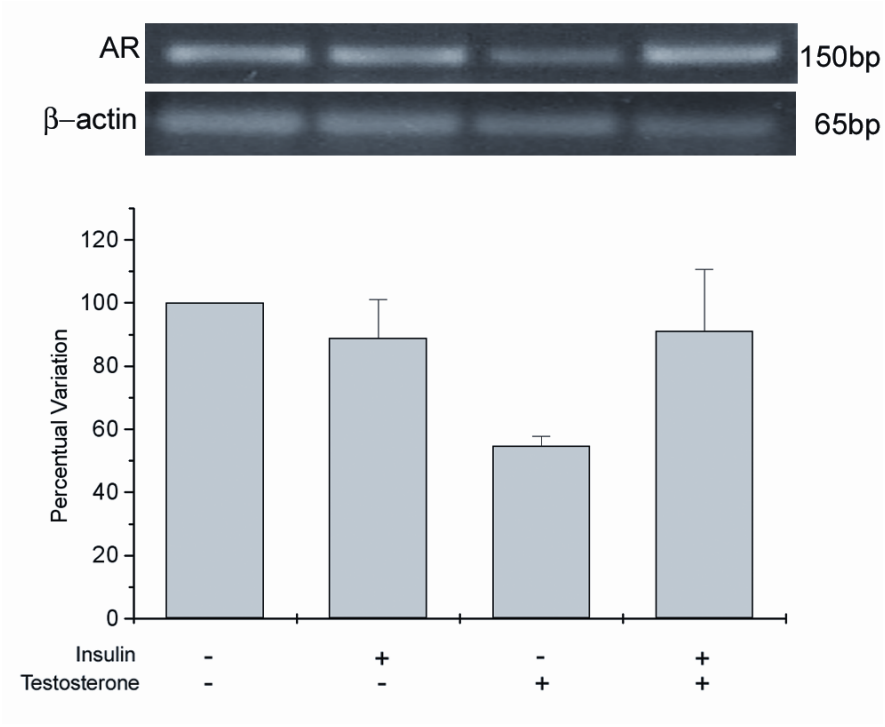
b*
Insulin - + - +
Testosterone - - + +

Figure 2


(A)
AR
150bp
(B)
β–actin
65bp
(C)
Percentual Variation
120
100
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Insulin - + - +
Testosterone - - + +

Figure 3

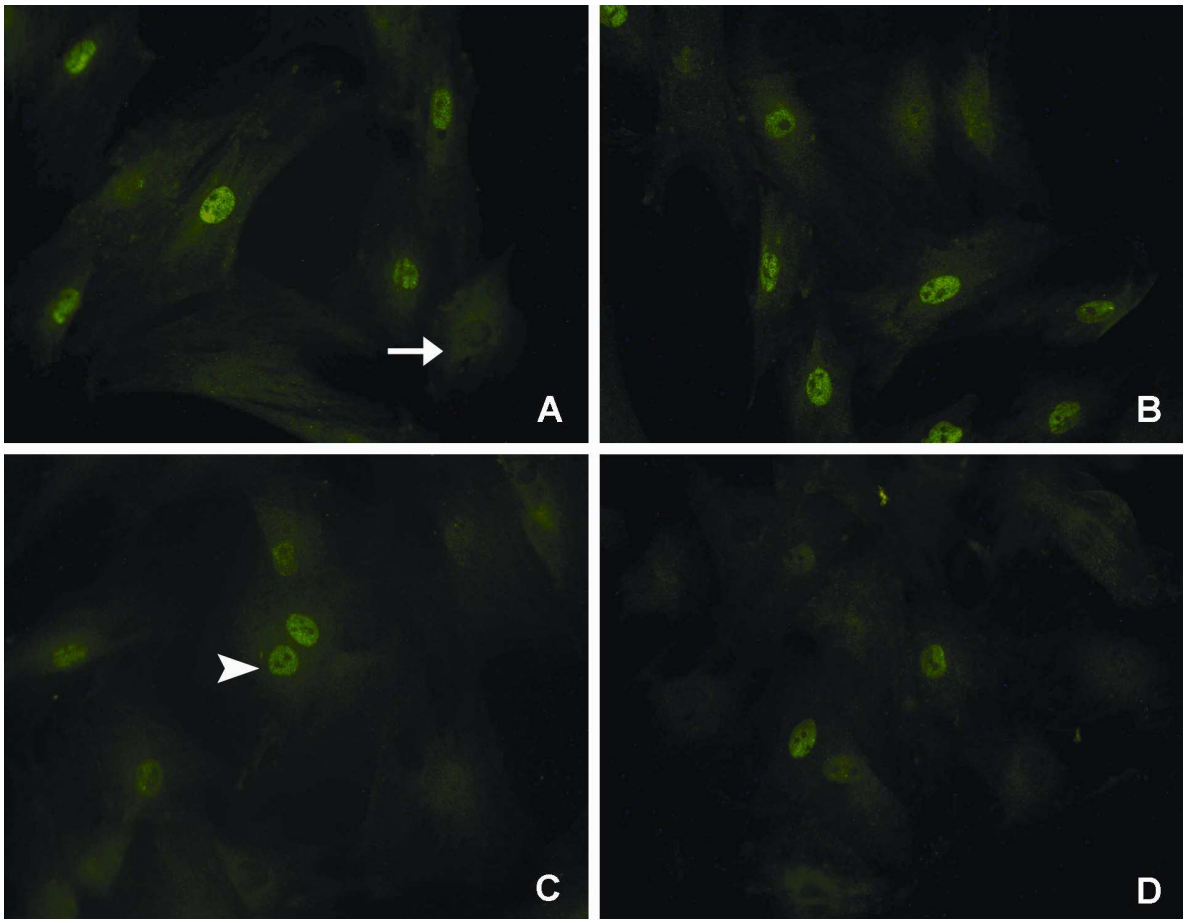
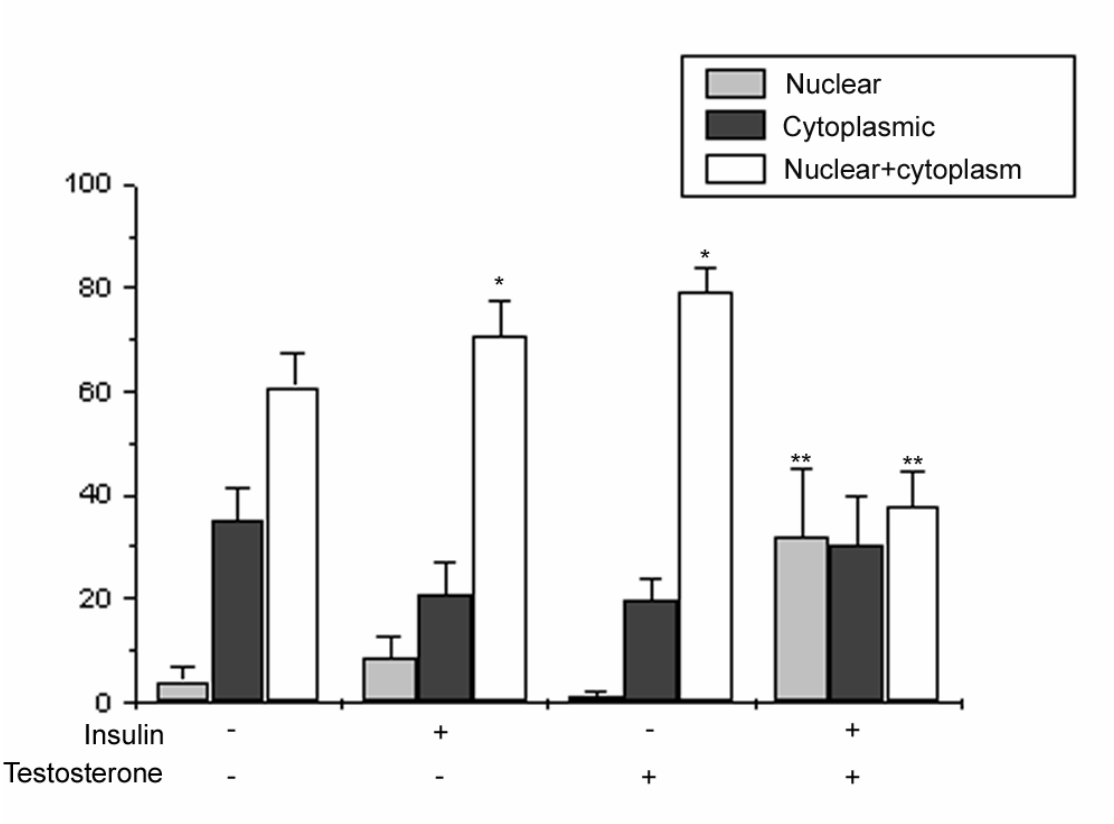


Figure 4


Nuclear
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Insulin - + - +
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4. CONSIDERAÇÕES FINAIS

As CML prostáticas tratadas com estradiol apresentaram alterações nos níveis de expressão do AR. O estradiol causou um efeito não monotônico nos níveis de expressão da proteína. Na presença da testosterona houve uma redução na expressão da proteína, porém sem o comportamento observado na ausência da testosterona.

Os níveis de RNAm do AR não foram afetados, sugerindo que o efeito do estradiol sobre a proteína é causado por um aumento da sua degradação.

O estradiol levou a uma localização predominantemente nuclear do AR independente da dose testada.

Os tratamentos feitos com insulina mostraram que sua presença no meio promove uma eficiente translocação nuclear do AR na presença da testosterona.

A presença da insulina parece levar a formação de um complexo andrógeno-AR menos estável, com uma sugestão de maior susceptibilidade proteolítica.

Os níveis de RNAm do AR não foram afetados pela presença da insulina no meio.

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