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Mobilização leucocitária: controle endógeno e efeitos de xenobióticos

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SUMÁRIO

	Pág.
1. RESUMO	01
2. SUMMARY	02
3. INTRODUÇÃO	03
3.1 Mobilização leucocitária	03
3.1.1 Interação leucócito-endotélio	04
3.2 Controle endógeno da mobilização leucocitária	10
3.2.1 Glicocorticóides endógenos	10
3.2.2 Óxido Nítrico	16
3.3 Efeitos de xenobióticos sobre a mobilização leucocitária	24
3.3.1 Compostos fenólicos derivados do metabolismo do benzeno	24
4. REFERÊNCIAS BIBLIOGRÁFICAS	33
5. ANEXOS	64

1. RESUMO

O presente trabalho apresenta estudos experimentais sobre os eventos responsáveis pela mobilização leucocitária na vigência de processos inflamatórios, especialmente os voltados às interações dos leucócitos ao endotélio vascular, eventos iniciais e fundamentais na migração celular para o foco de lesão. Duas linhas trilham esta tese, a saber: os mecanismos de modulação endógena e os efeitos de substâncias tóxicas.

Dentro do primeiro enfoque, demonstramos que os glicocorticóides endógenos modulam o recrutamento de células brancas, por atuarem, pelo menos em parte, sobre os fenômenos de interação leucócito-endotélio. A deficiência hormonal acarreta aumento no número de leucócitos "rolling" e leucócitos aderidos à parede de vênulas pós-capilares *in vivo*. A interferência sobre a maturação leucocitária e a expressão de moléculas de adesão em leucócitos e célula endotelial são efeitos importantes no processo. Nesta mesma linha mostramos, ainda, que o efeito da inibição prolongada da síntese de óxido nítrico (NO; L-NAME, 20 mg/kg/dia/14 dias) promove ação antiinflamatória, visualizada pela diminuição do influxo de leucócitos para o foco de lesão, dependente de prejuízos na interação leucócito-endotélio e na expressão de moléculas de adesão.

Na segunda linha de investigações, estudamos os mecanismos envolvidos na toxicidade causada pela exposição a compostos fenólicos, derivados da metabolização do benzeno. Mostramos que a exposição à hidroquinona por período prolongado de tempo (50 mg/kg; via i.p.; 1 vez ao dia com intervalos de 2 dias a cada 5 doses; 13 ou 16 doses) afeta distintamente o recrutamento celular na vigência de resposta inata ou adquirida, por mecanismos complexos que envolvem ações na maturação, estado de ativação e função leucocitária. Apesar do fenol ser endogenamente metabolizado à hidroquinona, a exposição ao fenol, nas mesmas condições que a exposição à hidroquinona, não altera os parâmetros inflamatórios.

2. SUMMARY

The present work shows experimental studies performed to clarify the mechanisms responsible for the control of leukocyte mobilization during inflammatory processes, specially to those related to leukocyte-endothelial interactions, fundamental and initial events to cellular migration into lesioned areas. Two lines of investigations lead this work, as follow: mechanisms of endogenous modulations and effects of toxic substances.

At the first line, we demonstrated that endogenous glucocorticoids modulate the white cells recruitment, by acting, at least in part, on leukocyte-endothelial interactions. Deficiency of hormones induces increment on number of rolling and adhered leukocytes to postcapillary venules *in vivo*. Modulations on leukocyte maturation phases and on adhesion molecules expressions on leukocytes and endothelial cells are important actions in these effects. At the same line of investigations, we also showed that chronicle inhibition of NO synthesis (L-NAME, 20 mg/Kg/day, during 14 days) reduces leukocyte influx into inflammatory site, dependent on impairment on leukocyte-endothelial interactions and on adhesion molecules expressions.

The second line of investigations comprises the mechanisms responsible for the toxicity provoked by exposing animals to phenolic compounds derived from benzene's biotransformation. We showed that hidroquinone exposure for a prolonged period of time (50 mg/kg; i.p. route; once a day, with interval of 2 days each 5 doses; 13 or 16 doses) affects the cellular recruitment during an innate or acquired inflammatory reaction, throughout complex mechanisms which involve actions on maturation, activation and functions of leukocytes. Despite fenol be endogenously transformed to hidroquinone, fenol exposure does not promote similar effects of hidroquinone administrations.

3. INTRODUÇÃO

3.1 Mobilização leucocitária

As associações da presença de leucócitos no tecido extravascular e a resposta a uma lesão foram inicialmente constatadas no final do século XIX por Dutrochet (1824), Addison (1843) e Waller (1846), sendo que este último também correlacionou o evento à formação de exsudato purulento. Subseqüentemente, Cohnheim (1867) descreveu que as bases fundamentais para a resposta tecidual à injúria dependiam da presença de leucócitos e da formação do exsudato inflamatório, sendo que alterações na parede microvascular favoreciam as interações celulares necessárias para o processo. Desta forma, foi descrita a primeira evidência da importância da **interação leucócito-endotélio**. Vinte e seis anos mais tarde, o brilhante Metchnikoff (1893) e seus discípulos postularam que o processo inflamatório era dependente da atividade de leucócitos em resposta à ação de **agentes químicos** extravasculares, com propriedades de induzirem a migração leucocitária. Assim, os primeiros conceitos da **quimiotaxia** foram inseridos (revisto em Rampart, 1994). Desde então, os mecanismos que regem as interações celulares, bem como as adesões entre as células e os componentes dos microambientes em cada etapa da migração leucocitária, são alvos de intensas investigações.

Desde o início até sua resolução, a reação inflamatória é caracterizada pela infiltração sucessiva dos diferentes subtipos de células brancas. A mobilização dos compartimentos de reserva e o recrutamento seqüencial no curso do processo - neutrófilos seguido pelas células mononucleares e eosinófilos - são eventos complexos e vitais para eficiência da imunidade inata e adaptativa. A complexidade do processo é decorrente da ação de inúmeros mediadores químicos, que desencadeiam sinalizações intracelulares diferentes para expressão de receptores e alterações morfológicas durante a ativação e locomoção leucocitárias. Interferências nestes eventos acarretam prejuízos

sérios à instalação, desenvolvimento e resolução de processos inflamatórios de diferentes origens.

3.1.1 Interação leucócito-endotélio

A mobilização dos leucócitos circulantes inicia-se com a mudança na localização das células e elementos figurados na corrente sangüínea. Em condições normais de fluxo, os leucócitos circulam no centro da corrente sangüínea. As interações das células brancas e a parede vascular são inexpressivas, com exceções do "pool" periférico de leucócitos aderidos à parede dos vasos da microcirculação, principalmente pulmonar, e de linfócitos no processo de recirculação, os quais interagem com células endoteliais especializadas de tecidos linfóides secundários. A posição central dos leucócitos na corrente sangüínea é dependente, pelo menos em parte, de seu maior tamanho em relação aos demais componentes sangüíneos. Na vigência de um processo inflamatório, uma série de eventos contribui para o contato inicial das células brancas com o endotélio vascular, entre os quais: 1) as alterações na hemodinâmica microvascular, representadas pela vasodilatação, aumento de permeabilidade vascular e redução de fluxo em capilares e vênulas pós-capilares, que em conjunto provocam modificações no estado de agregação e velocidade de eritrócitos na circulação, e 2) a expressão de receptores, denominados de **moléculas de adesão** (Schmid-Schönbein e cols., 1980; Pearson e Lipowsky, 2000; revisto em Sun e cols., 2003).

Comportamento "rolling" foi a denominação conferida ao contato inicial dos leucócitos ao endotélio, pela habilidade destas células de deslizarem sobre a parede vascular com velocidade menor que o fluxo sangüíneo e aderirem, momentaneamente. Com a progressão do processo inflamatório, o tempo de aderência à parede vascular aumenta e os leucócitos aderem firmemente ao endotélio. Estes eventos, denominados em conjunto de interação leucócito-endotélio, são iniciais e fundamentais para a subsequente diapedese e migração para o interstício adjacente.

A expressão coordenada e sucessiva de moléculas de adesão nas membranas celulares durante os eventos de interação leucócito-endotélio é um paradigma que foi atribuído após um grande número de estudos experimentais, que demonstraram que o bloqueio na síntese, expressão, interação com ligante(s) específico(s) ou na sinalização intracelular induzida pela molécula prejudicava especificamente cada etapa do processo. Estes estudos descreveram a gênese de doenças, até então desconhecidas, caracterizadas pela inabilidade de resposta a infecções, e denominadas de LAD (*leukocyte adherence deficiency*) (revistos em Roos e Law, 2001; Bunting e cols., 2002; Wild e cols., 2002; Alon e Etzioni, 2003).

A descrição das moléculas de adesão, desde o final da década de 1980, tem contribuído efetivamente para o conhecimento das bases fisiológicas e patológicas das interações celulares em processo vitais como fertilização, embriogênese, formação e manutenção da integridade tecidual, angiogênese, defesa, cicatrização, reparo e na formação de metástases. Nos últimos anos tem sido amplamente demonstrado que o papel das moléculas de adesão vai muito além da mediação das interações celulares, uma vez que vias de sinalizações intracelulares são ativadas após a expressão e/ou ligação destas moléculas. No que concerne à resposta inflamatória, as sinalizações conferem amplificação do processo, entre outros efeitos, pela síntese/expressão de moléculas de adesão, redistribuição de receptores na membrana celular, reorganização intracelular e síntese de mediadores inflamatórios. Desta forma, estas moléculas têm sido alvo para marcadores de doenças e para intervenções farmacológicas (revistos em Kluger, 2004; Bochner, 2004; Szekanecz e Koch, 2004; Ehrhardt e cols., 2004).

Dentro da cascata de ativação das moléculas de adesão, as moléculas pertencentes à família das **selectinas** são fundamentais para o comportamento "rolling" de leucócitos. Três moléculas desta família foram descritas e agrupadas pela sua capacidade de ligar-se a carboidratos sialilados, fucosilados e sialomucinas, presentes constitutivamente nas membranas celulares (Taylor e Drickamer, 1993; Vestweber e Blanks, 1999). A semelhança destes carboidratos às lectinas de plantas denominou esta família de LEC-CAM (*lectin-cell adhesion molecules*). A deficiência de síntese de carboidratos,

principalmente fucosilados, determina prejuízo no "rolling" de leucócitos e, conseqüentemente, na aderência ao endotélio e no influxo de células brancas para o foco de lesão. Este padrão de comportamento tem sido demonstrado em animais "knockout" para síntese de carboidratos e em indivíduos com LAD tipo II, caracterizada por deficiência de carboidratos fucosilados desencadeada por mutações gênicas em um sistema de transporte de fucose localizado no complexo de Golgi (para revisões Roos e Law, 2001; Bunting e cols., 2002). Tem sido demonstrado que a administração oral de fucose restaura a ligação celular às selectinas e corrige a neutrofilia detectada nestes pacientes. Se o tratamento é interrompido, após alguns dias, o mesmo padrão de doença reaparece (Lühn e cols., 2001).

A **L-selectina (CD62L)** ou **Mel-14** ou ***lectin-like cell adhesion molecule-1 (LECAM-1)*** medeia o "homing" de linfócitos às células endoteliais especializadas dos linfonodos (Gallatin e cols., 1983; Butcher e Picker, 1996) e o "rolling" de leucócitos em respostas inflamatórias agudas e crônicas (Tedder e cols., 1995; Winn e cols., 1998). Uma peculiaridade da L-selectina é sua rápida clivagem da membrana após ativação celular pela ação de metaloproteinases presentes na parede celular, gerando um ectodomínio solúvel e um pequeno fragmento retido na membrana (Zhao e cols., 2001; Smalley e Ley, 2005). O mecanismo de clivagem é dependente de vias de sinalização intracelulares, que envolvem proteína quinase C e inibição de calmodulina (Fors e cols., 2001; Smalley e Ley, 2005). O significado biológico da clivagem protéica tem sido demonstrado, experimentalmente, pela contínua migração de linfócitos para os linfonodos e pelo maior influxo e por tempo mais prolongado de neutrófilos para o peritônio inflamado na vigência de bloqueio enzimático (Venturi e cols., 2003).

A **P-selectin (CD62P)** ou ***granule membrane protein 140 (GMP140)*** é expressa constitutivamente nos grânulos α de plaquetas (Stenberg e cols., 1985) e nos corpúsculos de Weibel-Palade no endotélio (Bonfanti e cols., 1989). Após ativação, a molécula é rapidamente transportada para as membranas celulares (Dunlop e cols., 1992; Michelson e cols., 1996; Hartwell e cols., 1998); no entanto, sua expressão é passageira. Em plaquetas, sofre clivagem; e no endotélio, grande parte é re-internalizada, sendo que o restante

é clivado (Burger e Wagner, 2003). A P-selectina participa da interação responsável pelo comportamento "rolling" de leucócitos e da agregação heterotípica de plaquetas a leucócitos. Desta forma, é uma molécula importante para conexão entre inflamação, trombose e coagulação em áreas de lesão (Palabrica e cols., 1992). Sua expressão em plaquetas presentes em trombos em crescimento facilita o recrutamento dos leucócitos circulantes, além de induzir a sinalização intracelular para síntese/secreção de diferentes mediadores envolvidos no processo (revistos em Andre, 2004; Geng e cols., 2004).

A **E-selectina (CD62E)** ou ***endothelial-leukocyte adhesion molecules (ELAM-1)*** é expressa na célula endotelial pela ação de vários mediadores inflamatórios, entre os quais o fator de necrose tumoral α (TNF- α) e a interleucina-1 (IL-1), com pico de expressão na quarta hora e declínio 24 horas após estimulação (Bevilacqua e cols., 1987). A regulação de sua transcrição é dependente de vários fatores, incluindo o fator nuclear kappa B (NF- κ B) e o ativador de proteína 1 (AP-1) (revisto em Ehrhardt e cols., 2004). Uma vez expressa, a molécula é clivada ou lentamente re-internalizada para os lisossomos, com subsequente degradação (Subramaniam e cols., 1993). A clivagem exacerbada de E-selectina e a consequente elevação da concentração de sua forma solúvel no plasma desencadeia sintomatologia semelhante à observada em pacientes com LAD e, desta maneira, foi proposto que esta poderia ser um tipo de manifestação da doença (DeLisser e cols., 1999).

As **integrinas** compõem uma família de moléculas que medeiam a interação leucócito-endotélio e leucócito-matriz extravascular. Sua denominação foi decorrente da habilidade de integrar o meio intra e extravascular e, assim, são importantes sinalizadoras em diferentes processos fisiopatológicos (revistos em Rossetti e cols., 2002; Hogg e cols., 2002). Todas as integrinas são heterodímeros compostos de uma subunidade α e uma subunidade β . A perfeita associação das diferentes subunidades α a uma subunidade β comum determinou a classificação destas moléculas em subfamílias. Desta forma, as integrinas são agrupadas em pelo menos 5 subfamílias (revisto em González-Amaro e Sánchez-Madrid, 1999; Arnaout e cols., 2005). A **subfamília β_2** ou

complexo CD18 é composta de 4 moléculas, a saber: **CD11a/CD18** ou **lymphocyte function associated 1 (LFA-1)**, **CD11b/CD18** ou **macrophage antigen-1 (MAC-1)**, **CD11c/CD18** ou **gp150,95** e **CD11d/CD18**. São expressas exclusivamente em leucócitos, medeiam a aderência ao endotélio e sua subsequente diapedese. Neste processo, os ligantes para as β_2 integrinas são as moléculas que compõem a família das imunoglobulinas, além da fração C3b do complemento e de proteoglicanos (revisto em González-Amaro e Sánchez-Madrid, 1999). A adesão celular mediada pelas β_2 integrinas é decorrente de expressão qualitativa, pela ativação de moléculas quiescentes nas membranas leucocitárias, ou quantitativa, proveniente de mobilização de estoques intracelulares e de síntese protéica (Chatila e cols., 1989; revisto em Gahmberg e cols., 1997). Assim, uma série de mediadores químicos e hormônios interferem na expressão das integrinas, por mecanismos transcricionais e pós-transcricionais ou por provocarem alterações estruturais em moléculas constitutivamente expressas (revisto em Kim e Yamada, 1997). A deficiência autossômica recessiva para a síntese da cadeia β_2 foi denominada LAD I e caracterizada pela inabilidade dos leucócitos aderirem ao endotélio e migrarem para o foco de lesão. Indivíduos portadores desta doença, classificada como leve, moderada ou grave de acordo com a porcentagem de expressão das β_2 integrina, apresentam leucocitose persistente e processos infecciosos freqüentes e graves (Crowley e cols., 1980; Arnaout e cols., 1982; Springer, 1985; Dana e Arnaout, 1988; revisto em Etzioni e cols., 1999). Recentemente, uma doença com sintomatologia semelhante à LAD I foi denominada de LAD III e sua gênese foi associada à inabilidade de ativação de β_2 integrinas por receptores de membranas ligados à proteína G (Alon e Etzioni, 2003).

As **imunoglobulinas** são membros de uma família de moléculas de adesão, compostas por um domínio extracelular constituído de várias unidades repetidas e homólogas a domínios de imunoglobulina G, uma região transmembrana e uma pequena cauda citoplasmática. São expressas em diferentes tipos celulares, mas como citado acima, são os ligantes no endotélio para as β_2 integrinas nos processos de aderência e diapedese. As expressões da **intercellular cell adhesion molecule-1 (ICAM-1)** e **vascular cell adhesion molecule-1 (VCAM-1)** no endotélio quiescente são baixas, mas na presença

de estímulos inflamatórios suas concentrações na membrana endotelial são extremamente aumentadas. Diferentemente, as expressões constitutivas da **intercellular cell adhesion molecule-2 (ICAM-2)** e **platelet endothelial cell adhesion molecule-1 (PECAM-1)** são significativas (revisto em Huo e Ley, 2001). Neste contexto, vale ressaltar que a localização constitutiva e predominante de PECAM-1 nas junções interendoteliais é fundamental para a manutenção fisiológica da junção interendotelial e para diapedese de leucócitos na vigência de processo inflamatório (revisto em Muller e Randolph, 1999; Luscinskas e cols., 2002).

Apesar da comprovada expressão seqüencial das moléculas de adesão citadas acima em processos inflamatórios de diferentes origens, vias alternativas têm sido propostas e parecem ser dependentes de características específicas dos leitos microcirculatórios dos tecidos lesados (Duncan e cols., 1999; Doerschuk e cols., 2000; Doerschuk, 2001). Recentemente, tem sido demonstrado que uma classe de enzimas, semelhantes às monoaminoxidases, chamadas **vascular adhesion protein -1 (VAP-1)**, medeia o "rolling" e a aderência de leucócitos em alguns modelos de inflamação e na recirculação de linfócitos. Apesar do mecanismo ainda não estar estabelecido, há evidência de uma ação própria, como moléculas de adesão, mediando diretamente o "rolling", ou pela expressão de P-selectina induzida pela ação do peróxido de hidrogênio gerado em decorrência de sua atividade enzimática na deaminação de aminas primárias (Salmi e Jalkanen, 2001). Sua participação na migração leucocitária tem sido amplamente demonstrada *in vivo* e *in vitro* em modelos de inflamação aguda e crônica e na rejeição de transplantes (Yegutkin e cols., 2004; Koskinen e cols., 2004; Martelius e cols., 2004; Merinen e cols., 2005; Stolen e cols., 2005).

Uma das características presente nas LAD é a leucocitose persistente. Este efeito é resultante da manutenção das células na circulação e da hematopoese alterada. A participação efetiva das selectinas e integrinas na manutenção celular durante as etapas de produção e diferenciação das células brancas na medula tem sido demonstrada, como revisto por Chan e Watt (2001). Na população mielóide, a expressão de L-selectina varia de acordo com suas etapas de maturação e sua clivagem é fundamental para mobilização

dos leucócitos para a circulação (van Eeden e cols., 1997; Dimitroff e cols., 2001; Carion e cols., 2002). Adicionalmente, a interação das selectinas com seus ligantes específicos induz sinalização para modificação da expressão, estado de ativação e função de integrinas envolvidas na hematopoese (revisto em Gonzalez-Amaro e Sanchez-Madrid, 1999). Neste contexto, é importante ressaltar o papel relevante das β_2 integrinas e imunoglobulinas na sinalização de síntese de citocinas, fatores de crescimento e outras moléculas de adesão durante a hematopoese (revisto em Verfaillie, 1998; Chan e Watt, 2001).

Do exposto fica evidente que as moléculas de adesão modulam a interação leucócito-endotélio e atuam como sinalizadores intracelulares em diferentes microambientes durante a mobilização leucocitária em condições fisiológicas e na gênese de diferentes doenças, entre as quais, as de origem inflamatória.

Nossos trabalhos investigam o controle endógeno da migração leucocitária durante respostas inflamatórias e os efeitos de xenobióticos sobre este processo. Desta forma, a expressão/ativação das moléculas de adesão são eventos abordados neste estudo.

3.2 Controle endógeno da mobilização leucocitária

3.2.1 Glicocorticóides endógenos

Os glicocorticóides (GC) são agentes antiinflamatórios e imunossupressores potentes utilizados amplamente na clínica médica. No entanto, seus efeitos adversos intensos e graves podem restringir suas aplicações, apesar do grande benefício terapêutico.

O uso terapêutico dos GC se iniciou na década de 1950 a partir de estudos que mostraram a importância dos hormônios secretados pelas glândulas adrenais na homeostasia. A partir destes trabalhos, foi demonstrada a ativação do eixo hipotálamo-hipófise-adrenal na vigência de estresse de diferentes origens. No que concerne à resposta inflamatória, é conhecido que **mediadores inflamatórios secretados localmente no processo, entre os quais**

IL-1, interleucina-2 (IL-2), interleucina-6 (IL-6), interferon- γ (INF- γ) e fator de necrose tumoral (TNF α), alcançam a circulação sanguínea e estimulam centros específicos no hipotálamo e na hipófise que induzem, respectivamente, a secreção do hormônio liberador de corticotrofina (CRH) e do hormônio adrenocorticotrófico (ACTH). As células do córtex adrenal, estimuladas pelo ACTH, secretam GC que atuam como moduladores endógenos do processo (Leme e Schapoval, 1975; revistos em Tilders e cols., 1994; Haddad e cols., 2002). Ainda, alguns mediadores podem estimular as células adrenais diretamente, provocando a secreção hormonal (revisto em Haddad e cols., 2002).

A maioria dos efeitos antiinflamatórios dos GC é desencadeada por ação genômica, que leva a uma regulação positiva ou negativa da síntese de proteínas participativas do processo. O mecanismo molecular de ação inicia-se com a interação dos esteróides com o receptor protéico citosólico, composto por uma proteína da família das *heat shock protein* (HSP 90), um membro da família das *heat shock family of stress induced protein* (HSP70) e uma imunofilina, integrante da família de proteínas que se liga a agentes imunossupressores. Esta conformação protéica parece ser necessária para a manutenção do estado inativo do receptor na ausência do agonista. Duas isoformas do receptor têm sido descritas. A isoforma α , que liga-se ao hormônio e ativa os genes responsivos aos GC, entre os quais os responsáveis pelos efeitos antiinflamatórios; e a isoforma β , que não se liga ao hormônio, mas liga-se ao DNA, e parece atuar como regulador negativo da ativação mediada pela isoforma α . Expressões deste tipo de receptor estão associadas aos casos de resistência a GC durante resposta inflamatória (revisto em Smoak e Cidlowski, 2004; Lu e Cidlowski, 2004; Rhen e Cidlowski, 2005). Após interação hormonal, o receptor α dissocia-se das proteínas adjacentes, sofre fosforilações que resultam na homodimerização do complexo receptor-agonista e na sua transmigração para o núcleo. Nesta localização, o complexo hormônio-receptor pode atuar das seguintes maneiras: 1) domínios da região N-terminal do receptor ligam-se a seqüências específicas do DNA (*glucocorticoid responsive element*, GRE), positivas ou negativas, nas regiões promotoras dos genes, com conseqüente iniciação ou repressão da

transcrição; 2) pela estabilização da estrutura do DNA, evitando a ligação de fatores de transcrição (revisto em Barnes, 1998); 3) pela indução de RNAases específicas, que diminuem a estabilidade das respectivas RNAs (Fessler e cols., 1996); 4) ou pela ligação com fatores de transcrição, como o AP-1 e o NF- κ B, inativando-os (McEwan e cols., 1997; Wissink e cols., 1998; Reichardt e Schutz, 1998; revisto em Goulding, 2004; Rhen e Cidlowski, 2005).

Adicionalmente, um outro mecanismo, conhecido como não genômico, tem sido proposto e parece estar relacionado a doses elevadas dos hormônios. Os efeitos são rápidos (segundos e minutos após terapia hormonal) e não são abolidos pela ação de inibidores da transcrição gênica e de síntese protéica. O mecanismo molecular ainda não é conhecido, mas há evidências da participação de um receptor de superfície (Hafezi-Moghadam e cols., 2002; Bartholome e cols., 2004) ou da simples interação dos GC com os fosfolípidos de membrana. Este último mecanismo poderia alterar as propriedades físicas e químicas de proteínas e receptores constitutivos na membrana (revisto em Goulding, 2004; Stellato, 2004). Neste último contexto, tem sido demonstrado que a capacidade metabólica de células imunes ativadas por citocinas é rapidamente reduzida após a administração de GC. As células apresentaram decréscimo no consumo de ATP e de substratos para reações de oxidação (Schmid e cols., 2000). A importância deste mecanismo não está bem estabelecida para os diversos efeitos dos GC, mas parece ter papel significativo em diferentes condições fisiopatológicas e terapêuticas, principalmente na artrite reumatóide após pulso-terapia (Hafezi-Moghadam e cols., 2002; revisto em Pitzalis e cols., 2002; Smoak e Cidlowski, 2004; Goulding, 2004; Rhen e Cidlowski, 2005).

Dentre os inúmeros efeitos antiinflamatórios, os GC secretados ou exógenos são moduladores importantes do recrutamento leucocitário para o foco de lesão, por interferirem diretamente sobre as propriedades de adesão e/ou locomoção celular ou por inibirem a secreção de mediadores inflamatórios envolvidos no processo (Flower e cols., 1986; Moraes e cols., 1987; Perretti e cols., 1989; Stenberg e cols., 1990; revistos em Morand e Leech, 2001; Pipitone e cols., 2001).

Nós demonstramos que os GC endógenos inibem a migração leucocitária por modularem as etapas iniciais da interação leucócito-endotélio. Ratos Wistar, machos, submetidos à adrenalectomia bilateral ou tratados com metopirona, inibidor da síntese de GC, apresentavam valores maiores de leucócitos “rollers” em vênulas pós-capilares da fáscia espermática interna *in vivo* que animais controles submetidos à falsa-cirurgia ou não manipulados. Modulação adicional do hormônio sobre o processo de aderência pôde ser inferida, já que o número de células aderidas à parede vascular na pós indução de resposta inflamatória local também foi maior em animais com deficiência hormonal e, ainda, a administração de anticorpo monoclonal anti $\beta 2$ integrina reverteu o efeito. O envolvimento do mecanismo genômico nestas modulações foi demonstrado pela similaridade dos quadros detectados em animais com deficiência hormonal e animais normais pré-tratados com RU 38486, antagonista de receptor citosólico de glicocorticóides, actinomicina D, inibidor da transcrição gênica, e cicloheximida, inibidor da síntese de proteínas (FARSKY, S.H.P.; SANNOMIYA, P.; GARCIA-LEME, J. **Secreted glucocorticoids regulate leukocyte-endothelial interactions in inflammation: A direct vital microscopic study.** *Journal of Leukocyte Biology*, 57:379-86, 1995).

Com a descrição do envolvimento primordial das moléculas de adesão na interação leucócito-endotélio, o papel dos CG sobre a expressão/atividade das mesmas começou a ser investigado. Reduções ou não alterações nas sínteses e/ou expressões destas moléculas *in vivo* foram observadas em condições de concentrações elevadas dos hormônios, determinadas por administrações de doses farmacológicas dos mesmos ou por diferentes estados de estresse (Leech e cols., 1998; Fassbender e cols., 1999; Torsteinsdottir e cols., 1999; Mori e cols., 2000; Kaname e cols., 2002; Weber e cols., 2001; 2004; Sale e cols., 2004).

Entretanto, a ausência de efeitos ou a inibição da expressão das moléculas parece não ser válida em todos os casos. Recentemente, um estudo com pacientes em choque séptico tratados com baixas doses de hidrocortisona quantificou as concentrações séricas de moléculas de adesão solúveis como

determinantes da ativação endotelial. O tratamento reduziu a concentração de E-selectina, aumentou significativamente a concentração de ICAM-1 e não alterou as elevadas concentrações de P-selectina e VCAM-1, indicando que, em baixas concentrações terapêuticas, os glicocorticóides controlam diferentemente a expressão de moléculas de adesão no endotélio e a presença de suas formas solúveis na circulação (Leone e cols., 2004). Estes efeitos discrepantes podem ser atribuídos às diferentes condições de ativação celular e às doses dos GC, uma vez que a eficácia do GC depende da sua ligação ao receptor. Mudanças na densidade, afinidade e atividade do receptor ocorrem em diferentes concentrações hormonais desencadeadas por doenças ou protocolos terapêuticos (Schaaf e Cidlowski, 2003).

Como já havíamos demonstrado que os GC endógenos poderiam modular a aderência por controlar a expressão/atividade das $\beta 2$ integrinas, passamos a utilizar nossos modelos animais para investigar os efeitos da secreção de GC sobre as demais moléculas de adesão responsáveis pela interação leucócito-endotélio.

Mostramos que um dos mecanismos dos GC endógenos envolvidos no recrutamento leucocitário é a modulação da expressão de L-selectina em leucócitos polimorfonucleares (PMN). A expressão da molécula em PMN obtidos da medula óssea de ratos adrenalectomizados foi significativamente menor que em células obtidas de ratos falso-operados ou não-manipulados, usados como controles. Este efeito pode ser um dos responsáveis pela aceleração na maturação destas células e pela conseqüente neutrofilia detectada em animais com deficiência hormonal. Inversamente, a expressão de L-selectina em PMN circulantes de animais adrenalectomizados estava aumentada, e esta alteração pode ser um dos determinantes para o aumento do número de leucócitos em comportamento "rolling" e pela maior migração para o foco de lesão por nós previamente demonstrados. Os efeitos moduladores sobre a expressão de L-selectina não foram decorrentes de modificações na expressão gênica, uma vez que ensaios de *reverse transcription-polymerase chain reaction* (RT-PCR) mostraram valores equivalentes de RNAm em leucócitos segmentados da medula óssea e

neutrófilos circulantes de animais adrenalectomizados e não-manipulados. Estes dados sugeriram que os GC endógenos modulam a clivagem da L-selectina. Cabe ressaltar que a modulação dos GC endógenos sobre as moléculas de adesão em PMN durante a interação leucócito-endotélio parece ser restrita à expressão de L-selectina, uma vez que a expressão de β_2 integrina em neutrófilos não foi alterada pela adrenalectomia e neutrófilos circulantes de animais adrenalectomizados possuem menor habilidade de aderirem à célula endotelial *in vitro* (CAVALCANTI, D.M.H.; LOTUFO, C.M.C.; BORELLI, P.; MARKUS, R.P.; FARSKY, S.H.P. *Adrenal deficiency alters mechanisms of neutrophil mobilization. Molecular and Cellular Endocrinology*, 2006).

No entanto, o controle sobre a aderência parece estar associado ao efeito dos GC sobre o endotélio. A habilidade da célula endotelial de animal adrenalectomizado aderir neutrófilos foi marcadamente maior que o endotélio de animais controles, falso-operados e não-manipulados. Esta maior habilidade foi reforçada pelas maiores expressões de ICAM-1, VACM-1 e PECAM-1 em células endoteliais da microcirculação do músculo cremaster de animais adrenalectomizados. Ainda, corroborando o controle preferencial sobre os PMN durante o comportamento "rolling", a expressão de P-selectina não foi modificada pela deficiência hormonal (CAVALCANTI, D.M.H.; LOTUFO, C.M.C.; MAIA, A.; CHUDZINSKI-TAVASSI, A.M.; MARKUS, R.P.; FARSKY, S.H.P. Endogenous control of glucocorticoids on endothelial adherence. Em preparação).

A modulação endógena dos glicocorticóides sobre a clivagem de L-selectina foi surpreendente, já que a maioria dos trabalhos sugere que a administração exógena dos hormônios promove inibição de sua síntese, especificamente por células circulantes (Weber e cols., 2001; 2004). Como já salientado anteriormente, a clivagem de L-selectina é dependente da ação de metaloproteinases de membranas (Zhao e cols., 2001; Smalley e Ley, 2005), dependente de vias de sinalização intracelulares, que envolvem proteína quinase C e a inibição de calmodulina (Fors e cols., 2001; Smalley e Ley,

2005). Assim, os efeitos dos glicocorticóides endógenos sobre as enzimas e as vias de sinalização citadas acima serão objetos de investigações futuras.

O envolvimento do mecanismo genômico nas ações dos GC sobre os eventos inflamatórios pelo bloqueio do seu receptor citosólico tem sido amplamente demonstrado. A estratégia mais utilizada é a administração do antagonista de receptor RU 38 486, comercialmente denominado de mifepristone (revisto em Pitzalis e cols., 2002). No que concerne às moléculas de adesão, tem sido amplamente demonstrado que os GC exógenos regulam a expressão gênica de diferentes moléculas de adesão pela interação com seu receptor citosólico (α) e subsequente interação com GRE no DNA, inibindo suas sínteses, ou por interferirem com ativação de fatores de transcrição, entre os quais o AP-1 e o NF κ B (revisto em Pitzalis e cols., 2002; Almawi e cols., 2002; Hayashi e cols., 2004; Caramori e Adcock, 2005).

Como tínhamos evidências da participação do receptor citosólico no controle da interação leucócito-endotélio *in vivo* (Farsky e cols., 1995), neste momento estamos investigando a importância deste receptor nos efeitos por nós detectados. Para tanto, estão sendo utilizados animais tratados com o RU 38486, sendo que os dados obtidos até o momento mostram que a neutrofilia e os efeitos sobre as moléculas de adesão (a clivagem da L-selectina e a super-expressão de ICAM-1, VCAM-1 e PECAM-1) são mediados pela interação dos hormônios ao receptor citosólico. Paralelamente, estamos avaliando se efeitos são decorrentes de modificações na sinalização mediada por AP-1 ou NF κ B. Verificamos que a translocação de NF κ B está reduzida em neutrófilos e aumentada na célula endotelial de animais adrenalectomizados em comparação aos mesmos tipos celulares de animais controles. Embora preliminares, estes dados sugerem diferentes vias de mediação intracelular nos efeitos dos GC endógenos em PMN e na célula endotelial.

3.2.2 Óxido Nítrico

O óxido nítrico (NO) é uma molécula importante na sinalização intra e intercelular em diferentes processos fisiológicos e patológicos. O NO regula o

tônus vascular, está relacionado à reatividade plaquetária, age como neurotransmissor e apresenta atividade citotóxica, particularmente em processos inflamatórios (Moncada e Higgs, 1991; Alderton e cols., 2001; Bogdan, 2001; Dawn e Bolli, 2002).

O NO é um radical livre de meia-vida muito curta. É sintetizado a partir da L-arginina e do oxigênio molecular, utilizando a nicotinamida adenina dinucleotídeo fosfato (NADPH) como doador de elétrons e grupamento heme, flavina mononucleotídeo (FMN), flavina adenina dinucleotídeo (FMD) e tetrahidrobiopterina (BH4) como cofatores da reação. A produção de NO é catalisada por um grupo de enzimas homodiméricas (130–160 kDa) denominadas óxido nítrico sintases (NOS) (revisto em Aktan, 2004). Na presença de oxigênio, o NO é rapidamente metabolizado em nitrito (NO_2^-) e nitrato (NO_3^-) (Mayer e cols., 1998).

Três isoformas distintas de NOS foram identificadas. Duas isoformas constitutivas (cNOS), com atividade regulada por cálcio e calmodulina, a saber: 1) NOS neuronal (nNOS) ou NOS I, descrita inicialmente no sistema nervoso central e periférico, e 2) NOS endotelial (eNOS) ou NOS III, presente nas células do endotélio vascular. Uma terceira isoforma denominada NOS induzível (iNOS) ou NOS II, cuja expressão é independente de cálcio, pode ser isolada de uma enorme variedade de células, após ação de mediadores inflamatórios e produtos bacterianos (revistos em Laroux e cols., 2000; Hickey e cols., 2001). As NOS variam quanto suas expressões e distribuições celulares e teciduais (revisto em Stuehr, 1999).

Inibidores e/ou doadores de NO são ferramentas amplamente empregadas em estudos experimentais que investigam a influência do mediador em diferentes processos fisiopatológicos, entre os quais, a resposta inflamatória. N^G -nitro-L-arginina metil éster (L-NAME) é um análogo da L-arginina que inibe de maneira competitiva e não específica as NOS (Moore e cols., 1990). Há evidências que os tempos de tratamentos e as dosagens de inibidores/doadores contribuem para as diferentes respostas nos incontáveis estudos experimentais, principalmente quanto ao efeito pressórico e no processo inflamatório (Navarro-Cid e cols., 1996; Forster e cols., 2001; Dedeoglu e cols., 2001; Quiroz e cols., 2001). Abordagens mais atuais, que

envolvem o emprego de agentes sintéticos capazes de inibirem especificamente cada uma das três formas enzimáticas e a utilização de animais geneticamente deficientes das NOS, têm fornecido dados complementares e mais sensíveis aos obtidos com os inibidores não seletivos e, ainda, corroboram uma complexa relação entre efeitos pró e antiinflamatórios e as diferentes atividades enzimáticas (Hickey e cols., 1997; Hobbs e cols., 1999; Ialenti e cols., 2000; Sanz e cols., 2001; Louin e cols., 2006; Jiménez-Altayó, 2006).

As ações pró-inflamatórias do NO estão relacionadas, possivelmente, à sua habilidade de aumentar a permeabilidade vascular e o edema por meio do incremento do fluxo sanguíneo local (revistos em Colasanti e Suzuki, 2000; Canty, 2000) e ao seu alto potencial lesivo, pela habilidade de interagir com espécies reativas de oxigênio (ERO), como ânion superóxido (O_2^-), levando à formação de peroxinitrito ($ONOO^-$). Este último mecanismo ocorre, predominantemente, em concentrações elevadas de NO proveniente da iNOS, e os produtos formados alteram a estrutura de proteínas por reagirem com biomoléculas, como tióis e aminas presentes em peptídeos e proteínas, resultando na formação de S-nitrosotióis (revistos em Bogdan, 2001).

As ações antiinflamatórias estão relacionadas à modulação da migração leucocitária, controlando as interações dos leucócitos circulantes ao endotélio vascular (Kubes e cols., 1991; Arndt e cols., 1993; Granger e Kubes, 1994). No entanto, os dados são controversos e não estão completamente elucidados.

Especificamente na artrite humana ou experimental, tem se demonstrado que o NO possui papel pró-inflamatório, uma vez que animais tratados com L-NAME apresentaram valores de células brancas nas cavidades articulares menores que os encontrados em animais controles (McCartney-Francis e cols., 1993; Stefanovic-Racic e cols., 1994; Mello e cols., 1997; Palácios e cols., 1999). O papel pró-inflamatório do NO foi confirmado pela maior produção de NO_2^-/NO_3^- no líquido sinovial de pacientes com artrite reumatóide quando comparados a pacientes portadores de doenças como osteoatrose (Novaes e cols., 1997) e pelo aumento de expressão de iNOS, principalmente nas células mononucleares desse infiltrado (Borderie e cols., 1999).

Os efeitos pró-inflamatórios do NO foram por nós corroborados no modelo de inflamação intraarticular induzida pelo veneno de serpentes *Bothrops jararaca* (VBj) em coelhos New Zeland, adultos. Animais tratados com L-NAME por período prolongado de tempo (14 dias, 20 mg/kg, via oral) ou agudamente (30mg/Kg, i.v., 30 min antes da injeção do VBj) apresentaram redução no extravasamento protéico e no recrutamento leucocitário para a cavidade articular 4 horas após a injeção do veneno em comparação com animais tratados com D-NAME, enantiômero inativo do L-NAME, ou com salina. As diminuições nos parâmetros inflamatórios foram acompanhadas pela redução parcial na concentração de $\text{NO}_2^-/\text{NO}_3^-$, metabólitos estáveis do NO, no fluido sinovial. Cabe ressaltar que o tratamento dos animais com D-arg-[Hyp³, Thi⁵, Dtic⁵, Oic⁸]-bradykinin (HOE 140), antagonista de receptor B2 de bradicinina (0,3 mg/kg; 30 min antes da administração do VBj), provocou o mesmo efeito antiinflamatório do L-NAME e redução na concentração dos metabólitos do NO no líquido sinovial. Em conjunto, os resultados sugeriram papel pró-inflamatório e interrelação da bradicinina e NO nos efeitos pró-inflamatórios na artrite induzida pelo veneno de serpentes *Bothrops jararaca* (GUZZO, M.L.; FARSKY, S.H.P.; DE NUCCI, G.; ANTUNES, E.; SILVA, M.A.G. VERÍSSIMO DE MELLO, S.B. Role of kinins and nitric oxide on the rabbit arthritis induced by *Bothrops jararaca* venom. Toxicon, 38(11):1535-1546, 2000). Posteriormente, o papel do receptor B2 de bradicinina e sua relação com a produção de NO na indução da artrite foram ratificados, sendo a relação positiva entre NO e prostaglandina E₂ (PGE₂) demonstrada pela redução da concentração deste eicosanóide no líquido sinovial dos animais tratados com L-NAME ou HOE-140 (VERÍSSIMO DE MELLO, S.B.; GUZZO, M.L.; LISBOA, L.F.S.; FARSKY, S.H.P. Pharmacological characterization of arthritis induced by *Bothrops jararaca* venom in rabbits. A positive cross-talk between bradykinin, nitric oxide and prostaglandin E₂ (BjV). Mediators of Inflammation 11 (1):13-6, 2002).

Os primeiros trabalhos relacionados aos efeitos do NO sobre a interação leucócito-endotélio mostraram que a superfusão do mesentério de gatos ou de ratos com doadores de NO acarretava diminuição nos fenômenos de "rolling" e

aderência e, que por outro lado, a superfusão com inibidores inespecíficos das NOS promovia aumento nos eventos de interação leucocitária. Este último efeito era revertido pela suplementação de L-arginina após a superfusão com L-NAME. Em conjunto, estes dados demonstravam que o NO produzido em condições basais atuava como modulador da interação leucócito-endotélio e, conseqüentemente, permitiam inferir o NO constitutivo como um modulador endógeno da migração leucocitária (Kubes e cols., 1991; Arndt e cols., 1993; Granger e Kubes, 1994). Os possíveis mecanismos estavam relacionados à inibição ou ao consumo de substâncias reativas de oxigênio, entre as quais o superóxido, com conseqüente diminuição na ativação de mastócitos (Kubes e cols., 1993; Gaboury e cols., 1993). Da mesma forma que o NO constitutivo, uma linha de evidências na literatura mostra que concentrações elevadas de NO, gerado a partir da ativação da iNOS em condições inflamatórias, inibem o recrutamento leucocitário para o foco de lesão. Um dos mecanismos envolvidos parece ser também a modulação sobre a interação leucócito-endotélio, já que ensaios em animais deficientes de iNOS ou tratados agudamente com inibidores específicos da iNOS mostraram valores aumentados de leucócitos “rollers” ou aderidos em relação aos animais controles (revisto em Hickey e cols., 2001; Secco e cols., 2003).

As demonstrações do papel do NO sobre a interação leucócito-endotélio geraram a investigação imediata do papel deste mediador sobre a expressão de moléculas de adesão envolvidas neste processo. Apesar das indicações do NO inibir a síntese/expressão destas moléculas, a análise dos dados da literatura mostra a discordância de resultados. A administração de anticorpos monoclonais antimoléculas de adesão em animais tratados agudamente com L-NAME e a determinação de suas expressões em células pré-incubadas *in vitro* com inibidores da eNOS ou com doadores de NO indicaram a habilidade do mediador obtido da NOS constitutiva modular a expressão de β_2 integrinas (Kubes e cols., 1991; 1994; Mitchell e cols., 1998), ICAM-1 (Niu e cols., 1994), VCAM-1 e P-selectina (Lefer e cols., 1999; Zampolli e cols., 2000). No entanto, os dados obtidos em animais deficientes de eNOS nem sempre confirmaram as observações descritas acima. Recentemente foi demonstrado que células endoteliais de camundongos geneticamente deficientes de eNOS não

apresentaram diferenças na aderência de leucócitos e na expressão de moléculas de adesão em relação à célula de animais selvagens. Ainda, após estimulação com citocinas, a expressão de moléculas de adesão e aderência de leucócitos foi equivalente nos dois tipos celulares, indicando que a deficiência de NO constitutivo não afetava a capacidade de adesão e nem suprimia de maneira autócrina a capacidade de ativação endotelial (Kuhlencordt e cols., 2004).

Visto a discrepância de resultados quanto aos efeitos do NO sobre os fenômenos que regem a interação leucócito-endotélio, e os resultados por nós obtidos anteriormente, passamos a investigar os mecanismos envolvidos no efeito facilitador do NO sobre o influxo leucocitário. O modelo animal passou a ser o rato Wistar, macho, adulto, tratado com L-NAME, com seu enantiômero inativo (D-NAME) ou com salina estéril (14 dias, 20 mg/kg, via oral). Da mesma forma que observado em coelhos, a migração leucocitária e o aumento de permeabilidade vascular no tecido subcutâneo dorsal inflamado (modelo da bolsa de ar 6 horas após injeção do VBj) estavam reduzidos nos animais tratados com L-NAME quando comparados aos animais controles (tratados com D-NAME ou salina). A eficácia do tratamento foi demonstrada pela redução parcial da concentração de $\text{NO}_2^-/\text{NO}_3^-$ no exsudato inflamatório. Estudos de microscopia intravital mostraram reduções significativas no número de leucócitos “rollers” e de leucócitos aderidos ao endotélio em vênulas pós-capilares da fáscia espermática interna, tanto em condições basais, como na vigência de estimulação com o VBj. A mensuração do fluxo sanguíneo de arteríolas e vênulas pós-capilares e da reatividade vascular frente à noradrenalina, acetilcolina ou histamina mostrou que os efeitos não eram decorrentes de modificações na hemodinâmica microvascular. A quantificação do número de mastócitos adjacentes às vênulas pós-capilares e sua capacidade de desgranulação frente ao composto 48/80 também descartou o possível papel do NO sobre estas células. No entanto, o número de leucócitos PMN circulantes estava significativamente aumentado em animais tratados com L-NAME, mas a quantificação dos números de leucócitos na medula óssea mostrou que a neutrofilia não era proveniente de alterações na produção e/ou

maturação destas células. Estes dados indicavam que a neutrofilia presente em animais tratados com L-NAME poderia ser decorrente da inabilidade de neutrófilos interagirem com a parede vascular, com sua conseqüente manutenção na circulação. Os resultados subseqüentes, que mostraram redução na expressão de L-selectina em PMN circulantes de animais tratados com L-NAME, reforçaram esta hipótese. É importante salientar que esta mesma redução foi observada em leucócitos segmentados na última etapa de maturação na medula óssea sob estimulação com Vβ₁, indicando que a ausência de NO por período de tempo prolongado altera as propriedades adesivas de PMN, facilitando seu recrutamento para circulação e sua manutenção no compartimento periférico. Entretanto, o efeito parece ser restrito à L-selectina, uma vez que a expressão de β₂ integrina não foi alterada em células de ambos os compartimentos (FARSKY, S.H.P.; BORELLI, P.; FOCK, R.A.; PROTO, S.Z.; FERREIRA, J.M.C.; MELLO, S.B.V. **Chronic blockage of nitric oxide biosynthesis in rats: effect on leukocyte endothelial interaction and on leukocyte recruitment.** Inflammation Research, 53:442-542, 2004).

Os dados dos estudos da interação leucocitária foram opostos aos encontrados na literatura. No entanto, estes foram obtidos em condições de inibição das NOS por período prolongado de tempo, protocolo de inibição enzimática até então não utilizado para este tipo de estudo. Propusemos que no protocolo experimental por nós empregado ocorrem alterações em leucócitos ainda na medula óssea e, portanto, a discrepância entre nossos dados e os da literatura, provém, possivelmente, de mecanismos de ação distintos entre os diferentes protocolos de inibição das NOS. Dados obtidos recentemente em nosso laboratório mostraram que a inibição de migração leucocitária por nós detectada na vigência de inibição da síntese de NO é dependente também da menor expressão de PECAM-1, já que animais tratados com L-NAME apresentaram valores significativamente menores da expressão da molécula no endotélio que animais controles (Hebeda e cols., 2005).

Ultimamente, tem-se verificado papel importante da nNOS na regulação da migração leucocitária, principalmente em condições de inibição da eNOS (Beck e cols., 2004). Neste contexto, camundongos deficientes de eNOS, nos quais a interação dos leucócitos circulantes ao endotélio de vênulas pós-capilares do músculo cremaster não foi diferente dos animais controles, quando tratados com inibidor seletivo da nNOS, apresentaram um aumento significativo no número de leucócitos aderidos à parede vascular, sugerindo que a nNOS seja responsável pela modulação da adesão de leucócitos na ausência de eNOS (Sanz e cols., 2001). Ainda, Beck e cols. (2004) demonstraram que, em modelo de colite em camundongos, a inflamação e a mortalidade em camundongos deficientes de nNOS foi maior se comparadas com as obtidas em animais controles. Por outro lado, em animais deficientes de eNOS ou de iNOS o processo inflamatório foi menos significativo que em animais controles. Desta forma, sugeriu-se que o NO proveniente da nNOS parece exercer efeito protetor sobre o desenvolvimento do processo inflamatório, enquanto o NO proveniente da iNOS ou eNOS parece exercer efeito facilitador do processo.

Em conjunto, os dados da literatura sugerem que NO proveniente das diferentes NOS está envolvido no controle da interação leucócito-endotélio e conseqüentemente, no recrutamento leucocitário. No entanto, a participação efetiva de cada enzima no processo, especialmente em condições de inibição específica de uma delas, não está determinada.

Como L-NAME é um inibidor inespecífico das NOS e nossos resultados mostram que o tratamento não abole a produção de NO (reduções de 50% no sangue e nos exsudatos inflamatórios), a continuidade destes estudos envolve avaliar o potencial do protocolo de tratamento com L-NAME de inibir cada uma das enzimas e correlacionar a fonte do NO remanescente em nossos ensaios. Para tanto, ensaios de RT-PCR estão sendo realizados em leucócitos e célula endotelial para se aquilatar a expressão de iNOS, eNOS e nNOS.

3.3 Efeitos de xenobióticos sobre a mobilização leucocitária

3.3.1 Compostos fenólicos derivados do metabolismo do benzeno

As intoxicações por solventes orgânicos, entre os quais o benzeno, afetam vários sistemas endógenos por diferentes mecanismos e intensidades. De maneira geral, os efeitos se manifestam por alterações no sistema nervoso central, comprometimento hepático e renal, doenças respiratórias, doenças do tecido conjuntivo, alterações marcadas na hematopoese e no sistema imune, com a conseqüente gênese de anemias e leucemias, além de neoplasias de diferentes origens (revisto em Schenker e Jacobs, 1996; Gralewics, 1999; Garabrant e Dumas, 2000; Nietert e Silver 2000; Waidynatha e cols., 2004).

O benzeno é derivado do petróleo e empregado em diferentes ramificações industriais, principalmente como solvente para tintas, colas, vernizes, materiais de limpeza e na indústria do couro. Pelos seus graves efeitos prejudiciais à saúde, o uso do benzeno passou a ser controlado na última década e sua utilização ficou restrita às indústrias que o produzem ou utilizam-no em processos de síntese química, na composição de combustíveis e derivados do petróleo, ou em análises laboratoriais; neste último caso, somente quando não for possível sua substituição (BRASIL, 2001).

Dentre as intoxicações pelo benzeno, as mais comuns são as ocupacionais. No entanto, não são irrelevantes os casos de acidentes domésticos e de contaminação ambiental, devido à sua volatilidade. Neste último caso, vale ressaltar que o benzeno é um contaminante comum do meio ambiente, comumente gerado pelo processamento, combustão e evaporação de petróleo, além de ser um componente da fumaça do cigarro (Rothman e cols., 1996; Muzyka e cols., 1998; Javelaud e cols., 1998; Bogadi-Sare e cols., 2000; Boscolo e cols., 2000; revisto em Nietert e Silver, 2000).

Uma vez absorvido, principalmente pelas vias aérea e dérmica, o benzeno é metabolizado extensivamente pelo fígado. O passo inicial da biotransformação do benzeno é a oxidação do benzeno a epóxido (óxido de benzeno) pela ação da enzima CYP2E1 (Chepiga e cols., 1991; revisto em Snyder, 2004). O óxido de benzeno é subseqüentemente metabolizado por três

vias, a saber: 1) conjugação com a glutathiona, formando o ácido pré mercaptúrico, que posteriormente é convertido a ácido fenil mercaptúrico; 2) rearranjo não enzimático, com formação de fenol (FE), e 3) metabolização pela epóxido hidrolase, gerando os metabólitos FE, catecol (CAT), hidroquinona (HQ) e 1,2,4, benzenotriol hidroxilados (Seaton e cols., 1994). Estes compostos fenólicos são detoxificados por conjugação com sulfato, glutathiona ou glucuronídeo. Adicionalmente, tem sido bem descrito que os compostos fenólicos formados no fígado são transportados através do sangue para a medula óssea, onde se constituem substratos para peroxidases, como a mieloperoxidase (MPO) e ciclooxigenase (COX). Nesta localização são hidroxilados e/ou oxidados a quinonas e semiquinonas, metabólitos com alto potencial tóxico, por reagirem com estruturas nucleofílicas como DNA, RNA e proteínas; ou por gerarem espécies reativas de oxigênio que causam o estresse oxidativo (Thomas e cols., 1990; Kolachana e cols., 1993; Ross e cols., 1996). Também na medula o processo de detoxificação envolve a conjugação com sulfatos, glutathiona ou glicuronídeos. No entanto, a sulfatação não é um mecanismo potente de detoxificação, devido à presença de níveis elevados de sulfatase na medula óssea, que podem quebrar os conjugados sulforados para fenóis livres (Low e cols., 1995). Contribuindo para detoxificação, a enzima NAD(P)H: quinona oxidoreductase (NQO1) presente na medula reduz as quinonas e semiquinonas a benzoquinonas (Ross e cols., 2000; Bauer e cols., 2003). Tem sido proposto que a relação entre a concentração de MPO e NQO1 pode representar o balanço entre bioativação e detoxificação, influenciando, desta forma, susceptibilidade à toxicidade por compostos fenólicos (Ganousis e cols., 1992). A biotransformação do benzeno é crucial para sua toxicidade. Estudos experimentais *in vivo* e *in vitro* têm demonstrado que as concentrações dos metabólitos do benzeno necessárias para os efeitos tóxicos ao sistema imune são menores que as requeridas pelo benzeno (Irons e cols., 1985; Ewens e cols., 1999; Fabiani e cols., 2001) e que polimorfismos nas enzimas envolvidas no processo determinam diferenças no quadro da intoxicação (Kim e cols., 2004; Ross, 2005; Fustinoni e cols., 2005)

Visto a relevância da toxicidade do benzeno, os métodos analíticos empregados para monitorização biológica devem ser precisos e apresentar alta

sensibilidade para identificação de biomarcadores, mesmo em baixas condições de exposição. O emprego de método analítico inadequado pode fornecer dados errôneos na determinação de parâmetros toxicológicos importantes, como a relação entre exposição, dose-resposta e o efeito ("end-point") (Ong e Lee, 1994). Desta forma, a investigação analítica caminha no sentido de desenvolver métodos mais sensíveis e precisos para determinação de marcadores biológicos que indiquem alterações fisiológicas precocemente.

Uma grande variedade de métodos analíticos é utilizada para separação, quantificação e identificação dos produtos de biotransformação do benzeno (Schad e cols., 1992). A determinação da concentração biológica do benzeno e de seus produtos de biotransformação pode ser realizada em diferentes matrizes biológicas, entre as quais o ar exalado, o sangue e a urina. Análises do benzeno na respiração podem ser conduzidas com tubo coletor de ar exalado e sua subsequente análise direta pode ser realizada por cromatografia gasosa (CG) ou cromatografia gasosa acoplada a espectrometria de massa (CGMS) (Brega e cols., 1990; Schad e cols., 1992; Lee e cols., 1993; Laurens e cols., 2002; Waidynatha e cols., 2004). Entretanto, análises em amostras de sangue ou urina são comumente empregadas (Ong e Lee, 1994). A preparação das amostras de urina e sangue para determinação dos compostos fenólicos geralmente envolve uma fase de hidrólise ácida ou enzimática, seguida de extração líquido-líquido em solvente orgânico. Nestes casos, os métodos espectrofotométricos são empregados para determinação de componentes fenólicos totais, enquanto que os cromatográficos são apropriados para determinação seletiva destes compostos. Acetilação, benzilação, benzoilação, alquilação, sililação são métodos que têm conquistado terreno na literatura para análises de cromatografia gasosa de fenóis (Fiamegos e cols., 2003).

Recentemente, desenvolvemos e validamos o método de CGMS para determinação simultânea de FE, HQ e CAT na urina, no qual a obtenção dos analitos da matriz foi realizada por hidrólise ácida dos compostos fenólicos conjugados e subsequente extração usando microextração em fase sólida. Para tanto, os analitos foram extraídos pela submersão de uma fibra de

coberta de poliacrilato polar (85 μm) na urina (pH 3,0, ajustado com ácido acético glacial) por 20 minutos com agitação magnética. Os compostos extraídos na fibra foram expostos ao reagente hexametildisilazano em fase de vapor por 20 minutos para obtenção dos derivados trimetilsililados. Este procedimento de derivação permitiu a formação de compostos mais apropriados para a análise cromatográfica, sem afetar a meia-vida da fibra. A espectrometria de massa foi realizada no modo de monitoramento seletivo de íons. Os limites de detecção foram 0,3 $\mu\text{g/mL}$ para o FE; 0,15 $\mu\text{g/mL}$ para a HQ e 0,02 $\mu\text{g/mL}$ para o CAT. As precisões inter e intra-ensaios também foram verificadas (coeficiente de variação menor que 8%) com o uso de padrões deuterados (LOURENÇO, E.L.B.; FERREIRA, A.; PINTO, E.; YONAMINE, M.; FARSKY, S.H.P. On-Fiber derivatization of SPME extracts of Phenol, Hydroquinone and Catechol with GC-MS detection. Chromatographia, 2006).

Como já salientado anteriormente, os efeitos da intoxicação pelo benzeno sobre o sistema imune são graves e complexos e envolvem efeitos na produção, maturação e nas diferentes funções das células envolvidas nas respostas imunes (Woo e cols., 1989; Morimoto, 1993; Robinson e cols., 1997; Farris e cols., 1997; Bogadi-Sare e cols., 2000; Velasco-Lezama e cols., 2001).

O benzeno e seus metabólitos, especialmente as quinonas, são estimuladores *in vitro* e *in vivo* da diferenciação de granulócitos e promovem supressão da hematopoese *in vivo* (revisto em De Caprio, 1999). Tais observações provêm de estudos clínicos ou experimentais que mostraram que a exposição ao benzeno ou a seus metabólitos reduz o número de células da linhagem mononuclear no sangue periférico (Gill e cols., 1980; Snyder e cols., 1980; Green e cols., 1981; Cronkite e cols., 1989; Farris e cols., 1997), na medula e em órgãos linfóides secundários (Robinson e cols., 1997; Farris e cols., 1997; Kalf e cols., 2000; Boscolo e cols., 2000; Bogadi-Sare e cols., 2000; Velasco-Lezama, 2001).

A granulocitose induzida pelo benzeno, demonstrada *in vivo* por neutrofilia, tem sido atribuída à incompleta diferenciação celular, de mieloblasto a mielócito, com aumento no número de mielócitos. Este efeito ocorre devido à

biotransformação do benzeno, sendo que a oxidação da HQ a p-benzoquinona pela MPO parece ser etapa fundamental. Os mecanismos envolvidos não estão bem determinados, mas parecem ser dependentes de diferentes vias de ativação do ciclo celular. Foi demonstrado *in vitro* que o solvente suprime o ciclo celular mediado por p53 e aumenta a expressão de p21, um inibidor de quinase ciclo dependente (Yoon e cols., 2002). Adicionalmente, o mecanismo da incompleta diferenciação granulocítica parece ser dependente da ativação de proteína quinase C e da subsequente ativação da via da 5-lipoxigenase e produção de leucotrieno D4 e sua interação ao receptor (Hazel e cols., 1996a; Hazel e Kalf, 1996). Adicionalmente, a HQ oxidada pela MPO também inibe a apoptose de mieloblastos, contribuindo para o efeito (Hazel e cols., 1996b). Além da modificação na produção e maturação de granulócitos, o benzeno e seus metabólitos fenólicos estimulam o “burst” respiratório de granulócitos humanos com geração de metabólitos ativos do oxigênio, por meio de um mecanismo que envolve fosfolipase C cálcio-dependente, fosfolipase D e proteína C quinase (Myhre e Fonnum, 2001). Este trabalho ratifica observações prévias de MacEachern e cols. (1992) que demonstraram que granulócitos e monócitos da medula óssea de camundongos intoxicados por benzeno apresentam produção de peróxido de hidrogênio e atividade quimiotóxica aumentadas.

Visto que os metabólitos fenólicos do benzeno alteram a maturação e as funções de leucócitos polimorfonucleares, investigamos se a exposição por período prolongado de tempo a estes compostos afeta o recrutamento destas células na vigência de resposta inflamatória inata.

Para tanto, ratos Wistar machos foram expostos à HQ, FE ou veículo pela via intraperitoneal, na dose de 50 mg/kg, 13 doses, uma vez ao dia com intervalos de 2 dias a cada 5 doses, sendo os ensaios realizados 24 horas após a última dose. Animais expostos ao veículo foram usados como controles. Quantificações dos números de leucócitos presentes na medula óssea ou na circulação mostraram que somente a exposição à HQ acarretou neutrofilia, devido à acelerada mobilização destas células da medula, já que o número de células segmentadas na última etapa de maturação foi menor em animais

expostos à HQ que animais expostos ao FE ou ao veículo. Imagens da rede microcirculatória do mesentério, obtidas por ensaios de microscopia intravital, mostraram que os leucócitos circulantes de ratos expostos à HQ possuíam maiores capacidades de rolar e aderir à parede de vênulas pós-capilares. A investigação da capacidade de contração e dilatação dos vasos da microcirculação frente à noradrenalina e acetilcolina, respectivamente; a quantificação do número de mastócitos adjacentes às vênulas e suas habilidades de desgranulação em resposta ao composto 48/80; e a determinação da expressão de moléculas de adesão no endotélio mostraram que as alterações na interação leucócito-endotélio não eram dependentes de modificações na reatividade vascular, nem da ação de mediadores químicos secretados por mastócitos, nem da expressão de ICAM-1, VCAM-1 ou PECAM-1 no endotélio. No entanto, a maior interação com a parede vascular parece ser dependente, pelo menos em parte, da ativação dos neutrófilos circulantes, visualizados pelo decréscimo e acréscimo das expressões de L-selectina e $\beta 2$ integrinas, respectivamente. A maior mobilização de neutrófilo da medula para o compartimento periférico e seu estado de ativação alterado pode ter contribuído pelo maior número de neutrófilos no exsudato inflamatório coletado de um processo agudo (6 horas após injeção de glicogênio de ostra no subcutâneo dorsal - modelo da bolsa de ar). Em conjunto, os dados obtidos mostraram que a HQ altera a mobilização e ativação de neutrófilos, que resultam em uma reação exacerbada em resposta ao trauma inespecífico. Cabe salientar que apesar do FE ser biotransformado à HQ, a exposição ao FE resultou somente em aumento no número de leucócitos em comportamento "rolling", o qual não foi suficiente para alterar o processo inflamatório (MACEDO, SM; LOURENÇO, EL; BORELLI, P.; FOCK, RA; FERREIRA JM Jr; FARSKY, SH. Effect of in vivo phenol or hydroquinone exposure on events related to neutrophil delivery during an inflammatory response. Toxicology, 2006).

Além dos efeitos sobre a série granulocítica, o benzeno e seus derivados fenólicos alteram a produção e/ou maturação de células da linhagem mononuclear, especialmente os diferentes tipos de linfócitos, prejudicando a

mobilização destas células para a circulação e suas funções específicas (MacEachern e cols., 1992; Farris e cols., 1997; Yoon e cols., 2001). O papel da HQ nestes efeitos tem sido demonstrado após sua adição a células precursoras da produção linfocitária em cultura (King e cols., 1987; 1989; Li e cols., 1996; 1997; 1998; Pyatt e cols., 1998; 2000; McCue e cols., 2000; 2003) e pela redução do número de células B progenitoras na medula e no baço de camundongos expostos à HQ ou ao catecol (Wierda e Irons, 1982). Os mecanismos envolvidos são complexos e não bem compreendidos, e parecem depender das concentrações dos metabólitos nos diferentes compartimentos de maturação e função celular. Tem sido demonstrado que a HQ inibe a progressão do ciclo celular (Li e cols., 1996, 1997; McCue e cols., 2003) e a atividade da ribonucleotídeo redutase (Li e cols., 1998; McCue e cols., 2000), bloqueando conseqüentemente, a linfoproliferação de linfócitos. Ainda, a administração *in vitro* de HQ reduz a produção de interleucina 2 (IL-2) e TNF por células T e B cells, respectivamente, em um mecanismo dependente de NF-kappa B em resposta a vários estímulos (Pyatt e cols., 1998, 2000).

Com as fortes evidências dos metabólitos fenólicos do benzeno, especialmente a HQ, sobre a série linfocitária, investigamos o papel deste metabólito sobre a capacidade de migração leucocitária, agora em vigência de um processo alérgico no pulmão.

Para tanto, os animais foram expostos à HQ (50mg/kg, i.p., 16 doses, diariamente, com intervalos de 2 dias a cada 5 doses) ou ao veículo, nas mesmas condições. No décimo dia após o início das exposições, os animais foram sensibilizados pela Ovalbumina (OVA; 10 µg/10 mg de hidróxido de alumínio, via i.p.) e desafiados 13 dias mais tarde pela inalação do antígeno (solução de OVA a 1% em tampão fosfato salina, durante 15 minutos). Os experimentos foram sempre realizados 24 horas após a última dose de HQ. A quantificação do número de leucócitos no lavado bronco-alveolar (LBA), coletado 24 horas após o desafio, mostrou que a exposição à HQ provocou redução acentuada no número de leucócitos, representada por decréscimos nos números de leucócitos mononucleares (MN) e PMN. A determinação da atividade da enzima MPO no tecido pulmonar, que reflete a quantidade de

neutrófilos no tecido, mostrou que a exposição à HQ comprometeu a chegada destas células no pulmão, uma vez que os valores encontrados em animais expostos à HQ e ao veículo foram equivalentes. Estes efeitos não foram dependentes de modificações no número de leucócitos circulantes, nem da expressão das moléculas de adesão no leucócito (L-selectina e $\beta 2$ integrina) e no endotélio (ICAM-1, VCAM-1 e PECAM-1). No entanto, os ensaios subsequentes mostraram que o efeito parece ser dependente de comprometimento na desgranulação de mastócitos mediada por IgE. Esta hipótese foi levantada pelas seguintes observações: 1) a contração da traquéia *in vitro* estava reduzida em tecidos provenientes de animais expostos à HQ e 2) a resposta anafilática cutânea foi menor em animais normais que receberam previamente soro de animais sensibilizados e expostos à HQ que em animais que receberam soro de animais sensibilizados e expostos ao veículo. É importante salientar que a proliferação de linfócitos do baço em resposta à OVA ou à Concanavalina A foi similar em células obtidas de animais expostos à HQ ou ao veículo, excluindo possíveis efeitos sobre os mecanismos intracelulares envolvidos nas respostas específicas ou não específicas ao antígeno. Em conjunto, nossos dados mostram que a exposição à HQ, em doses que não modificam o leucograma, diminui o recrutamento leucocitário durante uma resposta alérgica, dependente da desgranulação mastocitária mediada por IgE (MACEDO, S.M.D; VAZ, S.C.M.; LOURENÇO, E.L.B.; DA GLÓRIA DE SOUZA, M.; CAVRIANI, G.; LIGEIRO-OLIVEIRA, A.P.; ALMEIDA, S.R.; TAVARES DE LIMA, W.; FARSKY, S.H.P. *In vivo hydroquinone exposure inhibits leukocyte migration during an allergic lung inflammation in rats. Toxicology and Applied Pharmacology*, enviado para publicação).

É de grande relevância ressaltar que, aparentemente, as doses dos compostos fenólicos usados nos trabalhos acima não foram tóxicas para os animais, uma vez que não observamos perda de peso corporal; o exame histológico do rim e fígado não evidenciou alterações em ambos os tecidos e as concentrações de alanina amino transaminase, aspartato aminotransferase

e creatinina foram equivalentes em animais expostos à HQ e ao veículo (estando dentro da faixa de normalidade).

Em conjunto, os dados dos trabalhos apresentados acima mostram que a exposição a agentes fenólicos por período prolongado de tempo interfere na migração leucocitária para o foco de lesão. No entanto, os efeitos são distintos, dependendo da origem do processo inflamatório e do tecido lesado. É possível que estas diferenças estejam associadas aos diferentes mediadores químicos e às sinalizações intracelulares dos processos. Neste sentido, umas das linhas que trilham nossos estudos é a investigação dos efeitos dos xenobióticos sobre a produção de óxido nítrico, uma vez que tem sido demonstrado que o mediador participa de reações oxidativas após exposição ao benzeno e a seus produtos de biotransformação (Laskin e cols., 1995; 1996; Wu e cols., 1998; Tuo e cols., 1999a,b; Fabiani e cols., 2001) e sobre a secreção de interleucina-4 (IL-4) e interleucina-13 (IL-13) por linfócitos T, mediadores envolvidos na sinalização da produção de anticorpos.

A literatura mostra que o FE não apresenta efeito sobre alguns parâmetros envolvidos na gênese da intoxicação por benzeno, mas que é capaz de atuar sinergicamente com a HQ (Kolachana e cols., 1993; Moran e cols., 1996; Henschler e cols., 1996). Como demonstramos que o FE não foi capaz de interferir com a migração neutrofílica na vigência de resposta inflamatória aguda inata, uma outra linha de investigação do laboratório visa avaliar os efeitos da associação dos compostos fenólicos nos parâmetros por nós observados. Para tanto, caracterizamos a menor dose de HQ capaz de interferir com o processo (5 mg/kg) e a injetaremos com a mesma dose de FE, utilizando os mesmos delineamentos experimentais descritos acima.

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5. ANEXOS

Pág.

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Secreted glucocorticoids regulate leukocyte-endothelial interactions in inflammation. A direct vital microscopic study

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Abstract: Leukocytes come into intimate contact with the venular endothelium as they extravasate from blood to the interstitium during inflammation. In exteriorized tissues, the number of leukocytes rolling along the vessel wall was markedly increased in adrenalectomized and metyrapone-treated animals, relative to sham-operated and normal animals. During the development of an acute, local inflammatory response, rollers were numerically decreased and a stronger adhesion of the cells to the endothelium, with a concomitant migration into tissues, was observed. Adhesion and migration were much more marked in adrenalectomized and metyrapone-treated animals than in controls, suggesting that secreted glucocorticoids exert a suppressive effect on leukocyte-endothelial interactions. The increased number of rolling leukocytes in adrenalectomized and metyrapone-treated animals apparently resulted in more cells available to migrate into inflamed tissues. The effect appears to involve receptor occupancy and induction of gene expression because normal animals receiving the steroid antagonist RU 38 486, actinomycin D, or cycloheximide behaved as adrenalectomized or metyrapone-treated animals. Administration to adrenalectomized animals of a monoclonal antibody to the membrane glycoprotein complex CD18 did not affect the number of rolling cells, but dramatically reduced the number of adherent or migrated leukocytes. It is suggested that secreted glucocorticoids, in addition to an effect on rolling behavior of circulating leukocytes, might also modulate the activity of the glycoprotein complex CD18 on white blood cells. The ultimate consequence is a restrictive effect on cell emigration in inflammation. *J. Leukoc. Biol.* 57: 379-386; 1995.

Key Words: adhesion molecules • CD11/CD18 • microcirculation • RU 38 486

INTRODUCTION

The principal cellular events that constitute the phenomena of inflammation have been clearly described in studies employing direct observation of microvessels. These studies indicate that leukocytes always become adherent to the vascular endothelium before penetrating the vessel wall and accumulating at sites of injury. It is now established that specific adhesion glycoproteins expressed on the surface of leukocytes and endothelial cells play a relevant role in the adhesion phenomenon [1-9]. Agents capable of interfering with leukocyte-endothelial interactions may, therefore, exhibit antiinflammatory properties.

Administration of glucocorticoids results in a suppressive action on neutrophil mobilization during inflammation. Because locomotion of leukocytes remains unaffected after exposure of the cells to glucocorticoids [10], the steroids, at

least in part, appear to inhibit leukocyte accumulation in an inflamed area by blocking the production of mediators, particularly cytokines, involved in the recruitment process [11-13]. More recent findings support the view that glucocorticoids are also likely to directly inhibit the expression of adhesive molecules by stimulated endothelium [8, 14].

Blood levels of glucocorticoids are consistently and markedly increased during the development of inflammation [15-19]. The steroids, secreted in larger amounts at the early stages of an inflammatory reaction, are thought to regulate the initiation and subsequent course of inflammatory responses [17, 20, 21].

These observations prompted us to investigate the influence of endogenous glucocorticoids on cellular events occurring in inflammation. The data to be presented suggest that glucocorticoids, through receptor-mediated genomic actions, modulate leukocyte-endothelial interactions and, consequently, leukocyte accumulation in an injured area.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing between 200 and 220 g at the beginning of the experiments were used. The animals were allowed a standard pellet diet and tap water ad libitum throughout the experimental period. Blood glucose concentrations, hematocrit values, plasma corticosterone levels, and total and differential blood leukocyte counts were determined in the different experimental groups. Blood glucose levels were evaluated with a blood glucose monitor in samples obtained from the cut tip of the tail of nonanesthetized animals. The hematocrit was determined at the moment of death in blood samples collected from the abdominal aorta while the animals were under anesthesia. Corticosterone levels were estimated by radioimmunoassay [22]. Total and differential leukocyte counts were carried out in blood samples obtained from the cut tip of the tail. Total counts were made in Neubauer chambers. Stained blood films were used for differential leukocyte counts.

Direct vital microscopy of the microcirculation

The animals were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally) and the internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination in situ [23]. This was done through

Abbreviations: HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; mAb, monoclonal antibody.

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a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum and opening of the cremaster muscle to expose the internal fascia. The procedure does not require extensive surgical manipulation for the observation of the vascular network, and provides a valuable means for transilluminating a tissue for quantitative studies of the microcirculation. The animals were maintained on a special board thermostatically controlled at 37°C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37°C) Ringer-Locke's solution, pH 7.2-7.4, containing 1% gelatin. The composition of the solution was (mM): NaCl 154, KCl 5.6, CaCl₂·2H₂O 2, NaHCO₃ 6, and glucose 5. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the preparation in continuous contact with a film of the liquid. A 500-line television camera was incorporated to a triocular Zeiss microscope to facilitate observation of the enlarged image (×3400) on the video screen. Images were recorded on a video recorder using a ×40 longitudinal distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope [24]. The image-splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image-splitter in the phototube the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. Vessels selected for study were third-order venules, defined according to their branch order location within the microvascular network [25, 26]. These vessels corresponded to postcapillary venules; their diameters ranged from 12 to 16 μm.

Interaction of leukocytes with the luminal surface of the venular endothelium was studied in a 100 μm segment of the vessels using recorded images. Leukocytes moving in the periphery of the axial stream, in contact with the endothelium, were considered to be rollers [27, 28], and their number was determined in 10-min periods. These leukocytes moved sufficiently slowly to be individually visible and were counted as they rolled past a selected point on one side of the vessel. The number of leukocytes adhering to the endothelium (stickers) was determined at the same intervals on the vascular segment. A given section of the vascular bed was tested only once, and no more than two determinations were performed on a single animal. These were averaged for each animal. The number of leukocytes accumulating at the connective tissue adjacent to a postcapillary venule was determined in a standard area of 1,000 μm². This area was defined on the video screen, 10 μm in tissue corresponding to 3.4 cm on the screen (magnification ×3400). Five different fields were evaluated on a single animal to avoid variability based upon sampling. Data were then averaged for each animal. A local inflammatory response was evoked by the injection of 0.1 ml of a solution containing 500 μg/ml carrageenan in physiologic saline into the scrotal chamber of the animals.

Erythrocyte velocity measurement in microvessels

Erythrocyte velocity was measured in postcapillary venules by the time delay between the photometric signals that originate from two windows positioned along the centerline of the televised vessel under study [29].

Neutrophil migratory assays in vitro

The migratory assays were performed using a multiwell chemotaxis chamber. In brief, aliquots of cell suspensions

containing 1.5×10^6 neutrophils were added to the upper compartment of the chamber separated from the chemotactic agent in the lower compartment by a cellulose nitrate filter, 8 μm average pore size. Hanks' balanced salt solution (HBSS) was substituted for the chemotactic agent to measure random migration [30]. The cells were incubated in humidified air for 60 min, followed by removal of the filters for fixation and staining of the cells. Neutrophil migration within the filter was determined under light microscopy by the leading front method, in which the distance was measured from the top of the filter to the farthest plane still containing two cells with a ×40 objective [30]. For each variable, duplicate wells were always run. Five fields were counted and averaged for each filter. Neutrophils were obtained after the intraperitoneal injection of 20 ml of 1% oyster glycogen (type II, Sigma, St. Louis, MO) in phosphate-buffered saline. The animals were anesthetized with ether and the cells collected by rinsing the abdominal cavity with HBSS containing 1 U/ml heparin. Cell viability was confirmed by the trypan blue exclusion method. Final cell suspensions contained 0.1% crystalline bovine albumin. To induce the generation of chemotactic activity, lipopolysaccharide (LPS, serotype 026:B6, Sigma) from *Escherichia coli* was incubated in rat plasma (65 μg/ml) for 30 min at 37°C. Varying concentrations of LPS-activated plasma were obtained with dilution in HBSS.

Surgical procedures

Bilateral adrenalectomy was performed with entrance into the peritoneal cavity through bilateral skin and muscle incisions [31]. Adrenalectomized rats were supplied with physiological saline in addition to water. In the set of sham-operated animals, surgical incisions, handling of the abdominal viscera, and closure of the incisions were of similar extent as those of actual surgeries. No special postoperative care was required in this case. Surgeries were performed under ether anesthesia; the animals used 6 days postoperatively. Intact controls were kept under the same conditions as operated rats.

Pharmacological treatments

Metyrapone (Ciba-Geigy, São Paulo, Brazil) dissolved in physiological saline was administered to block synthesis of glucocorticoids without causing a typical deficiency of mineralocorticoids [32]. Two daily doses of 30 mg/kg each were given orally at 12-h intervals for 6 days. Animals were used 2 h after the last dose of the drug. Dexamethasone (Merck, Sharp & Dohme, São Paulo, Brazil) was given intraperitoneally to adrenalectomized rats 6 days postoperatively. A single dose of 1 mg/kg was used and the animals tested 3 h later. To investigate the mechanisms by which endogenous glucocorticoids influence leukocyte-endothelial interactions and cell migration, animals were treated with the steroid antagonist RU 38 486 (Roussel UCLAF, Romainville, France), with actinomycin D (Sigma), cycloheximide (Sigma), or with a monoclonal antibody (mAb) against the membrane glycoprotein complex CD18 (Immunotech, Marseille, France, clone BL5). The steroid antagonist RU 38 486, dissolved in absolute ethanol, was administered by the intraperitoneal route and the animals tested 2 h later. Actinomycin D and cycloheximide, dissolved in physiological saline, were given intraperitoneally 2 h before testing. The anti-CD18 mAb, dissolved in physiological saline, was given intravenously 30 min or 2 h before testing. In all cases, controls received the same volume of the vehicle used to dissolve the drugs, by the same route.

TABLE 1. Blood Glucose Concentrations, Hematocrit Values, and Corticosterone Levels in Different Treatment Groups

Treatment group ^a	Blood glucose (mg/dl)	Hematocrit (%)	Corticosterone (ng/ml)
Normal	98 ± 2.0 (38)	47.5 ± 0.6 (12)	61.6 ± 5.0 (10)
Sham-operated	98 ± 2.3 (34)	48.2 ± 0.8 (13)	52.1 ± 7.0 (9)
Adrenalectomized	94 ± 1.3 (35)	51.0 ± 0.7 (13)	0.08 ± 0.02 (10)*
Metyrapone-treated	96 ± 2.1 (21)	47.8 ± 0.8 (13)	1.63 ± 0.42 (6)*

^aAnimals submitted to surgical procedures were used 6 days postoperatively. Metyrapone was given as two daily doses of 30 mg/kg each at 12-h intervals for 6 days and the animals were used 2 h after the last dose.

**P* < 0.001 vs values in normal and sham-operated groups. Figures within parentheses indicate number of animals used.

Data analysis

Means and standard errors of means (SEM) of all data are presented and were compared by Student's *t*-test or analysis of variance with significant probability levels of less than 0.01.

RESULTS

To investigate the influence of endogenous glucocorticoids on leukocyte-endothelial interactions, we used adrenalectomy, and administration of metyrapone, which blocks synthesis of glucocorticoids without causing a typical deficiency of mineralocorticoids. The procedures did not cause significant changes in blood glucose concentrations or hematocrit values in comparison with values determined in sham-operated and normal animals. Plasma corticosterone levels, however, were sharply reduced by adrenalectomy or treatment with metyrapone (Table 1).

Leukocyte-endothelial interactions

Slow-rolling leukocytes and firmly adhered blood cells are absent in most normal tissues. However, rollers can be observed in surgically prepared tissues for investigation of the microcirculation such as the exteriorized internal spermatic fascia. In the absence of an inflammatory stimulus, adherence of leukocytes to the vessel wall or migration of cells into tissues did not occur. Nevertheless, leukocytes could be seen rolling along the endothelium with a velocity much lower than that of plasma. The phenomenon was particularly prominent in postcapillary venules. Quantitation of the rolling cell flux in the different groups of animals showed that previous adrenalectomy or administration of metyrapone resulted in a marked increase in the number of rolling leukocytes, relative to sham-operated or normal animals. Treatment of adrenalectomized animals with dexamethasone 3 h before testing, caused a full recovery of cell behavior to match control values. Results are presented in Figure 1. The observed changes were not dependent on the number of circulating leukocytes or blood flow: 1) total and differential counts in the peripheral blood were equivalent in normal, sham-operated, adrenalectomized, and metyrapone-treated animals (Table 2) and 2) erythrocyte velocity determined in adrenalectomized and sham-operated rats was not significantly different from that of controls. Values obtained ranged from 1.80 ± 0.13 mm/s to 2.11 ± 0.63 mm/s, indicating comparable blood flow rates in the different experimental groups.

Tests performed 2 h after the injection of an irritant (carrageenan dissolved in physiological saline) into the scrotum to induce a local inflammatory response, showed a reduction

in the number of rolling leukocytes. The levels attained were equivalent in all groups studied. Since the tissue cannot be exposed for such an interval without damage, the determination of levels of rolling leukocytes before the initiation of the inflammatory stimulus is impractical under this condition. For comparison, see data in Figure 1. The decrease in rolling was paralleled by the observation of cells adhering to the vessel wall and accumulation of leukocytes in perivascular tissues. Adherence was particularly noticeable in adrenalectomized and metyrapone-treated rats, in which much of the endothelial surface of randomly chosen venules was covered by firmly adhered cells. Similarly, accumulation of leukocytes in a standard area ($1,000 \mu\text{m}^2$) of the connective tissue adjacent to the observed postcapillary venule was more intense in these groups of animals than in controls (Fig. 2).

Fig. 1. Rolling leukocytes in postcapillary venules of adrenalectomized (A), metyrapone-treated (M), sham-operated (S), adrenalectomized given dexamethasone (Ad), and normal (N) rats. Animals submitted to surgical procedures were used 6 days postoperatively. Metyrapone was given as two daily doses of 30 mg/kg each at 12-h intervals for 6 days and the animals were used 2 h after the last dose. Dexamethasone was given as a single dose (1 mg/kg) 6 days after adrenalectomy and the animals used 3 h later. Figures within bars indicate number of animals in each group. **P* < 0.001 vs. values in other groups.

TABLE 2. Leukocyte Counts in the Peripheral Blood of Different Treatment Groups

Treatment group ^a	Cells/mm ³				
	Total	Lymphocytes	Monocytes	Neutrophils	Eosinophils
Normal (8)	13,237 ± 507	8,364 ± 384	1,752 ± 172	3,038 ± 231	83 ± 32
Sham-operated (6)	13,151 ± 612	8,408 ± 317	1,319 ± 129	3,335 ± 241	89 ± 26
Adrenalectomized (9)	12,383 ± 485	7,903 ± 491	1,477 ± 140	2,816 ± 224	187 ± 38
Metyrapone-treated (7)	12,699 ± 610	8,089 ± 464	1,779 ± 144	2,696 ± 197	135 ± 54

^aAnimals submitted to surgical procedures were used 6 days postoperatively. Metyrapone was given as two daily doses of 30 mg/kg each at 12-h intervals for 6 days and the animals were used 2 h after the last dose. Figures within parentheses indicate number of animals in each group.

Essential changes, capable of altering cell locomotion, did not occur in adrenalectomized animals. Cells collected from normal and sham-operated rats, as well as from adrenalectomized rats were tested *in vitro* for their capacity to respond to varying concentrations of LPS-activated normal plasma, using the micropore filter system. Equivalent responses were obtained with either cell population. Migration ranged from $59 \pm 2 \mu\text{m}$ to $68 \pm 3 \mu\text{m}$ at a 1% concentration of activated plasma and from $100 \pm 3 \mu\text{m}$ to $108 \pm 2 \mu\text{m}$ at a 10% concentration, when the different cell populations were compared. Similarly, the capacity to generate chemotactic factors upon interaction with LPS was comparable in plasma samples obtained from adrenalectomized, sham-operated, and normal animals. This was assessed by quantitating the response of normal cells in the presence of varying concentrations (1 and 10%) of LPS-activated plasma obtained from either group of animals. Migration ranged from $61 \pm 7 \mu\text{m}$ to $70 \pm 10 \mu\text{m}$ and from $104 \pm 2 \mu\text{m}$ to $114 \pm 2 \mu\text{m}$ at each concentration of activated plasma, when the different plasma samples were compared.

Effect of RU 38 486

The steroid antagonist RU 38 486 was administered intraperitoneally to normal rats 2 h before testing. Treatment resulted in a dose-dependent increase in the number of rolling leukocytes whereas no alteration was observed in controls receiving the vehicle alone (Fig. 3). The values attained

Fig. 2. Cells sticking to the endothelium of a 100- μm segment of postcapillary venules (open bars), accumulation of leukocytes in a standard area (1000 μm^2) of the perivascular tissue (hatched bars), and rolling leukocytes (inset) in adrenalectomized (A), metyrapone-treated (M), sham-operated (S), and normal (N) rats. Results were obtained 2 h after the initiation of a local inflammatory response induced by the injection of carrageenan. For comparison, values for rolling leukocytes in animals not treated with carrageenan are indicated in Fig. 1. Animals submitted to surgical procedures were used 6 days postoperatively. Metyrapone was given as two daily doses of 30 mg/kg each at 12-h intervals for 6 days and the animals were used 2 h after the last dose. Five animals were used in each group. * $P < 0.001$ vs. corresponding values in other groups. No significant difference was observed between values for rolling leukocytes.

Fig. 3. Rolling leukocytes in postcapillary venules of untreated rats (C) and rats receiving the glucocorticoid antagonist RU 38 486. The antagonist was administered intraperitoneally as a single injection 2 h before testing. Figures within bars indicate number of animals in each group. * $P < 0.01$ and ** $P < 0.001$ vs. value in control group.

matched those observed in adrenalectomized and metyrapone-treated animals. Total and differential leukocyte counts performed before and 2 h after drug administration showed no changes in the number of circulating leukocytes (data not shown).

During the development of a local inflammatory response evoked by carrageenan as referred to above, RU 38 486 given immediately before the irritant caused a marked enhancement in the number of adherent and migrated cells. Rolling leukocytes were concomitantly reduced as compared with data presented in Figure 3. Normal animals receiving the steroid antagonist behaved strictly as adrenalectomized or metyrapone-treated rats. Results are presented in Figure 4.

Effect of actinomycin D and cycloheximide

Actinomycin D, which blocks the transcription of DNA by RNA polymerase, and cycloheximide, which inhibits protein synthesis, were given intraperitoneally to normal animals 2 h before testing. The doses were 1 mg/kg and 2 mg/kg, respectively. Treatment with both drugs resulted in a significant increase in the number of rolling leukocytes ($P < 0.001$) in comparison with values in control groups. The levels attained matched those observed in adrenalectomized and metyrapone-treated animals. The number of rolling leukocytes in actinomycin D-treated rats were 237 ± 5 , $n = 5$ versus 120 ± 10 , $n = 5$ in control animals. In cycloheximide-treated rats and matching controls the number of rolling leukocytes were 224 ± 7 , $n = 6$ and 110 ± 7 , $n = 6$, respectively. Total and differential leukocyte counts were not al-

Fig. 4. Cells sticking to the endothelium of a 100-μm segment of postcapillary venules (open bars), accumulation of leukocytes in a standard area (1000 μm²) of the perivascular tissue (hatched bars), and rolling leukocytes (inset) in untreated normal rats (C) and rats receiving 10 mg/kg of the glucocorticoid antagonist RU 38 486. Results were obtained 2 h after the initiation of a local inflammatory response induced by injection of carrageenan. The steroid antagonist was administered intraperitoneally as a single injection, immediately before the injection of carrageenan. Five animals were used in each group. * $P < 0.01$ vs. corresponding values in control group.

Fig. 5. Rolling leukocytes in postcapillary venules of untreated adrenalectomized rats (A) and adrenalectomized rats receiving 1 mg/kg anti-CD18 mAb (A + mAb) intravenously. Results were obtained 30 min after the injection of the anti-CD18 mAb. Five animals were used in each group.

tered by treatment of the animals with the drugs (data not shown).

During the development of a local inflammatory response evoked by carrageenan as referred to above, actinomycin D and cycloheximide given immediately before the irritant caused an enhancement in the number of migrated cells to a standard area in the perivascular tissue. Values were 18 ± 1.2 , $n = 5$ and 19 ± 0.9 , $n = 6$ cells/1,000 μm² in actinomycin D- and cycloheximide-treated animals, respectively. In matching controls, values were 8 ± 0.6 , $n = 5$ and 9 ± 0.7 , $n = 6$, respectively ($P < 0.01$ by comparing values in treated and untreated animals).

Effect of a monoclonal antibody against the membrane glycoprotein complex CD18

A commercially available anti-CD18 mAb was given intravenously to adrenalectomized animals 30 min before testing. Untreated (adrenalectomized) controls received the same volume of the vehicle used to dissolve the mAb. Treatment did not influence the number of rolling leukocytes (Fig. 5), which is increased in these animals as shown above.

During the development of a carrageenan-induced local inflammatory response the number of rolling leukocytes remained high in adrenalectomized animals receiving the anti-CD18 mAb immediately before the injection of the irritant. However, a dramatic decrease in the number of leukocytes adhering to the endothelium and in the number of cells accumulating at the interstitial tissue was observed. Data are presented in Figure 6.

DISCUSSION

Exteriorization of a tissue for the investigation of the microcirculation or its exposure to inflammatory stimuli causes marked changes in the behavior of leukocytes at the microcirculatory level. In the former circumstance cells rolling along the venular endothelium of the microcirculation network are observed. This early type of leukocyte-endothelial interaction is a prerequisite for stronger adhesion. During the development of an inflammatory response, leukocytes eventually become adherent to the venular endothelium and then extravasate to the perivascular tissue.

We show here that the number of leukocytes rolling along the venular endothelium was markedly increased in adrenalectomized and metyrapone-treated animals relative to sham-operated and normal animals. Plasma corticosterone levels were sharply reduced by adrenalectomy or treatment with metyrapone. Metyrapone, through inhibition of the 11β -hydroxylation reactions, markedly decreases the secretion of cortisol, corticosterone, and aldosterone, while the secretion

of 11 -deoxycorticosteroids is relatively unimpaired. Accordingly, the administration of metyrapone results in a compensatory increase in the secretion of adrenocorticotrophic hormone and in enhanced secretion of 11 -deoxycortisol, a relatively inert steroid, and 11 -deoxycorticosterone, a mineralocorticoid. Therefore, the drug blocks the production of glucocorticoids but does not typically cause a deficiency of mineralocorticoids [32]. The relative lack of glucocorticoids in adrenalectomized and metyrapone-treated animals appears to be the major determinant for the increased number of rollers in these animals. Total and differential leukocyte counts in the peripheral blood were equivalent in all groups tested. Comparable blood flow rates, as assessed by erythrocyte velocity measurements, were observed in adrenalectomized, sham-operated, and normal animals. Furthermore, adrenalectomy followed by treatment with dexamethasone caused a full recovery of cell behavior to match control values.

Two h after the initiation of a local inflammatory process induced by the injection of carrageenan, the number of rollers at the microcirculation network was reduced to comparable levels in all groups of animals. This was accompanied by the appearance of cells adhering to the vessel wall and accumulation of leukocytes in perivascular tissues. An inverse relationship between adhesion and rolling is demonstrated, indicating that when a greater number of stationary white blood cells prevails a reduction in the number of rolling leukocytes is observed [28, 33]. The adhesion phenomenon was particularly prominent in adrenalectomized and metyrapone-treated animals, in which much of the endothelial surface was covered by firmly adhered cells. Similarly, accumulation of leukocytes in a standard area of the connective tissue adjacent to the venule was more intense in adrenalectomized and metyrapone-treated animals. Leukocytes collected from adrenalectomized or normal animals, however, exhibited comparable chemotactic responses in a concentration gradient of LPS-activated plasma. In addition, the capacity to generate chemotactic factors upon interaction with LPS was equivalent in plasma samples obtained from adrenalectomized and normal animals. Accordingly, adrenalectomy does not influence cell locomotion per se. Secreted glucocorticoids, therefore, appear to exert a suppressive effect on leukocyte-endothelial interactions.

The effect apparently involves receptor occupancy and induction of gene expression. Normal animals receiving the steroid antagonist RU 38 486 behaved as adrenalectomized or metyrapone-treated animals. A dose-dependent increase in the number of rolling leukocytes was observed in animals given the antagonist. Furthermore, RU 38 486 caused a marked enhancement in the number of adherent and migrated cells during the development of a local inflammatory response. RU 38 486 stabilizes the association of steroid receptors with heat shock protein 90 in the presence of ligand, thereby preventing translocation of glucocorticoid receptors to the nucleus and transcription of genes containing glucocorticoid-responsive elements [34-38]. The finding was corroborated by experiments in which actinomycin D, which blocks the transcription of DNA by RNA polymerase, and cycloheximide, an inhibitor of protein synthesis, were used. Both drugs, given to normal animals, markedly enhanced the number of rolling leukocytes. In addition, the drugs increased the amount of cells migrating into the interstitial tissue during the development of an inflammatory reaction.

Therefore, acting at their cellular receptors, glucocorticoids influenced leukocyte rolling along the venular endothelium and leukocyte adhesion to vessel wall. The in-

Fig. 6. Cells sticking to the endothelium of a 100- μ m segment of postcapillary venules (open bars), accumulation of leukocytes in a standard area (1000 μ m²) of the perivascular tissue (hatched bars), and rolling leukocytes (inset) in adrenalectomized rats (A) and adrenalectomized rats receiving 1 mg/kg anti CD18 mAb (A + mAb). Results were obtained 2 h after the initiation of a local inflammatory response induced by the injection of carrageenan. The mAb was administered intravenously as a single injection, immediately before the injection of carrageenan. Four animals were used in each group. * $P < 0.001$ vs. corresponding values in adrenalectomized animals.

creased number of rolling leukocytes in adrenalectomized and metyrapone-treated animals apparently resulted in more cells available to respond to chemoattractants and therefore in more migration into inflamed tissues. Glycoproteins of the $\beta 2$ -integrin family, collectively termed CD11/CD18 complex [39, 40], are known to be required for the firm adhesion of rolling cells to the endothelium [4, 8, 9, 41-43]. Administration of an anti-CD18 monoclonal antibody to adrenalectomized animals caused a marked reduction in the number of cells adhering to the endothelial surface of postcapillary venules and an almost complete block of leukocyte migration to the perivascular inflamed tissue without affecting the number of rolling leukocytes. Members of the selectin family of cell adhesion molecules are thought to mediate leukocyte rolling along the walls of the microvasculature [1, 43-46]. Recent observations demonstrate that glucocorticoids, at nanomolar concentrations, inhibit the expression of interactive molecules by endothelial cells that either act as counter-receptors for CD11/CD18 complex or recognize molecular constituents in leukocyte glycolipids and glycoproteins [8, 14]. Accordingly, secreted glucocorticoids, in addition to an effect on rolling behavior of circulating leukocytes possibly involving molecules of the selectin family, might also modulate the activity of the CD11/CD18 complex on white blood cells.

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Molecular and Cellular Endocrinology xxx (2006) xxx–xxx

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Adrenal deficiency alters mechanisms of neutrophil mobilization

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Abstract

Deficiency of adrenal hormones promotes exacerbated neutrophil influx into inflammatory sites. We investigated the effect of adrenal deficiency on neutrophil mobilization comparing adrenalectomized (ADX) male Wistar rats to sham-operated (SO) or non-manipulated (N) animals, as controls. Seven days after surgeries, the number of neutrophils in peripheral blood was increased in ADX rats, by accelerating neutrophil maturation steps in the bone marrow. The investigation of adhesive properties on neutrophil membranes indicated reduced and increased expressions of L-selectin on cells present in the bone marrow and circulating blood, respectively. Similar levels of L-selectin mRNA in both cells from ADX or non-manipulated rats suggest that these effects do not depend on gene expression. Even though no differences in the expression of β_2 integrin by neutrophils were detected, modulation on subsequent PMN activation may occur by adrenal hormones, since circulating neutrophils from ADX exhibit lower in vitro adherence to the endothelium. We conclude that adrenal hormones control the adhesive interactions of neutrophils with the bone marrow microenvironment and with the vascular endothelium chiefly by modulation of L-selectin on PMN membrane in a mechanism independent of L-selectin gene expression.

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Keywords: Adrenal deficiency; Neutrophils; Bone marrow; Adhesion molecules; L-selectin mRNA

1. Introduction

Glucocorticoids are well known antiinflammatory substances when used in pharmacological concentrations, and the adrenal cortex hormone production is increased during the course of an inflammatory response. In contrast, the effect of typical diurnal concentrations of these hormones on the mechanisms that support inflammatory responses is not well understood.

Leukocyte recruitment, one of the first steps in mounting an inflammatory response, is known to be altered in adrenalectomized (ADX) rats, in which a higher influx of neutrophils into injured areas is observed, compared to control subjects (Flower et al., 1986; Abe et al., 1995; Rovai et al., 1998; Leech et al., 2000). This higher influx is probably due to the loss of glucocorticoid negative control on the rolling and adhesion

of leukocytes to postcapillary venules (Farsky et al., 1995). Adrenalectomy of normal rats leads to an increase in rolling and adhesion of leukocytes to postcapillary venules in vivo. Similar results are obtained after inhibition of the corticosterone synthesis with metyrapone or inhibition of glucocorticoid receptors with mifepristone (RU 38486), suggesting that the corticosterone synthesized by the adrenal gland plays a role in negatively modulating the migration of blood cells from the vascular bed (Farsky et al., 1995).

The initial rolling behavior and the subsequent adherence to the vessel wall are influenced by the expression of adhesion molecules on the membranes of leukocytes and endothelial cells and changes in hemodynamic behavior of circulating cells (Gonzalez-Amaro and Sanchez-Madrid, 1999; Pearson and Lipowsky, 2000; Marshall and Haskard, 2002; Sun et al., 2003). Transient expression of selectins on leukocytes and endothelial cells mediates rolling behavior (Zhao et al., 2001; Rosen, 2004), whereas the subsequent expression of β_2 integrin on leukocytes and its binding to immunoglobulin receptors on the endothelium

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2

D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology* xxx (2006) xxx–xxx

are responsible for adhesion (Hubbard and Rothlein, 2000; Hogg et al., 2002; McIntyre et al., 2003). This same set of molecules contribute to maintaining leukocytes in the bone marrow compartment during the maturation phase of these cells, and changes in their expression or cleavage can alter the adhesion either to the bone marrow microenvironment or to the vascular endothelium (Van Eeden et al., 1997; Dimitroff et al., 2001; Carion et al., 2002).

Recent data strongly suggest that adhesion molecules also mediate signal transduction. Initial interaction between leukocytes and the endothelium induces bi-directional signaling, which mediates events that impel leukocytes onward through microcirculatory vessels, as well as their responsiveness, differentiation states and survival at sites of inflammation (Wang and Doerschuk, 2002; Kluger, 2004; Puri et al., 2004; Nourshargh and Marelli-Berg, 2005).

Glucocorticoids interfere with the expression of adhesion molecules, and this is thought to be the basis for the inhibition of rolling and adhesion of leukocytes to endothelial cells (Nakagawa et al., 1998; Waisman et al., 1998; Torsteinsdottir et al., 1999; Fassbender et al., 1999; Weber et al., 2001, 2004; Kaname et al., 2002). However, these results were obtained with very high doses of natural or synthetic hormones, and may not represent the normal mechanism responsible for the effects of cortical adrenal hormonal production.

The aim of the current study is to determine the influence of adrenal cortical hormones on the distribution of leukocytes between the bone marrow and the blood compartment, and on leukocyte migration to peripheral tissues independently of the occurrence of an injury. We show that adrenal hormones deficiency accelerates the maturation of bone marrow neutrophils resulting in a faster migration of these cells to the peripheral compartment. A reduced expression of L-selectin on bone marrow segmented-quietest polymorphonuclear (PMN) cells may underlie the facilitation of cell migration to peripheral blood. In addition, the increased expression of the same molecule in blood PMN cells may favor the migration of leukocytes from blood vessels when adrenal glands are removed. Both alterations on L-selectin on PMN membranes were not generated by modifications on gene expressions. On the other hand, adrenal hormones seem to alter the subsequent PMN activation, given that β_2 integrin on its membranes was not altered and its *in vitro* ability to adhere to the endothelium was reduced.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 180–250 g at the beginning of the experiments were used. The animals were allowed a standard pellet diet and water *ad libitum*. During experimental procedures the animals were anesthetized with pentobarbital sodium (65 mg/kg; intraperitoneally) to avoid any stress condition. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

2.2. Surgical procedures

Bilateral adrenalectomy was performed with entrance into the cavity through bilateral skin and muscle incisions. Adrenalectomized rats (ADX) were supplied

with physiological saline solution in addition to water. In the set of sham-operated animals, surgical incisions, handling of the abdominal viscera, and closure of the incisions were of similar extent as those of actual surgery. No special postoperative care was required in this case. Surgical procedures were performed with pentobarbital sodium anaesthesia; the animals were used for 7 days postoperatively. Intact controls were kept under the same conditions as operated rats.

2.3. Determination of corticosterone concentration by radioimmunoassay

The concentration of corticosterone was determined by radioimmunoassay (RIA) after extraction from plasma using ethanol (Lopez-Jimenez et al., 1989). The mean assay sensitivity and intra and interassay coefficients of variation were 0.4 $\mu\text{g/dL}$ and 6.7%. The percentage of recovery after extraction was 80%. All the samples obtained from each experiment were analyzed in duplicate in the same assay.

2.4. Hematological parameters

2.4.1. Blood

Animals were anaesthetized with pentobarbital sodium and whole blood samples were obtained via abdominal aorta punctures using EDTA (1 mg/mL, Sigma, St. Louis, MO, USA). Total cells were quantified using a Neubauer chamber. Differential leukocyte counts were performed on blood smears stained with May Grunwald–Giemsa solution (Sigma, St. Louis, MO, USA).

2.4.2. Bone marrow

Bone marrow cells were obtained by flushing the femoral cavity with McCoy's 5A medium (Sigma, St. Louis, MO, USA) with EDTA (1 mg/mL). The number of total cells was quantified in Neubauer chamber. Differential counts were performed on the basis of 500 cells per slide in cytocentrifuge smears stained with May-Grunwald and Giemsa dye.

2.5. Flow cytometry

Leukocytes were isolated from blood collected from the abdominal aorta or from the bone marrow to quantify L-selectin or β_2 integrin expressions. Blood was collected using EDTA solution 2% and bone marrow content was harvested by perfusion with Hank's balanced solution (HBSS) containing BSA 1% and EDTA 10%. In brief, erythrocyte lysis was performed using ammonium chloride solution (0.13 M) in both samples and leukocytes were recovered after washing with HBSS. Cells (1×10^6) were incubated with or without *N*-formylmethionyl-leucyl-phenylalanine (fMLP 10^{-8} M/15 or 30 min).

After washing, leukocytes were further incubated for 20 min at 4 °C in the dark with 10 μL of monoclonal antibody against L-selectin (FITC conjugated, mouse anti-rat L-selectin, BD Pharmingen Technical, CA, USA, 50 mg/mL) or β_2 integrin (FITC conjugated, mouse anti-rat β_2 integrin, BD Pharmingen Technical, CA, USA, 50 mg/mL). Immediately after incubation, cells were analyzed on a FACScalibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). Data from 10,000 cells were obtained and only the morphologically viable leukocytes were considered for analysis. Results were expressed as fluorescence units.

2.6. Determination of L-selectin mRNA levels

2.6.1. RNA extraction

Total RNA was extracted from neutrophils by the TRIzol[®] Reagent method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. RNA was quantified by reading the absorbency at 260 nm according to the methods described by Sambrook et al., 1989.

2.6.2. RT-PCR

The cDNAs were synthesized from 2 μg of total RNA using oligo(dT)₁₅ primer (20 $\mu\text{g/mL}$) (Promega), after incubation at 70 °C for 5 min, dNTP mix (2 mM) (Promega), RNasin Inhibitor (20U) (Promega) and M-MLV-RT

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D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology* xxx (2006) xxx–xxx

3

(200U) (Promega) were added with final volume of 25 μ L in buffer M-MLV-RT (Promega). The reverse transcription occurred by incubation at 42 °C for 60 min. For the polymerase chain reaction (PCR), the cDNA obtained was incubated with 2.5U Taq DNA polymerase (Promega), 0.4 μ M 3' and 5' specific primers and 200 μ M dNTP mix in buffer-thermophilic DNA polymerase (Promega), containing 1.5 mM MgCl₂. The annealing temperature for L-selectin and GAPDH was 51 and 54 °C, respectively, obtained in 30 cycles. The primers used were: L-selectin sense: 5'AACGAGACTCTGGGAAGT'3; L-selectin antisense: 5'CAAAGGCTCATTGGAT'3; GAPDH sense: 5'TATGATGACATCAAGAAGGTGG'3; GAPDH antisense: 5'CACCACCCTGTTGCTGTA'3. The PCR products were detected on 2% agarose electrophoresis stained with ethidium bromide using 100 or 50 bp ladder (Promega) as nucleotide size markers. A FLA-3000 scanner (Fuji film) scanned the gels and the densitometric analyses were obtained using the Total Lab Software. The target gene was amplified and the level of each expression was represented by the ratio of densities of the target genes versus the band corresponding to the GAPDH, a constitutive control gene.

2.7. In vitro neutrophil-endothelium adherence

2.7.1. Microvascular endothelial primary cultures

Primary cultures of microvascular endothelial cells were obtained from the rat cremaster muscle using the method described by Chen et al. (1995). Rats were anesthetized and non-coagulated by intraperitoneal injection of heparin (Liquimine®, Roche, 1000 units per animal). The animals were exsanguinated by cutting the bilateral carotid arteries. Subsequently the cremaster muscles were isolated, cut into pieces of approximately 2 mm $\times$ 2 mm, and two pieces were placed into a flask and cultured in DMEM supplemented with 20% fetal calf serum and 1% penicillin/streptomycin. After 48h, the tissues were discarded and the medium changed. The flask contained only endothelial cells and blood cells. The cells were sub-cultured with 10% pancreatin in PBS solution and used for an adherence assay on the 8th day, when they achieved confluence. The endothelial identification was performed by adding monoclonal antibody against PECAM-1 (Becton and Dickinson, San Jose, CA, USA, 50 mg/mL).

2.7.2. PMN cell-enriched leukocytes

Blood was collected from the abdominal aorta of anesthetized rats using EDTA 2%. Cell separation was performed by adding 3 mL of Percoll (56% in sterile PBS) to 5 mL of blood samples. After centrifugation (1000 $\times$ g, 40 min), the interface containing mainly PMN cells was collected and erythrocyte lysis was promoted by adding lysis solution (NH₄Cl 8.02 g/L, NaHCO₃ 0.84 g/L). An amount of 10⁶ cells/mL were suspended in HBSS with 0.5% BSA and used for an adherence assay.

2.7.3. Adherence assay

First-passage endothelial cells were plated in 6.4 mm diameter wells at 10⁴ cells/mL in DMEM supplemented with 20% fetal calf serum. Cells were washed with three-well volume exchange of HBSS and PMN cells (10⁵ cells/50 μ L/well) were then added in HBSS containing 0.5% BSA. Endothelial and PMN cells were incubated for 5 min (37 °C, 5% CO₂). Non-adherent PMN cells were washed with a two-well volume exchange of HBSS. A colorimetric tetramethylbenzidine (TMB) assay was then applied to detect

neutrophils adhering to the monolayers, using TMB as a peroxidase substrate. Fifty microlitres of acetate buffer (0.05 M, pH 5.8) containing 0.25% of dodecyltrimethylammonium bromide as peroxidase solubilizing and 25 μ L of TMB (16 mM in dimethylsulfoxide) were added and incubated for 5 min at room temperature. Subsequently, 100 μ L of hydrogen peroxidase (final concentration of 2.5 nM) were added. After 2 min, the peroxidase reaction was stopped by the addition of 50 μ L of sulfuric acid (1 M). The absorbance was determined at 420 nm using an ELISA microplate reader (spectraMAX 250, Molecular Devices). PMN adherence was calculated using a calibration curve obtained by performing the peroxidase reaction in wells containing known amounts of neutrophils.

2.8. Statistical analysis

Means and standard errors of means (S.E.M.) of data are presented and were compared by analysis of variance (ANOVA) or Tukey Multiple Comparisons Test with a significant probability of less than 0.05. L-selectin mRNA levels are expressed as mean of two assays performed in duplicate.

3. Results

3.1. Corticosterone levels

Adrenalectomy (7 days) reduced plasma concentration of corticosterone from 6.43 μ g/dL $\pm$ 0.71 ($n=9$; normal) to 0.43 μ g/dL $\pm$ 0.03 ($n=9$, ADX, $p<0.05$), confirming the efficacy of adrenal removal surgery. In addition, no significant difference in corticosterone levels was detected between normal and sham-operated animals (6.92 μ g/dL $\pm$ 0.69, $n=9$) after 7 days of surgery, indicating that corticosterone returned to basal levels in sham-operated rats.

3.2. Effects of adrenal deficiency on the production and maturation of segmented and mononuclear cells

Adrenalectomy increased the number of total circulating leukocytes (Fig. 1A), which reflected in an increment in the number of PMN cells (Fig. 1B). The adrenal ablation did not modify the number of mononuclear (MN) leukocytes (data not shown). No changes in the numbers of progenitor cells of MN or PMN lineages in the bone marrow compartment were observed (data not shown), although a shunting line to the left in the PMN sector was detected. In fact, this deviation was compensated by an increased number of band PMN cells and a reduced number of immature PMN cells (Fig. 2). No modification in the number of PMN cells in the last phase of maturation was detected (Fig. 2), probably due to their enhanced migration to the circulating compartment, as shown in Fig. 1A.

Fig. 1. Number of total leukocytes (A) and PMN cells (B) in the circulation compartment of normal (N), sham-operated (SO) or adrenalectomized (ADX) rats. Cells were collected 7 days after surgeries. Data are expressed as mean $\pm$ S.E.M. of values obtained in six animals in each group. * $p<0.01$ vs. SO or N values.

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4

D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology xxx (2006) xxx–xxx*

Fig. 2. Number of immature (A), band (B) or segmented PMN cells (C) from bone marrow of normal (N), sham-operated (SO) or adrenalectomized (ADX) rats. Cells were collected 7 days after surgeries. Data are expressed as mean $\pm$ S.E.M. of values obtained in six animals in each group. * $p < 0.05$ vs. SO or N values.

3.3. Effects of adrenal deficiency on L-selectin and β_2 integrin expressions on bone marrow leukocytes

To evaluate a possible role of adhesion molecules on accelerated PMN maturation, the expressions of L-selectin or β_2 integrin were investigated. Immature and band PMN cells from ADX animals presented L-selectin expression comparable to cells from control animals (data not shown). However, segmented PMN cells obtained from ADX rats presented reduced expression of L-selectin when compared with cells collected from SO or N rats (Fig. 3A). In contrast, in vitro stimulation (fMLP 10^{-8} M) promoted similar increase on L-selectin expression in all groups of cells tested (Fig. 3A). Expression of β_2 integrin was not altered, neither under quiescent nor stimulated conditions, in all phases of PMN maturation (Fig. 3B). In addition, adrenal deficiency did not modify the expressions of both molecules on any stage of maturation of MN cells (data not shown).

3.4. Effects of adrenal deficiency on L-selectin and β_2 integrin expression on circulating leukocytes

Adrenalectomy promoted an increase on L-selectin expression on the membrane of total leukocytes (Fig. 4A), which reflected a marked increased on PMN population (Fig. 4B). The enhanced expression of L-selectin by PMN after in vitro addition of fMLP was similar in the three groups of cells tested (Fig. 4B). Conversely, adrenalectomy did not modify the expression of L-selectin on MN cells, since expression levels were not different than those from control cells, even on quiescent or fMLP stimulated cell membranes. Additionally, results presented in Fig. 5A

showed that the expression of β_2 integrin on total circulating leukocytes, such as on PMN (Fig. 5B) and on MN cells (data not shown), did not differ among cells obtained from the three groups of animals.

Fig. 3. Expression of adhesion molecules on segmented PMN cells from bone marrow. Data were obtained by blood flow cytometry analysis. Panel (A) represents L-selectin expression and panel (B) represents β_2 integrin expression. White columns show values obtained in non-stimulated cells and striped columns show values obtained after stimulation by fMLP (10^{-8} M). Cells were collected 7 days after surgeries. Results express mean $\pm$ S.E.M. of cells collected from six to eight non-manipulated (N), sham-operated (SO) and adrenalectomized (ADX) rats. ^a $p < 0.05$ vs. SO or N non-stimulated values; ^b $p < 0.05$ vs. respective non-stimulated condition.

+ Model

D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology xxx (2006) xxx–xxx*

5

Fig. 4. Expression of L-selectin on total leukocytes (panel A) and on PMN circulating cells (panel B). Data were obtained by blood flow cytometry analysis. White columns show values obtained in non-stimulated cells and striped columns show values obtained after stimulation by fMLP (10⁻⁸ M). Cells were collected 7 days after surgeries. Results express mean $\pm$ S.E.M of cells collected from six to eight non-manipulated (N), sham-operated (SO) and adrenalectomized (ADX) rats. ^a $p < 0.05$ vs. non-stimulated SO or N values; ^b $p < 0.05$ vs. respective non-stimulated condition.

3.5. Effects of adrenal deficiency on L-selectin mRNA levels

To investigate if modifications on membrane expression of L-selectin were dependent on alterations in gene expression, levels of mRNA were evaluated in bone marrow segmented cells and circulating PMN. Data obtained showed equivalent levels of L-selectin mRNA in both types of cells from ADX and non-manipulated rats (Fig. 6).

3.6. Effects of adrenal deficiency on neutrophil adherence

We performed in vitro assays using circulating neutrophils and cultured endothelium to investigate whether the deficiency

of adrenal hormones affected the ability of neutrophils to adhere to the endothelium. Our data showed that the number of neutrophils from ADX animals adhering to normal endothelium was significantly smaller than the number of neutrophils collected from SO or N rats (Fig. 7).

4. Discussion

The present study described the alterations elicited by deficiency of adrenal hormones on neutrophil behavior during their mobilization steps. Most importantly, they suggest that adrenal hormones modulate adhesive properties promoted by L-selectin in the bone marrow and circulation in a mechanism independent of L-selectin gene expression.

Recruitment of neutrophils from bone marrow is a characteristic phenomenon of different types of stress, and it is modulated by elevated concentrations of glucocorticoids. Neutrophilia has been fully detected in experimental animals and humans after dexamethasone injection (Burton et al., 1995; Nakagawa et al., 1999; Weber et al., 2004) or during special physiological conditions, such as periparturient cows and neonatal calves (Lee and Kehrl, 1998; Preisler et al., 2000). The mechanisms involved are associated to demargination of immature cells (Meagher et al., 1996) or to impairment of neutrophil apoptosis (Liles et al., 1995; Chang et al., 2004). However, to our knowledge it has not hitherto been demonstrated that depletion of endogenous adrenal hormones induces neutrophilia in the absence of stress stimulus, by inducing accelerated delivery of cells from bone marrow to blood, as suggested here by adrenalectomy. Our data also show that the mechanisms involved may be related to the expression of adhesion molecule on neutrophils, since impairment on L-selectin expression on segmented cells at the last phase of maturation in the bone marrow compartment was observed in cells from ADX animals. The modulation of L-selectin expression on bone marrow neutrophils by elevated concentrations of glucocorticoids had already been suggested (Weber et al., 2001), and inferred as a possible determinant of neutrophilia. However, this effect was recently questioned

Fig. 5. Expression of β_2 integrin on total leukocytes (panel A) and on PMN circulating cells (panel B). Data were obtained by blood flow cytometry analysis. White columns show values obtained in non-stimulated cells and striped columns show values obtained after stimulation by fMLP (10⁻⁸ M). Cells were collected 7 days after surgeries. Results express mean $\pm$ S.E.M of cells collected from six to eight non-manipulated (N), sham-operated (SO) and adrenalectomized (ADX) rats. ^b $p < 0.05$ vs. respective non-stimulated condition.

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6

D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology xxx (2006) xxx–xxx*

Fig. 6. Representative gel of PCR products and result of L-selectin:GAPDH ratios. (A) Agarose gel visualised under ultraviolet light using the fluorescent properties of ethidium bromide and scanned using FLA-3000 scanner (Fuji film). PCR products for L-selectin from a control bone marrow PMN in lane 1 and ADX bone marrow PMN in lane 2, with their respective GAPDH in lanes 3 and 4 are shown. (B) Densitometric analysis of L-selectin RT-PCR products standardized according to the corresponding GAPDH RT-PCR product levels. Data represent two independent experiments performed in duplicate.

in a bovine experimental model. It was suggested that dexamethasone downregulates L-selectin expression predominantly on the membrane of circulating neutrophils, through activation of glucocorticoid receptor and subsequent effects on intracellular reservoirs of L-selectin mRNA (Weber et al., 2004). It is interesting to note that the impairment on L-selectin expression on segmented bone marrow cells from ADX detected here, does not reflect a defect on the synthesis or expression machineries of L-selectin, given that L-selectin mRNA levels and the enhanced expression of the molecule under *in vitro* fMLP stimulation was similar in PMN cells from ADX and controls rats. Van Eeden et al. (1997) showed that L-selectin shedding occurs in PMN moving from the hematopoietic compartment, and suggested that this could control the release of PMN from the bone marrow. Thus, endogenous adrenal hormones may control L-selectin shedding on the segmented bone marrow, modulating its delivery to the circulation.

Fig. 7. Number of polymorphonuclear cells from ADX, SO or N rats adhered to cultured endothelium. Data express the mean $\pm$ S.E.M. of values obtained from four animals in each group. Cells were collected 7 days after surgeries. * $p < 0.05$ vs. SO or N values.

Interestingly, data herein show that on the circulating PMN cells, L-selectin expression is inversely modulated by adrenal hormones in comparison to bone marrow cells, as demonstrated by an increment on expression of L-selectin on peripheral neutrophils collected from ADX rats. These data corroborate the action of glucocorticoids on the molecule expression, since impairment on its expression is observed by elevated endogenous glucocorticoids concentrations during several pathophysiological conditions (Filep et al., 1997; Fassbender et al., 1999; Nakagawa et al., 1998; Torsteinsdottir et al., 1999; Weber et al., 2001, 2004; Kaname et al., 2002). The mechanism proposed is mainly based on a genomic hormonal action that induces reduction on L-selectin mRNA levels (Weber et al., 2001, 2004). However, the equivalent levels of L-selectin mRNA in circulating PMN from ADX and non-manipulated rats found here do not confirm the role of adrenal hormones on L-selectin induced synthesis. In addition, this result may indicate the induction of L-selectin shedding as a physiological action of adrenal hormones on the control of leukocyte-neutrophil interactions. Consistent with our hypothesis, Strausbaugh and Rosen (2001) showed that annexin I, a protein suggested as responsible for some anti-inflammatory effects of glucocorticoids, induced a dose-response decrease in L-selectin on neutrophil and monocyte membranes, by evoking its shedding. Also, it was shown that clinical concentrations of dexamethasone reduce the shedding of L-selectin induced by PAF (Filep et al., 1997).

It has been shown that the blockage of L-selectin shedding on PMN membranes during an inflammatory response increase the number and transit time of rolling cells on activated endothelium, resulting in elevated adherence and transmigration (Walcheck et al., 1996; Hafezi-Moghadam and Ley, 1999; Hafezi-Moghadam et al., 2001; Venturi et al., 2003; Smalley and Ley, 2005). A similar pattern of response was observed after inducing a local inflammatory reaction in ADX and metyrapone-treated rats (Farsky et al., 1995).

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D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology xxx (2006) xxx–xxx*

7

Cross-linking of L-selectin in an inflammatory process initiates intracellular signaling and participates of PMN activation during adherence and transmigration by enhancing neutrophil ability to deform and β_2 integrin activation (Crockett-Torabi et al., 1995; Simon et al., 1995, 1999). We expected that in ADX animals the expression of β_2 integrin would be increased, with consequent cellular activation. However, our results do not confirm this hypothesis because of the following observations: (1) adrenalectomy did not modify β_2 integrin expression on membrane of PMN cells and (2) circulating neutrophils from ADX animals lost their capacity to adhere to normal cultured endothelium. Our results may indicate that physiological control of subsequent steps of rolling behavior by adrenal hormones may be different during disease or after exogenous therapy. In this context, it has been shown that the effects of glucocorticoid hormones on adherence and on β_2 integrin expression are controversial, and seem to be dependent on hormonal concentrations. In vitro or in vivo assays carried out with pharmacological doses of dexamethasone inhibited β_2 integrin expression (Youssef et al., 1995, 1996; Filep et al., 1997; Lim et al., 2000), but the blockage of glucocorticoid synthesis in rheumatoid patients, with elevated concentration of cortisol, did not alter the pattern of integrin expression (Torsteinsdottir et al., 1999). Additionally, House et al. (1997) showed that differences on corticosteroid concentrations induced by the circadian rhythm alters the adherence of leukocyte to postcapillary venule walls in vivo throughout the day. Higher adherence of leukocytes to postcapillary venules was detected at elevated concentrations of corticosteroids.

Taken together, our data demonstrate the physiological control by adrenal hormones of neutrophil events responsible for cellular interactions during their mobilization. L-selectin expression on PMN membranes seems to be essential to maintain these cells at their specific compartment during production and distribution, independently of its gene expression. Our results provide a better understanding of physiological modulation of adrenal hormones on mechanisms involved on leukocyte recruitment.

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8

D.M.H. Cavalcanti et al. / *Molecular and Cellular Endocrinology xxx (2006) xxx–xxx*

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Role of kinins and nitric oxide on the rabbit arthritis induced by *Bothrops jararaca* venom

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Abstract

The effects of the nitric oxide synthase inhibitor N^ω-Nitro-L-arginine-methyl ester (L-NAME) and of the bradykinin B₂ receptor antagonist HOE 140 were evaluated in the inflammatory reaction induced by *Bothrops jararaca* venom (BjV) in New Zealand White rabbits. Arthritis was induced by injecting 0.5 ml of a sterile solution of BjV (1–64 µg/ml) into the knee intraarticular cavity. The contralateral joint was injected with bovine serum albumin (BSA) diluted in sterile saline. At selected times thereafter (4, 24 and 48 h), the vascular permeability and the leukocyte influx in both the synovial fluid and synovium were evaluated. BjV caused a dose-dependent increase in both leukocyte influx and protein extravasation which reached a maximal response at 16 µg. *Bothrops jararaca* venom also induced the increase in the leukocyte accumulation in the synovium and in the concentration of both NO₂/NO₃ in the synovial fluid. Chronic administration of L-NAME (20 mg/kg/day in the drinking water for 2 weeks) markedly reduced the leukocyte accumulation (90%), protein leakage (44%), and NO₂/NO₃ (50%) levels in the synovial fluid, measured at the 4th h. Hoe 140, given i.v. (0.3 mg/kg, 30 min before) also reduced leukocyte accumulation (75%), protein leakage (48%), and NO₂/NO₃ (79%) levels in the synovial fluid, measured at the 4th h. Similar results were obtained with acute

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administration of L-NAME (30 mg/kg, i.v., 30 min before). These results indicate that arthritis induced by BjV is triggered by kinin formation and that the increase in both vascular permeability and leukocyte accumulation is modulated by NO release. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Bothropic snakes are responsible for about 90% of ophidic envenomation in Brazil and their venoms evoke a pronounced local tissue lesion characterised by haemorrhage, necrosis, inflammatory process and pain (Rosenfeld, 1971; Gutiérrez and Lomonte, 1989). The acute inflammatory reaction induced by *Bothrops jararaca* venom (BjV) in the rat paw (Trebien and Calixto, 1989), rat pleurisy (Búrigo et al., 1996) and rat air-pouch (Farsky et al., 1997) is determined by oedema and leukocyte influx. These effects are multimediated with participation of histamine, serotonin, products of arachdonic acid metabolism, platelet activating factor (PAF) and factors derived from the complement system (Trebien and Calixto, 1989; Búrigo et al., 1996; Farsky et al., 1997). Although BjV induces bradykinin (Bk) formation (Rocha e Silva et al., 1949), there is no evidence of its contribution to the local lesion. Additionally, to several systemic effects, Bk stimulates the nitric oxide (NO) generation by endothelial cells or leukocytes at the site of the injury (Radomski et al., 1990). Since, the rat kinin system is different from the other species, including man, (Bhoola et al., 1992), we have investigated the interaction of Bk and NO in a rabbit model of arthritis.

The participation of NO in the intraarticular inflammatory process has been fully described in arthritic patients (St Clair et al., 1996; Novaes et al., 1997). Additionally, NO is produced by chondrocytes (Stadler et al., 1991; Palmer et al., 1993; Stefanovic-Racic et al., 1994a; Grabowski et al., 1996), synoviocytes (Grabowski et al., 1996; Stefanovic-Racic et al., 1994b), and osteoblasts (Grabowski et al., 1996). The administration of nitric oxide synthase (NOS) inhibitor in the experimental arthritis models is followed by a reduction of intraarticular accumulation of leukocytes and a reduction in swelling as well as in histopathological abnormalities (McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1994c; Stefanovic-Racic et al., 1995; Verissimo de Mello et al., 1997). Moreover, endogenous release of NO enhances macrophage cyclooxygenase (COX) activity that may result in the production of pro-inflammatory prostaglandins (Corbett et al., 1993; Salvemini et al., 1993).

Despite the fact that BjV activates the kinin system and Bk releases NO (De Nucci et al., 1988), the contribution of both inflammatory mediators has not been evaluated in this process. Then, in this study the participation of these mediators

in the BjV-induced arthritis was evaluated using the NO synthase inhibitor, L-NAME and the bradykinin B₂ receptor antagonist HOE 140.

2. Material and methods

2.1. Venom

Lyophilised BjV was supplied by the Butantan Institute (Brazil). The venom was maintained at -20°C , dissolved in sterile saline and filtered through a sterilising membrane (ester cellulose filter, 0.22 μm pore size) before joint injection.

2.2. Induction of arthritis

The experimental protocol was approved by the Animal Ethics Committee of the Brazilian College of Experimental Animals. Arthritis was induced in the knee joint of adult male New Zealand White rabbits (2 kg) by injecting BjV (1–64 $\mu\text{g}/\text{ml}$; 0.5 ml) into the articular cavity. The contralateral joint was injected with the same amount of bovine serum albumin (BSA) diluted in sterile saline and previously filtered. The amount of protein present in the BjV or BSA solution was spectrophotometrically assessed and an equivalent protein concentration was injected in each joint. After 4, 24 or 48 h of the articular challenge, the animals were anaesthetised (xylazine 5 mg/kg, i.m. and ketamine 50 mg/kg, i.m.) and then sacrificed with 20% KCl (2 ml, i.v.).

2.3. Treatments

Both L-NAME and D-NAME (20 mg/kg/day) were administered in the drinking water for 2 weeks prior to the induction of arthritis. In a separate set of animals, L-NAME (30 mg/Kg, i.v.) was given as a single dose, 30 min prior to the venom administration. HOE 140 (D-Arg-[Hyp³, Thi⁵, Dtic⁵, Oic⁸]-bradykinin), a specific B₂ receptor antagonist (Rhaleb et al., 1992; Brenner et al., 1993) (0.3 mg/kg, s.c.) was injected 30 min before BjV administration.

2.4. Sampling of synovial fluid and measurement of leukocytes

Immediately after sacrifice, 2 ml of saline containing 0.1% EDTA was injected into the knee joint. The synovial fluid was aspirated, the joint opened and the remaining synovial fluid and the synovial membrane removed. Total leukocyte count was performed in a Neubauer chamber and the differential white cell was evaluated under light microscopy following Giemsa staining.

2.5. Assessment of vascular permeability response with Evans blue dye

Briefly, before venom injection into knee joints, the animals received i.v. 20 mg/kg of Evans's blue dye as a 2.5% solution in saline. The dye binds to plasma proteins resulting in a tagged macromolecule which passes the endothelial barrier in a negligible extent under normal conditions. However, it is raised in the synovial fluid under circumstances of increased vascular permeability. The synovial fluid was centrifuged and the optical density colorimetrically assessed at 630 nm. The concentration of dye bound to protein in the synovial fluid was estimated from a standard graph recording the optical density of serial dilutions of a known sample of Evans blue in NaCl. Results are expressed as μg of protein/ml of synovial fluid.

2.6. Synovial membrane histology

The synovial membrane sections (5 μm) were stained with hematoxylin and eosin for the quantification of PMN cell infiltrate under light microscopy. The results were expressed as cells/field by an observer blinded to the experimental groups.

2.7. Total NO_2/NO_3 determination

Total NO_2/NO_3 determination was realised by high performance liquid chromatography (HPLC) analysis (Muscará and De Nucci, 1996). After the passage of synovial fluid through a strong anion exchange column, two on line post column reactions occur. In the first, nitrate anions (NO_3) are reduced to nitrite (NO_2) on a copper-plated cadmium filled column, and in the second, a reaction with the Griess reagent takes place. The absorbance of the chromophore is read at 504 nm. The method has a sensitivity of 30 pmol for both anions.

2.8. Materials

Crystalline bovine albumin (fraction V), L-NAME and D-NAME were acquired from Sigma (St Louis, USA). Ester cellulose filters (0.22 μm pore size) was bought from Millipore (USA); xylazine from Bayer (Brazil); ketamine from Parke Davis (Brazil); EDTA and Evans blue dye from Merck (Brazil). Hoe 140 was kindly provided by Hoechst (Germany).

2.9. Statistical analysis

Results were presented as mean $\pm$ SEM and $p < 0.05$ as assessed by the analysis of variance followed by the Newman-Keuls test was considered significant.

3. Results

3.1. Characterisation of the arthritis induced by the intraarticular injection of BjV

The intraarticular injection of BjV (1–32 µg/ml) dose-dependently increased both the leukocyte accumulation and the protein leakage into the synovial fluid as measured at the 4th h after the venom administration (Fig. 1a). The leukocyte infiltration was mainly due to polymorphonuclear cells (Fig. 1a). A higher dose (64 µg) did not further increase leukocyte accumulation or protein leakage and evoked erythrocyte contamination (not shown). The intraarticular injection of BjV (16 µg) led to a time dependent increase in the cellular infiltrate and protein leakage that was maximal at the 4th h, decreasing thereafter until 48 h, when it was abolished (Fig. 1b).

3.2. Effects of NOs inhibition and bradykinin antagonism

L-NAME given either acutely or chronically significantly reduced leukocyte accumulation (Fig. 2a) and protein leakage (Fig. 2b) as measured at the 4th h after arthritis induction. Chronic administration of the inactive enantiomer D-NAME had no effect on these parameters as compared to control animals, which drank only water (not shown). The previous administration of HOE 140 (0.3 mg/kg) promoted a reduction of cellular migration and protein leakage to the inflamed area at the 4th hour of arthritis. The magnitude of this inhibitory effect on both parameters was similar to that evoked by L-NAME treatment (Fig. 2a and b).

3.3. NO₂/NO₃ measurement in the synovial fluid

The level of total NO₂/NO₃, at the 4th hour of arthritis, was significantly reduced in the synovial fluid, collected from the BjV injected joint, of L-NAME and HOE 140 treated animals when compared with the level obtained in the control animals (Table 1). The NO₂/NO₃ levels in the contralateral joint, injected

Table 1

NO₂/NO₃ levels in the synovial fluid of Control, L-NAME and HOE 140 treated animals at 4th h of BjV (16 µg) arthritis induction. The contralateral joint was injected with the same amount of BSA

Treatment	Control (n = 4)	L-NAME (n = 5) Chronic	L-NAME (n = 4) Acute	HOE 140 (n = 4)
BjV NO ₂ /NO ₃ (µM)	18.08 ± 3.05	9.63 ± 2.03*	9.8 ± 2.0*	5.75 ± 3.59*
BSA NO ₂ /NO ₃ (µM)	9.88 ± 0.58*	9.95 ± 0.81*	8.0 ± 0.7*	4.83 ± 0.98*

* $p < 0.05$ compared with values obtained of BjV injected joint in control animals.

1540

M.L. Guzzo et al. / *Toxicon* 38 (2000) 1535–1546

Fig. 1. Characterisation of the arthritis induced by *Bothrops jararaca* venom in rabbits. (a) Dose dependent cellular influx ($\square$ = polymorphonuclear; $\blacksquare$ = mononuclear) and protein leakage (line) induced by *BjV* injected intraarticularly. The animals were killed after 4 h of the induction of arthritis. (b) Represents the time-course of the arthritis induced by the injection of 16 μg of the *BjV* diluted in 0.5 ml of sterile saline. The animals were killed at 4, 24 and 48 h of *BjV* administration. Each point represents the mean $\pm$ SEM of 4–8 rabbits.

Fig. 2. (a) Total and differential cell count (▨ = PMN; ■ = MN) in the synovial fluid of rabbits injected with BjV (16 µg) 4 h after challenge. Animals were chronically (20 mg/kg/day/2 weeks) or acutely (30 mg/kg i.v.) treated with L-NAME or with HOE 140 (0.3 mg/kg/s.c., 30 min before the arthritis induction). Each column represents the mean ± SEM of 4–6 joints injected with BjV. * $p < 0.001$ by comparison with control animals. (b) Vascular permeability in the joint fluid collected from rabbits at 4 h of BjV induced arthritis (striped columns). The solid column represents the protein leakage assessed in the contralateral joint, injected with albumin. * $p < 0.01$ compared with the values obtained in respective controls.

with BSA did not differ from those evoked by saline injection (Veríssimo de Mello, 1997) and were not altered by the any drug employed. Treatment with D-NAME did not affect the synovial fluid levels of NO metabolites, when compared with the results obtained in animals, which drank only water (data not shown). Interestingly, the decrease in NO₂ and NO₃ levels observed with HOE 140 was significantly larger as compared to that induced by the chronic L-NAME treatment.

3.4. Synovial membrane PMN cell infiltrate

The synovial membrane of animals that received the venom intraarticularly had significant PMN cell infiltrate at the 4th hour, 12 cells/23 μm^2 (Fig. 3a). Chronic administration of L-NAME, but not D-NAME, greatly reduced the cell infiltration in the synovial membrane, 2 cells/23 μm^2 (Fig. 3b). The pattern of the synovial membrane of HOE 140 treated animals was similar to that observed in the synovial membrane of the L-NAME treated animals (data not shown).

4. Discussion

The findings that HOE 140 and L-NAME treatment abolished the inflammatory reaction induced by BjV clearly implicates kinins and NO in this phenomenon. Since bradykinin is known to release NO from various cells, including polymorphonuclear cells (Catz and Sterin-Speziale, 1996), it could be concluded that this kinin plays a pivotal role in the venom-induced arthritis.

Other mediators, such as histamine, 5-hydroxytryptamine, PAF, cyclooxygenase and lipoxygenase products have been considered to play a role in the BjV-induced inflammatory process. The contribution of eicosanoids is relevant to oedema formation and leukocyte recruitment during BjV-induced pleurisy and air-pouch inflammatory reaction (Búrigo et al., 1996; Farsky et al., 1997). Since bradykinin causes eicosanoid release via both phospholipase A₂ (Hong and Deykin, 1982) and phospholipase C (De Nucci et al., 1988) activation, it is possible that bradykinin also plays a key role on eicosanoids mediation during BjV envenomation. Additionally, bradykinin may be responsible for NO release (De Nucci et al., 1988) which suggests the importance of the interaction between both mediators in the BjV inflammatory response. The intense reduction of NO₂/NO₃ levels in the synovial fluid by HOE 140 and L-NAME suggests that NO production in the BjV inflammatory reaction is mediated by bradykinin.

The role of NO as a mediator of inflammatory reaction is controversial. The participation of NO in the vascular and cellular aspects of inflammation was demonstrated by their attenuation in L-NAME treated animals (Salvemini et al., 1995; Veríssimo de Mello et al., 1997; Stefanovic-Racic et al., 1995; Ianaro et al., 1994; Salvemini et al., 1996). Acute administration of L-NAME reduces protein leakage in response to different inflammatory agents; however, this reduction was attributed to a reduction in local microvascular blood flow rather than vascular

Fig. 3. Histopathological characteristics of rabbit synovial membrane at 4th h after BJV injection. The animals were treated before the induction of arthritis with D-NAME (a) or L-NAME (b). Note the reduction of PMN cell infiltration in B (bar = 23 μ m).

permeability (Hughes et al., 1990; Ialenti et al., 1992; Teixeira et al., 1993; Mariani-Pedroso et al., 1995). Interestingly, L-NAME treatment increases the plasma protein influx in certain microcirculatory beds including rat large airways, liver, spleen, pancreas, kidney, stomach, duodenum, mesenteric circulation, coronary circulation and mesenteric venules (Filep and Foldes-Filep, 1993; Kurose et al., 1993). The inhibition of oriented leukocyte locomotion induced by L-NMMA is overcome by L-arginine or dibutyl cGMP (Kaplan et al., 1989), indicating an important role of NO on cell migration. On the other hand, intravital microscopy study has demonstrated that NOS inhibitors greatly enhance leukocyte–endothelial interactions, initial events involved in leukocyte recruitment (Kubes et al., 1991).

The present study clearly implicates NO in the acute phase of BjV induced arthritis in the rabbit as suggested by the following observations: first, treatment with the NOS inhibitor L-NAME evoked a marked reduction in some signs of acute articular inflammation such as synovial membrane PMN cell infiltrate and vascular permeability in the synovial fluid; second, a reduction in cellular infiltration was also observed in the synovial membrane; third, NO₂/NO₃ level was reduced in the synovial fluid of L-NAME treated animals. D-NAME treatment did not induce any attenuation of the studied parameters. Our results show that in the articular environment NO seems to play a pro-inflammatory effect and corroborate our previous results in the antigen-induced arthritis in rabbits. These data show the effect of L-NAME treatment promoting a decrease in leukocyte infiltration, prostaglandin E₂ and interleukin-1 β levels in the synovial fluid (Veríssimo de Mello et al., 1997).

Bradykinin induces an increase in the blood flow in the rabbit synovium via activation of bradykinin B₂ receptors (Cambridge and Brain, 1998). HOE 140 has been tested in vivo and in vitro assays as a highly potent B₂ antagonist receptor (Wirth et al., 1991; Feletou et al., 1994). The magnitude of the inhibition of the inflammatory reaction induced by BjV in animals treated with the bradykinin B₂ receptor antagonist (HOE 140), further supports the idea that the B₂ receptor is the major receptor involved in the inflammatory action of bradykinin in this experimental model.

In conclusion, the present results suggest that endogenous NO is liberated at the BjV inflammatory site, probably in response to bradykinin, with consequent leukocyte recruitment and oedema formation. These new findings indicate that these mediators play a pivotal role in the early phase of the BjV inflammatory process.

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BACKGROUND: Our previous results showed that nitric oxide (NO) and bradykinin (BK) mediate the arthritis induced by *Bothrops jararaca* venom (BjV) in rabbits. In this study, we investigated the contribution of each receptor of BK as well as the inter-relationship between NO and eicosanoids in BjV-induced arthritis.

Methods: The arthritis was induced in rabbits with 16 µg of BjV injected intra-articularly. Prostaglandin E₂ (PGE₂), thromboxane B₂ (TxB₂), leukotriene B₄ (LTB₄) (radioimmunoassay) and nitrite/nitrate concentrations (NO₂/NO₃) (Griess reaction) were evaluated in the synovial fluid 4 h later. The animals were prior treated with NO synthase inhibitor (L-NAME; 20 mg/kg/day for 14 days), the B2 antagonist of BK (HOE-140) and the B1 antagonist of BK (des-Arg⁹[Leu⁸]-bradykinin), both at a dose of 0.3 mg/kg, 30 min prior to the venom injection.

Results: Data show that L-NAME and HOE-140 treatment were equally able to reduce PGE₂ and NO₂/NO₃ levels without interfering with TxB₂ and LTB₄ production. On the contrary, the B1 antagonist of BK inhibited TxB₂ and LTB₄ production, and did not alter PGE₂ and NO metabolites levels in the inflamed joint.

Discussions: The results presented clarify the contribution of the kinin system, mainly through the B2 receptor, to the local inflammatory response induced by BjV, as well as its positive interaction with PGE₂ and NO production.

Key words: *Bothrops jararaca* venom, Inflammatory reaction, Arthritis, Bradykinin, Nitric oxide, Eicosanoids

Pharmacological characterisation of arthritis induced by *Bothrops jararaca* venom in rabbits: a positive cross talk between bradykinin, nitric oxide and prostaglandin E₂

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Introduction

Endogenous nitric oxide (NO) has been described as a pro-inflammatory molecule showing interactions with cytokines¹⁻³ and inflammatory products of the cyclooxygenase pathway.⁴⁻¹⁰ Studies have shown that NO increases interleukin-1β and eicosanoid products, which may result in an exacerbated inflammatory response.^{2,3} In addition, inhibition of NO biosynthesis by an L-arginine antagonist reduced prostaglandin E₂ (PGE₂) production in different models of inflammatory reactions *in vivo*.¹¹⁻¹³

Additionally, bradykinin (BK) and NO have been implicated on eicosanoids and cytokine secretions in several experimental inflammatory processes.^{8,12,13-15} BK acts on the acid arachidonic metabolism via both phospholipase A₂¹⁶ and phospholipase C¹⁷ activation. Also, BK is known to release NO from various cells, including polymorphonuclear¹⁸ and endothelial cells at the site of injury.¹⁹

The acute inflammatory reaction induced by *Bothrops jararaca* venom (BjV) is determined by oedema and leukocyte influx.²⁰⁻²² These effects are

mediated by histamine, serotonin, products of arachidonic acid metabolism, platelet-activating factor and factors derived from complement system.²⁰⁻²² The eicosanoids contribution is relevant to oedema formation and leukocyte recruitment during BjV-induced pleurisy and air-pouch inflammatory reaction.^{21,22} Recently, to determine the participation of kinins and NO in the local inflammatory reaction induced by BjV, we developed an experimental model of arthritis by injecting the venom intra-articularly. The findings that B2 BK antagonist (HOE-140) and NO synthase inhibitor (L-NAME) treatment greatly reduced the cellular influx and protein leakage in the arthritis induced by BjV clearly implicate kinins and NO in this phenomenon.²³

Since eicosanoids, BK and NO are important mediators involved in the inflammatory reaction evoked by BjV, and since there is strong evidence regarding the interaction between these mediators on different models of the inflammatory process, the aim of this study was to evaluate the participation of each receptor of BK in NO and eicosanoid production in the acute inflammatory response evoked by BjV.

S.B.V. Mello et al.

Material and methods

Induction of arthritis

The Animal Ethics Committee of COBEA (Brazilian College of Experimental Animals) approved all experimental procedures, performed on animals in accordance with procedures set by The Universities Federation for Animals Welfare. The arthritis was induced in the knee joint of Male New Zealand White rabbits (2 kg) by the injection of BjV provided by the Laboratory of Herpetology, Butantan Institute, Brazil (16 µg diluted in 0.5 ml of saline). The venom was filtered in sterilising membrane (ester cellulose filter, 0.22 µm pore size; Millipore, São Paulo, Brazil) before joint injection. The contralateral joint was injected with the same amount of bovine serum albumin (BSA) (Sigma, St Louis, MO, USA). The amount of protein present in BjV or BSA solution was spectrophotometrically assessed and the equivalent protein concentration was injected in each joint. Four hours after intra-articular challenge, the animal was anaesthetised with a mixture of xylazine (5 mg/kg; Bayer, São Paulo, Brazil) associated with ketamine (50 mg/kg; Parke Davis, São Paulo, Brazil) by intramuscular injection and killed by intravenous injection of 2.0 ml of 20% KCl solution. Immediately after the sacrifice, 2 ml of saline containing ethylenediamine tetraacetic acid (1 mg/ml) was injected into the knee joint. Synovial fluid was aspirated, the joint was opened and the remainder of the synovial fluid was recovered and stored at -70°C.

Pharmacological treatments

A group of animals were randomly treated with L-NAME (Sigma), 20 mg/kg/day mixed with drinking water, administered for 2 weeks prior to the induction of arthritis. Treatments with 0.3 mg/kg of B1 or B2 BK antagonists des-Arg⁹[Leu⁸]-bradykinin (Sigma) and HOE-140 (Hoechst, Frankfurt, Germany), respectively, were performed subcutaneously, 30 min before the venom injection. Control animals received an equivalent volume of sterile saline by the same route.

Determination of total NO₂⁻/NO₃⁻ levels in the synovial fluid

The concentration of the total amount of nitrate and nitrite (NO₂⁻/NO₃⁻) anions (stable NO breakdown products *in vivo*) present in the synovial fluid samples collected 4 h after venom injection was determined by Griess reaction. The absorbance was monitored at 546 nm.

Determination of eicosanoids in the synovial fluid

PGE₂, thromboxane B₂ (TxB₂), leukotriene B₄ (LTB₄) levels were assayed in the synovial fluid collected 4 h

after venom injection using commercial kits (NEN Life Science products, Boston, MA, USA) as previously described.^{24,25} The competitive binding radioimmunoassays were performed in polypropylene tubes with the reagents diluted in phosphate-buffered saline (pH 6.8) with gelatine and thimerosal. One hundred microlitres each of unknown samples, of tracers (¹²⁵I-PGE₂ and ¹²⁵I-TxB₂ or ³H-LTB₄), and of rabbit antiserum to each eicosanoids were combined and incubated overnight at 8°C for LTB₄ determination, and were then incubated for 2 h at 25°C in a water bath for PGE₂ and TxB₂ determinations. Unbound radiolabelled PGE₂ and TxB₂ was removed by the addition of 250 µl of donkey anti-rabbit serum coated onto magnetisable polymer particles, and LTB₄ was removed by the addition of 250 µl of 2% charcoal suspension coated with 0.4% dextran to the tubes. After centrifugation, the residual bound activity was measured in each tube by counting ¹²⁵I-PGE₂ and ¹²⁵I-TxB₂ in a γ-scintillation counter, and ³H-LTB₄ in a β-scintillation counter. Cross-reactivities of the antiserum with other eicosanoids were below 0.05%. The assay sensitivities were 1.25–160 pg/tube (PGE₂), and 1.6–200 pg/tube (LTB₄).

Statistics

Results are expressed as mean ± standard error of mean. Results were analysed by Student's *t*-test or by repeated-measures analysis of variance. When appropriate, the data were analysed by the Newman-Keuls test. The chosen level of significance was 0.05.

Results

Eicosanoids and NO₂⁻/NO₃⁻ levels in the synovial fluid

The synovial fluid collected from animals pre-treated with L-NAME, HOE-140 and des-Arg⁹[Leu⁸]-bradykinin and intra-articularly injected with venom, and that from respective controls, was extracted to PGE₂, LTB₄ and TxB₂. The determinations were performed by radioimmunoassay. NO₂⁻/NO₃⁻ levels in diluted joint wash were also determined by Griess reaction. Figure 1A shows the PGE₂ level measured in the synovial fluid obtained 4 h after the BjV. Prior treatment of the rabbits with L-NAME or B2 antagonist of BK HOE-140 significantly reduced it when compared with values obtained in samples from animals, which did not receive pharmacological treatments. It is important to note that des-Arg⁹[Leu⁸]-bradykinin treatment did not interfere with the production of PGE₂. On the contrary, levels of LTB₄ and TxB₂ (represented in Fig. 1B and Fig. 1C, respectively) exhibited a different pattern of alteration. They were not altered after treatment with L-NAME or HOE-140, but they were greatly reduced

Mediators involved in arthritis induced by *B. jararaca* venom

FIG. 1. Effects of L-NAME, HOE-140 and des-Arg⁹[Leu⁸]-bradykinin treatments on local eicosanoid and NO production 4 h after intra-articular injection of BjV. (A) PGE₂ levels, (B) LTB₄ levels (C) TxB₂ levels and (D) NO₂/NO₃ levels following treatments with L-NAME (n = 5), HOE-140 (n = 4) and des-Arg⁹[Leu⁸]-bradykinin (n = 5). Open column, the level of the eicosanoid in control animals (n = 6). Dotted line, the values of each eicosanoid in the BSA-injected joint. Each column expresses data as the mean (±s.e.m.). *p < 0.05 by comparison with control animals.

after pre-treatment with the B1 antagonist of BK des-Arg⁹[Leu⁸]-bradykinin. Also, values observed in samples from B1 antagonist-treated animals were significantly lower than that obtained in samples from control animals. At the fourth hour after the injection

of BSA, the contralateral joint exhibited low levels of the eicosanoids, similar to the observed in the saline-injected joint (data not shown).^{12,26}

Similar to the observed pattern of alteration promoted in PGE₂ production, the level of the total amount of the NO metabolites (NO₂/NO₃; Fig. 1D) was significantly reduced in the synovial fluid collected from L-NAME-treated and HOE-140-treated animals when compared with the level observed in control animals. Differently, the treatment with the B1 antagonist of BK, des-Arg⁹[Leu⁸]-bradykinin, did not affect NO production in the joint, which exhibited values of NO₂/NO₃ obtained 4 h after BjV injection equivalent to those found in control animals.

Discussion

The data presented in this paper regarding BjV local effects corroborate the positive cross-talk between BK, NO and the cyclooxygenase (COX) product PGE₂ described in the inflammatory reaction induced by other different agents,^{8,12,13} and additionally show the main participation of the B2 BK receptor in this axis. This last observation is in accordance with recent experimental studies in rats that demonstrated HOE-140 treatment reduced the oedema induced by local injection of *Bothrops lanceolatus* snake venom and abolished the hyperalgesic effect of BjV.^{27,28} Additionally, the employment of specific receptor antagonists and enzymatic inhibitors in experimental studies has demonstrated the participation of BK receptors and COX metabolites on the inflammatory process evoked by BjV through measurement of its symptomatic effects such as oedema,^{20,21,29} leukocyte recruitment^{21,22,29} and pain.²⁸ However, in the experimental model employed in this study, it was possible to establish the link between each mediator involved in the kinin/NO/COX axis at the inflammatory site after pharmacological treatments.

We have clearly demonstrated the contribution of the kinin system, via the B2 receptor, on NO production, with subsequent stimulation of PGE₂ secretion. Also, our results showed that the B2 receptor and NO do not participate in LTB₄ and TxB₂ secretions, which were affected by inhibition of the B1 receptor. These conclusions are demonstrated by the following observations: (1) HOE-140 and L-NAME treatments equally abolished the inflammatory reaction,²³ PGE₂ and NO₂/NO₃ levels without interfering with LTB₄ or TxB₂ production; and (2) alternatively, des-Arg⁹[Leu⁸]-bradykinin treatment did not alter the PGE₂ and NO metabolites, and greatly reduced LTB₄ and TxB₂ secretions. It is important to note that our previous results showed that the NO synthase inhibitor (L-NAME) and the B2 receptor antagonist of BK (HOE-140) treatments promoted a great reduction of the leukocyte influx (90% of the control animals) to the articular cavity.²³ These data associated with the

verified low levels of LTB₄ in the joint, suggest the participation of other(s) chemoattractant(s) in this phase of BjV inflammatory reaction. Recent experimental studies have demonstrated that *Bothrops* snake venoms activate the complement system, with a consequent contribution of its metabolites on leukocyte recruitment.^{22,30} The participation of the complement system, BK and NO has been described in several models of inflammation.^{31,32} The possibility that the complement system participates in or may be affected by BK/NO cascade in the bothropic envenomation will be further investigated. In addition, the treatment with the B1 receptor antagonist of BK, which promoted reduction of LTB₄, was less effective than HOE-140, promoting 40% of reduction in the cellular influx to the inflamed area (data not shown).

In conclusion, our results suggest that the NO generation in arthritis induced by BjV is produced mainly after BK stimulation through B2 receptors. Also, the endogenous release of NO enhances COX activity with further increase of PGE₂ production, demonstrating the inter-relation between the kinins system with NO and PGE₂ in the BjV inflammatory process.

This paper, together our previous results,²³ would be useful to pharmacological interventions to modulate the imminent inflammatory response evoked by BjV, which is not neutralised by specific antivenom.

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Original Research Papers

Chronic blockade of nitric oxide biosynthesis in rats: effect on leukocyte endothelial interaction and on leukocyte recruitment

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Abstract. *Introduction:* Previous studies showed that animals chronically treated with NG-nitro-L-arginine methyl ester (L-NAME) have a reduced inflammatory reaction. Now the role of L-NAME treatment (20 mg/Kg/day/14 days) on leukocyte mobilisation was assessed in rats.

Methods: In vivo leukocyte recruitment evoked by *Bothrops jararaca* venom (BjV) and nitrite/nitrate ($\text{NO}_2^-/\text{NO}_3^-$; Griess reaction) were evaluated in the air pouch cavity. Haematological parameters were evaluated in the bone marrow and in the peripheral compartment. Microcirculatory blood flow, number of rolling and adhered leukocytes, vascular reactivity and mast cell activity were studied by intravital microscopy. Blood pressure was measured by the tail-cuff method. L-selectin and β_2 integrin expressions on peripheral and bone marrow leukocytes were quantified by flow cytometry.

Results: When compared with control rats (D-NAME) L-NAME treated rats had reduced PMN cell infiltrate (50%) and $\text{NO}_2^-/\text{NO}_3^-$ (27%) in the air pouch cavity. Rolling leukocytes were decreased (70%) in L-NAME-treated animals, which was reversed by topical application of NO donor (SIN-1). BjV stimulation increased the number of rolling and adhered leukocytes only in control rats. Systemic blood pressure, microcirculatory blood flow and microvascular reactivity was not altered by the treatment. Only the vessel response to acetylcholine was delayed in treated rats. Peripheral PMN cells were increased by L-NAME treatment (100%), but the number of bone marrow cells was not altered. The treatment reduced L-selectin expression on circulating leukocytes, by either with (16%) or without (26%) stimulation with BjV; PMN cells were more affected (32–37%). Impairment of L-selectin expression was also verified in bone marrow cells under stimulation with BjV.

Conclusions: Results show that this schedule of L-NAME treatment promotes a decrease on L-selectin expression. This effect may promote the standstill of leukocytes in the blood compartment and may be responsible, at least in part, for the observed deficient leukocyte-endothelium interactions with subsequent impairment of leukocyte migration to the inflammatory site.

Key words: Nitric oxide – Leukocyte-endothelial interactions – Microcirculation – Inflammation – Haematological parameters

Introduction

The effect of nitric oxide (NO) in the inflammatory reaction is complex since an effective role has been established on the production and mobilisation of leukocytes and on vascular reactivity. Different experimental conditions, doses and administration routes, as well as the tissue employed have determined opposite effects. Thus, NO has been suggested to act either as a pro or anti-inflammatory mediator.

Enhanced production of NO by the inducible form of nitric oxide synthase (iNOS) is associated with the development of inflammatory diseases. It has been demonstrated that in vivo non-specific pharmacological inhibition of nitric oxide synthases (NOS) reduces leukocyte migration to inflammatory sites promoting amelioration of pathological conditions [1–8]. The reduction is not likely to depend on the hypertensive effect evoked by these inhibitors, because pharmacological normalisation of blood pressure does not prevent the decrease in the number of leukocytes migrating to the inflamed area [9].

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In apparent conflict with these studies, it has been fully demonstrated that both constitutive and inducible generation of NO by competent cells modulates leukocyte-endothelium interaction within the microcirculatory network. Superfusion or bolus injection of non-specific NOS inhibitors, as NG-monomethyl-L-arginine (L-NMMA) or NG-nitro-L-arginine methyl ester (L-NAME), evokes marked increases in the attachment of leukocytes to postcapillary venules and their emigration into the extravascular tissue [10–12]. In addition, treatment with aminoguanidine, a specific iNOS inhibitor, promoted an increase in the number of rolling leukocytes in cat mesentery superfused with TNF- α [13]. A plausible explanation for the effect was interference with superoxide production and mast cell degranulation [14–16]. However, the NO effect on mast cell degranulation is also controversial [17–20].

It is well known that leukocyte-endothelial interaction is dependent on the expression of adhesion molecules. The role of NO on expression and/or activity of these receptors has not been fully elucidated. In this context, in vivo studies have suggested a correlation between inhibition of NO production and increment of endothelial adhesion molecules expression [12, 21]. Additionally, the effect of L-NMMA or L-NAME superfusion in cat or rat microcirculatory vessels is mediated by CD11/CD18 complex expression [22]. The effect of NOS inhibitors directly on blood flow may contribute to the CD18 expression [22]. In vitro studies corroborate the inhibitory effect on vascular adhesion molecules [10, 23–26], but do not confirm the participation of CD18 complex detected in vivo through L-NAME superfusion [10].

A role for NO modulating haematopoiesis has been proposed since several studies have demonstrated its interference on progenitor cells, on maturation steps, apoptosis, and bone marrow blood flow [27]. L-NAME treated mice presented lymphopenia and neutrophilia, which were partially reversed by L-Arg administration [28]. These effects may interfere with leukocyte/endothelial interactions, since up and down modulation of adhesion molecules are observed during the intermediate stages of leukocyte maturation [29].

We have previously shown that chronicle L-NAME treatment (14 days, 20 mg/kg, oral route) reduces albumin leakage and leukocyte migration to the articular cavity of rabbits in which an arthritic lesion was induced, either immunologically or by the injection of *Bothrops jararaca* venom (BjV) [9, 30, 31]. The efficacy of NO synthesis blockade was confirmed by a significant reduction in levels of NO metabolites (nitrate/nitrite) in the synovial fluid [9, 30–32].

The results presented herein show that oral L-NAME chronicle treatment employed in our laboratory promotes impairment in L-selectin expression firstly in PMN. This effect may be responsible, at least in part, for the in vivo deficient leukocyte/endothelium interaction, which may determine a reduced recruitment to the inflamed area and their standstill in the peripheral compartment.

Materials and methods

Animals

Male Wistar rats, weighing 200–250 g at the beginning of the pharmacological treatment were employed. The animals were allowed a stan-

dard pellet diet and water *ad libitum*. During experimental procedures the animals were anaesthetised to avoid any stress condition. The local Committee for Ethical Surveillance in Animal Experimentation (COBEA) approved the experimental protocol.

L-NAME and D-NAME treatments

A group of animals were randomly treated with L-NAME or its inactive enantiomer D-NAME (Sigma, St Louis, MO, USA; 20 mg/Kg/day) mixed in the drinking water. The drugs were administered during two weeks and the experiments, in vivo, in vitro or in situ were then performed.

Bothrops jararaca Venom (BjV)

Lyophilised crude BjV was a venom pool obtained from several specimens of *Bothrops jararaca* snakes and was supplied by the Laboratory of Herpetology, Institute Butantan, Brazil. The venom was maintained at -20°C , dissolved in sterile saline solution at the moment of the use, the solutions were filtered through a cellulose ester membrane of 0.22 μm average pore size.

In vivo leukocyte migration: Air Pouch model

Animals were anaesthetised with pentobarbital sodium (Cristália, Brazil; 65 mg/Kg; intraperitoneally) and 10 mL of sterile air was injected subcutaneously in the dorsal region. Seven days thereafter the pouch was reinstalled with approximately 5 mL of air. At the 14th day following the first air injection, BjV (10 μg or 25 μg dissolved in 1.0 mL of sterile saline, $n = 6$ for each dose of BjV) was injected into the pouch. Control animals received the same volume of sterile saline. Six h later the animals were anaesthetised and sacrificed. The pouches were washed with 3 mL of Hank's balanced salt solution and the total leukocyte number in the harvested material was determined in Neubauer chambers. Differential cell counts were performed in smears stained with Giemsa dye.

Total NO_2/NO_3 determination

The concentration of the total amount nitrite/nitrate anions presented in the pouch wash samples collected 6 h after BjV injection was determined by Griess reaction. The absorbance was monitored at 646 nm.

Intravital microscopy studies

Animals were anaesthetised with pentobarbital sodium (65 mg/Kg; intraperitoneally) and the internal spermatic fascia or cremaster muscle of the wall of the scrotal chamber were exteriorised for microscopic examination in situ [33, 34]. After surgery, the animals were maintained on a special board thermostatically controlled at 37°C , which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed Ringer-Locke solution (pH 7.2–7.4 $\times$ NaCl 154 mM; KCl 5.6 mM; CaCl_2 2 mM; H_2O 2 mM; NaHCO_3 6 mM and glucose 5 mM) containing 1% gelatine. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the preparation in continuous contact with a film of the liquid. Transilluminated images were obtained by optical microscopy (Axioplan II, Carl-Zeiss equipped with $\times 5.0/0.30$ plan-neofluar or $\times 10.0/0.25$ Achromplan longitudinal distance objectives/numeric aperture and $\times 1.0$, 1.25 or 1.60 optovar). The images were captured by a video camera (ZVS, 3C75DE, Carl-Zeiss) and were transmitted simultaneously to a TV monitor and to a computer. Images obtained on the TV monitor were recorded on a video, and digitised in the computer and analysed through an image analyser soft-

ware (KS 300, Kontron). An optical doppler velocimeter was incorporated to the microscope to assess erythrocyte velocity [35, 36].

Chronicle administration of L-NAME or D-NAME on leukocyte-endothelial interactions, microvascular reactivity, blood flow and mast cell number and degranulation ability were evaluated at 14th day of the beginning of treatments.

Leukocyte-endothelial interaction

The number of rolling and adhered leukocytes in postcapillary venules (20–30 μm diameter; 200 μm length) of internal spermatic fascia was determined at intervals of 10 min before and after the topical application of BjV (0.25 $\mu\text{g}/10 \mu\text{L}$, $n = 6$ for L-NAME and control groups). In another group ($n = 4$) of L-NAME and D-NAME treated animals the effect of topical application of a nitric oxide donor, 3-morpholinosydnonimine (SIN-1, 10 mM) on leukocyte-endothelial interactions was observed.

Microvascular reactivity

The constrictor response to noradrenaline (NA, 1, 2.5, 5 $\mu\text{g}/\text{mL}$, Sigma, St Louis, MO, USA) and vasodilatation to acetylcholine (Ach, 300 $\mu\text{g}/\text{mL}$, Sigma, St Louis, MO, USA) and histamine (50 $\mu\text{g}/\text{mL}$, Sigma, St Louis, MO, USA) were tested. Drugs dissolved in Ringer-Locke's solution were topically applied to vessels (20–30 μm) of the internal spermatic fascia or cremaster muscle in a standard volume of 10 μL and removed by washing with Ringer-Locke's solution. Animals of each group ($n = 4-6$) were tested for the different stimulus. The latency time required to the arteriole stasis in response to each dose of NA and the subsequent re-flow in response to Ach were recorded. Changes in venule diameter, expressed as percentage of increasing of the initial diameter in response to histamine was addressed. Results are expressed as mean $\pm$ S.E.M of 4–6 rats for each group, in which were evaluated 5 fields.

Mast cell: number and activity

Other set of experiments was designed to assess the interference of L-NAME treatment on mast cell. The degranulation of mast cells under topical application of 10 μg of 48/80 compound (Sigma, St Louis, MO, USA) was determined in the adjacent tissue to postcapillary venules of internal spermatic fascia. The number of mast cell degranulated in an area of 7,200 μm^2 and the time of latency required to begin the process was recorded in 10 vessels of each animal. The number of constitutive mast cells was evaluated after 5 min of topical application of blue toluidin dye (Sigma, St. Louis, MO, USA) and 10 areas were evaluated. It was employed 4–6 animals for each group, in each assay.

Blood pressure measurement

Arterial blood pressure was measured in 6 μm anaesthetised animals of each group by tail plethysmography using a programmed E&M Instrument Co electrophygmomanometer (Narco Bio System, Texas, USA). The rats were placed in a warming chamber maintained at 37°C for 10 min to allow pulses to be recorded. The cuff pressure was controlled automatically and the systolic pulses detected by the pulse transducer were monitored with the audio signal. The average of five successive measurements was taken as the mean systolic pressure value.

Haematological parameters

Blood

Animals (6–8 for each group) were anaesthetised with pentobarbital sodium (65 mg/Kg, intraperitoneally) and whole blood samples with

EDTA (1 mg/mL, Sigma, St. Louis, MO, USA) were obtained via abdominal aorta punctures and collected concomitantly with bone marrow cells. The haemoglobin concentration [37] and hematocrit were [38] determined by standard methods. Total cells were quantified using a Neubauer chamber. Differential leukocytes counts were performed on blood smears stained May Grunwald- Giemsa solution (Sigma, St. Louis, MO, USA).

Bone marrow and splenic cells

Bone marrow cells were obtained by flushing the femoral cavity with the McCoy's 5A medium (Sigma, St. Louis, MO, USA) with EDTA (1 mg/mL). Spleens were removed, placed in Petri dishes containing McCoy's 5A medium (Sigma Chemical Co., St. Louis, MO) with EDTA (1 mg/mL) and gently dissociated. The number of total cells was quantified in Neubauer chamber. Differential counts were performed on the basis of 500 cells per slide in cytocentrifuge smears stained by May-Grumwald and Giemsa dye.

Flow cytometry

Leukocytes were isolated from blood collected from the orbital plexus using EDTA or from bone marrow (4 L-NAME and 4 control animals) to quantify L-selectin or β_2 integrin expression. Briefly, erythrocyte lysis was performed using ammonium chloride solution (0.13 M) and leukocytes were recovered after washing with Hank's balanced solution (HBSS). Cells (1×10^6) were incubated with or without BjV (1–5 $\mu\text{g}/10$ or 30 min). After washing, leukocytes were further incubated for 30 min at 4°C in the dark with 10 μL of IgG 1-FITC and 10 μL of primary monoclonal antibody against L-selectin (FITC conjugated, hamster anti-rat L-selectin, Seikagaku corporation, Tokyo, 50 $\mu\text{g}/\text{mL}$) or β_2 integrin (FITC conjugated, mouse anti-rat CD18, BD PharMingen Technical, CA, USA, 50 $\mu\text{g}/\text{mL}$). Immediately after incubation, cells were analysed on a FACScalibur flow cytometer (Becton & Dickinson – San Jose, CA, USA). Data from 10,000 cells were obtained and only the morphologically viable leukocytes were considered for analysis.

Statistical analysis

Means and standard error of means (SEM) of all data are presented and were compared by student's *t*-test or Mann & Whitney test with significant probability levels of less than 0.05.

Results

In vivo leukocyte migration

To investigate the effect of L-NAME treatment on in vivo leukocyte recruitment, groups of L-NAME or D-NAME treated animals were injected with increasing doses of BjV. Results presented in figure 1 show that oral L-NAME administration impaired the leukocyte migration 6 h after BjV injection. The number of polymorphonuclear cells (PMN) in the inflammatory reaction induced by BjV was reduced in L-NAME treated rats when compared with control animals (D-NAME). Total amount of nitrite/nitrate anions presented in the control pouch wash samples was $26 \pm 0.68 \mu\text{M}$ and $19.53 \pm 0.85 \mu\text{M}$ in L-NAME treated animals ($n = 5$; $p < 0.001$).

Leukocyte-endothelial interactions

The contribution of NO to leukocyte-endothelial interactions was examined by determining the number of rolling and

Fig. 1. Leukocyte influx into rat air pouch cavity, 6 h after 10 or 25 µg of Bjv injection. ■ = L-NAME (20 mg/Kg/day for 14 days); ● = D-NAME treated rats (20 mg/Kg/day for 14 days). Results are expressed as mean ± S.E.M. of six rats in each group. *p < 0.05 vs. control.

Fig. 2. Number of rolling (Panel A) and adhered leukocytes (Panel B) in postcapillary venules of the internal spermatic fascia of L-NAME (20 mg/Kg/14 days; n = 6) and D-NAME (20 mg/Kg/14 days; n = 6) treated rats. Black columns represent the number of rollers before BjV and striped columns represent values obtained after the topical administration of 0.25 µg of BjV. *P < 0.05 vs initial values #P < 0.001 vs. control.

adhered cells in postcapillary venules of the internal spermatic fascia of L-NAME treated rats. As shown in figure 2 A, L-NAME reduced the number of rolling leukocytes, either before or after BjV topical application. Adhered cells, in basal conditions, were quantitatively equivalent in both groups of animals (Figure 2 B). However, the increment in the number of adhered cells induced by BjV was much lesser in L-NAME treated rats than in controls.

To assess the contribution of NO in L-NAME treated rats, a set of L-NAME- or D-NAME-treated animals received a topical application of the NO donor SIN-1 (10 mM). Data obtained demonstrated that SIN-1 reversed the decrease in the number of rolling leukocytes previously observed in L-NAME-treated animals. Twenty min after SIN-1 addition, the number of rolling leukocytes was similar to that observed in control animals (Figure 3). In both group of animals was detected decreasing in blood flow, however the reduction was more intense in L-NAME treated rats (control: 10%; L-NAME treated: 23%; n = 4).

Fig. 3. Number of rolling leukocytes in postcapillary venules of the internal spermatic fascia of rats treated with L-NAME (■ 20 mg/Kg/14 days; n = 4) and D-NAME (● 20 mg/Kg/14 days; n = 4) before and after the topical application of nitric oxide donor (SIN-1, 10 mM). *p < 0.05 vs. control.

Fig. 4. Effect of L-NAME treatment (20 mg/Kg/14 days) on in vivo arteriolar response to noradrenaline and acetylcholine. Data represent the time required to cessation of blood flow. White columns show mean ± S.E.M. of values obtained in 4-6 control animals (D-NAME 20 mg/Kg/14 days) and the striped column values obtained in 4-6 L-NAME treated rats. *p < 0.05 vs. control values.

Table 1. Haemodynamic parameters (erythrocyte velocity in postcapillary venules and systemic blood pressure) in L-NAME (20 mg/Kg/day for 14 days) and D-NAME treated animals (20 mg/Kg/day for 14 days). n = number of animals in each group. Results are expressed as mean $\pm$ S.E.M.

Treatment	Erythrocyte velocity (mm/seg)		Mean blood Pressure (mmHg)
	Before BjV	After BjV	
Control (n = 6)	2.9 $\pm$ 0.15	2.8 $\pm$ 0.30	118 $\pm$ 13.4
L-NAME (n = 6)	3.2 $\pm$ 0.37	2.6 $\pm$ 0.05	125 $\pm$ 9.7

Fig. 5. Mast cells activity and number. Panel A and D show normal venules (20 μ m) in the spermatic fascia of control and L-NAME treated animals (20 mg/Kg/14 days) respectively. Panels B and E exhibit the degranulation of perivascular mast cells under topical application of 10 μ g of 48/80 compound in control and L-NAME treated animals, respectively. Panels C and F show the number of perivascular mast cells 5 min after topical application of blue toluidin dye in control (D-NAME 20 mg/Kg/14 days) and L-NAME treated animals, respectively. Bar = 40 μ m.

Microvascular response to Noradrenaline, Acetylcholine and Histamine

At resting conditions, diameters of arterioles and venules of L-NAME and D-NAME treated rats were not different (data not shown). Results presented in figure 4 shows that L-NAME treatment did not interfere with arteriolar reactivity in response to NA. Similar dose-response was observed in both groups of animals studied. On the other hand, the subsequent re-flow induced by Ach was significantly de-layed in L-NAME treated animals in comparison to controls (Figure 4).

In addition, vessel response to histamine was not altered by L-NAME treatment. Equivalent dilation was recorded in both groups of animals, 20% for venules and about 10–15% for arterioles (n = 4–6 for each group of animals).

Mast cell: number and activity

Results depicted in figure 5 show that chronicle L-NAME treatment, here employed, does not interfere with the number or degranulation ability of mast cells adjacent to venules. The average of mast cell number was similar in L-NAME (16.26 ± 2.11) and control animals (12.38 ± 0.45). Topical application of 48/80 compound evoked equivalent mast cell degranulation in both groups. The time required to degranulate was about 30 s, and the numbers of degranulated cells in adjacent area of postcapillary venules were 13.26 ± 0.57 for L-NAME treated rats and 15.25 ± 1.1 for control animals (4–6 animals in each group).

Haemodynamic parameters

Results presented in table 1 indicate that L-NAME administration, as currently employed, did not significantly affect systemic blood pressure or erythrocyte velocity in postcapillary venules. Therefore the impairment in leukocyte-endothelial interactions could not be ascribed to haemodynamic factors.

Haematological parameters

L-NAME administration did not modify the total cellularity of the bone marrow and spleen or the distribution of the different cellular populations (Table 2). Shunting line to the left in the granulocyte sector was not observed. However L-NAME treatment promoted a markedly increase (111%) in the absolute number of circulating neutrophils (Table 3), being that this rise did not reflect the appearance of band cells. Others blood parameters such as erythrocyte, reticulocyte and platelet numbers, haemoglobin concentration and hematocrit were not modified by L-NAME treatment (data not shown).

L-selectin and CD18 expression

Surface expressions of L-selectin and CD18 were investigated in circulating and bone marrow leukocytes using immunofluorescence flow cytometry. Images presented in figure 6 B and 6 C demonstrate that L-NAME, but not D-NAME, reduced L-selectin expression, particularly noticeable in PMN cells. The reduction was detected in cells even stimulated or not with BjV (Fig. 6A). Pre incubation of cells, collected from control rats, with BjV increases the expression of both adhesion molecules by about 30% (not shown). On the other hand, β_2 integrin expression was not altered by L-NAME treatment even with or without stimulation (data not shown).

Additionally, impairment on L-selectin expression may be present in bone marrow cells. The treatment did not alter the basal expression of L-selectin on membrane of early, mature PMN cells or lymphocyte and blastocytes (figure 7, panels A and C). On the other hands, under stimulation with BjV the expression was highly increased in cells obtained from control animals (early PMN = 40%; mature PMN = 100% and lymphocyte and blastocytes = 100%, figure 7, panels A and B), but cells collected from bone marrow of L-NAME treated animals were not able to respond to the stimulus (figure 7, panels C and D).

Table 2. Cell counts in bone marrow and spleen of control and L-NAME treated rats.

Treatments	Total cells ($\times 10^3/\text{mL}$)		Granulocytes ($\times 10^3/\text{mL}$)		Lymphocytes ($\times 10^3/\text{mL}$)		Erythroblasts ($\times 10^3/\text{mL}$)	
	Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen
Control (n = 6)	4.70 ± 0.29	4.32 ± 0.59	170.7 ± 16.93	11.68 ± 3.45	81.84 ± 6.51	349.1 ± 59.48	141.0 ± 27.29	36.54 ± 7.82
L-NAME (n = 8)	4.42 ± 0.23	3.88 ± 0.39	150.3 ± 15.53	12.06 ± 3.23	67.68 ± 7.17	323.0 ± 33.56	135.9 ± 15.08	35.29 ± 5.95

Data are expressed as mean $\pm$ S.E.M.

Table 3. Total and differential leukocytes counts in blood of control and L-NAME treated rats. Results are presented as mean $\pm$ S.E.M.

Treatments	Leucocytes (mm^3)	Neutrophils (mm^3)	Lymphocytes (mm^3)	Monocytes (mm^3)
Control (n = 6)	$4,819 \pm 516$	471 ± 77	$3,763 \pm 495$	139 ± 42.5
L-NAME (n = 7)	$5,714 \pm 469$	$993 \pm 136^*$	$4,031 \pm 548$	185 ± 31.9

* $P < 0.05$ vs. respective value in control animals.

Fig. 6. L-selectin expression on peripheral leukocytes obtained from L-NAME and D-NAME treated rats. Data were obtained in blood flow cytometry. (A) Represents percentage of reduction of L-selectin in L-NAME treated animals (20 mg/Kg/14 days; n = 4) in relation to D-NAME (20 mg/Kg/14 days; n = 4), in basal and following stimulation by BjV (10 µg of BjV). Panel B shows images observed in total leukocytes and C demonstrate images from polymorphonuclear (PMN) and mononuclear (MN) cells. *p < 0.05 vs. control.

Discussion

In the current study we have clearly show that chronic unspecific NOS inhibition evokes impairment on leukocyte-endothelial interactions, visualised by decreasing on the number of rolling and adhered leukocytes to the postcapillary venule endothelium. The reversal of rolling behaviour following local application of the NO donor SIN-1 indicates that the relative lack of NO in L-NAME treated rats appears to be the major determinant for the effect. These data are surprising since the literature has determined NO as a modulator of leukocyte-endothelium interactions. However, these studies were performed using higher doses and acute treatments with NOS inhibitors or NO donors [10, 11, 13–16]. Possibly, differences obtained between studies are attributed to the amount of NO produced as an outcome of pharmacological treatments. This hypothesis is based on observations that NO is recognised as a protective or injurious agent in physiological and pathological conditions depending on the amount produced, the site of production, and the presence of other reactive species [25, 39, 40]. It is important to emphasise that our treatment does not induce a total blockage in NO synthesis, since nitrite/nitrate levels in air pouch inflammatory exudate was reduced by about 30%.

The alterations on leukocyte behaviour on microvessels, observed in basal conditions, persisted during the development of an inflammatory response evoked by the topical application of BjV. The increase in number of rolling and adhered leukocyte evoked by the venom in control animals was not observed in L-NAME treated rats.

The migration of leukocytes from the vascular system to the injured area is a key event in inflammation. In this context, tethering and rolling of leukocytes on the vessel wall is the initial and fundamental event, following by firmly adherence to the endothelium. These cellular interactions in vivo are influenced by alterations on microcirculatory haemodynamics, leukocytes and platelet aggregation as well as other blood component, and by endogenous mediators liberated by interstitial cells. Our results indicate that inhibition on leukocyte-endothelial interaction by L-NAME treatment does not reflect haemodynamics modification, since erythrocyte velocity on postcapillary venule and arteriolar or venular response to vasoactive substances, such as noradrenaline and histamine are not altered. As expected, a delayed response to acetylcholine was recorded in L-NAME treated rats. Systemic blood pressure is also similar in control and L-NAME treated animals. The observed effect is not surprisingly since

Fig. 7. L-selectin expression on bone marrow leukocytes obtained from L-NAME and D-NAME treated rats. Figures represent L-selectin expression in non-stimulated (A) and stimulated with BjV (B) leukocytes collected from control animals. Panels C and D represent equivalent results obtained in cells collected from L-NAME treated rats (20 mg/Kg/14 days; n = 4). R1 = mature PMN; R2 = early PMN; R3 = lymphocyte and blastocytes.

it has been shown that increment on blood pressure by L-NAME treatment is achieved by higher doses and during a longer time of treatment than here employed [41–44].

Mediators secreted by mast cells, mainly histamine, are clearly associated to induction of rolling behaviour [45, 46]. It has been suggested that NO plays a role in the in vivo modulation of mast cell degranulation and histamine release [17, 18, 20, 47]. The possibility that our treatment could inhibit mast cell activity and consequently impair the leukocyte-endothelial interactions was excluded by the observations that the number of mast cells on adjacent tissue to postcapillary venule and their ability of degranulating in response to 48/80 compound was not modified.

The impaired leukocyte-endothelium interaction in L-NAME-treated rats may be responsible, at least in part, by the reduction of leukocyte influx to the air pouch in response to BjV. We had already demonstrated that rabbits submitted to the same pharmacological treatment had reduction in cellular recruitment and vascular permeability on arthritis induced even by BjV or by serum albumin bovine [9, 30–32]. Additionally, the reduced inflammatory response observed in L-NAME treated rats is in agreement with other experimental studies which employed different schedules of treatments and stimulus [48–51]. Although we demonstrated that L-NAME treatment promotes leukocyte-endothelial impairment, a direct effect on leukocyte locomotion should not be excluded. Previous studies demonstrated that in vitro incubation with NOS inhibitors attenuates subsequent chemotaxis of polymorphonuclear cells [52–55]. It is important to note that this protocol of treatment also promoted reduction on IL1 and eicosanoids levels on inflammatory exudates, which may contribute to the impairment of the inflammatory reaction [9, 30, 32].

During the course of an inflammatory reaction, neutrophils first bind loosely to endothelial cells and this is due to the interaction between selectin molecules expressed, such as on endothelium and leukocytes, and their ligands. Neutrophils then roll on the vessel wall in the direction of the blood stream. Subsequent adherence is dependent on leukocyte integrin expression and the intervention of immunoglobulin adhesion molecules on the endothelial cell membrane. Downregulation of endothelial molecules has been observed in cultures of normal cells submitted to the action of exogenous NO donors [23, 24, 26, 56, 57]. The participation of NO derived from constitutive NOS and the exclusion of NO derived from iNOS on basal up expression of P-selectin was ascertained with the use of knockout mice [58]. Additionally, In vitro incubation of human neutrophils with L-NAME promoted increment on function of β_1 integrin, but did not modify their expression [59]. The role of NO on in vivo adhesion molecule expression is now suggested, by the expression of L-selectin on peripheral leukocytes. Nonspecific chronic blockade of NO synthesis promoted a reduction in the expression of this component of the selectin family in circulating PMN, which are the predominant cells in the air pouch infiltrate. Interestingly, the impairment on molecule expression was detected even in basal conditions. On the other hand, the treatment did not affect the expression of β_2 integrin. However, modification on activity of this molecule cannot be excluded, and it will be further investigated.

Different growth factors, cytokines, hormonal action and plasmatic and cellular mediators of the inflammatory response, including NO, influence haematopoiesis [27, 60–62]. NO is a potent inhibitor of haematopoiesis, but the levels required for the inhibition in vitro may not be physiologically important. Additionally, erythroid and myeloid progenitor cells appear to have differential sensitivity to NO [63, 64]. Determination of myeloid and erythroid cell numbers at different stages of maturation in the bone marrow demonstrated that haematopoiesis was not altered by L-NAME treatment, however the number of circulating neutrophils is markedly increased. The analysis of blood and medullary erythropoiesis in L-NAME treated animals excluded haemoconcentration or haemolytic nature in the effect, which suggesting the standstill of PMN in the peripheral compartment.

It has been fully demonstrated that each stage of cell maturation is associated with the expression of a particular combination of adhesion molecules, which may therefore play general roles in compartmentalisation and development of leukocytes [65–67]. Here we showed that basal expression of L-selectin on early or mature PMN, lymphocyte and blastocytes of bone marrow was not modified by L-NAME treatment. However, under stimulatory condition, all lines of cells obtained from treated rats were completely incapable of increasing L-selectin expression.

Together, these data suggest that treatment with L-NAME here employed promotes impairment of L-selectin expression, even in the medullary compartment. The continued effect on the leukocyte circulating pool may be responsible for the standstill of neutrophils in the peripheral compartment and for the deficient leukocyte/endothelium interaction, which may underlie the reduced recruitment to the inflamed area.

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On-Fiber Derivatization of SPME extracts of Phenol, Hydroquinone and Catechol with GC-MS Detection

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Abstract

A gas chromatography-mass spectrometry (GC-MS) method for the simultaneous determination of phenol (PHE), hydroquinone (HQ) and catechol (CAT) in urine was developed and validated. The method was based on the acidic hydrolysis of conjugated phenolic compounds and further extraction of analytes using solid-phase microextraction (SPME). Analytes were extracted by submersing the polar polyacrylate coated fiber (85 μm) into urine (adjusted to pH 3.0 with glacial acetic acid) for 20 min with magnetic stirring. The extracted compounds on the fiber were exposed to hexamethyldisilazane reagent in the vapor phase for 20 min to yield the corresponding trimethylsilylated derivatives. This on-fiber derivatization procedure allowed the formation of more amenable compounds for GC analysis, without adversely affecting the lifetime of the fiber. The MS was operated in the selected ion monitoring mode (SIM). The limits of detection were 0.3 $\mu\text{g mL}^{-1}$ for PHE, 0.15 $\mu\text{g mL}^{-1}$ for HQ and 0.02 $\mu\text{g mL}^{-1}$ for CAT. Inter and intra-assay precisions were also verified (coefficient of variation < 8%) with the use of deuterated internal standards. This method of GC-MS analysis can be readily utilized to monitor PHE and its metabolites (HQ and CAT) in urine samples.

Keywords

Gas chromatography-mass spectrometry
Solid-phase microextraction
On-fibre derivatisation
Phenol, hydroquinone and catechol

Introduction

Phenols are compounds frequently monitored in urine and other biological samples to obtain an indication of occupational exposure or exposure to environmental contamination [1, 2]. Phenol (PHE) itself is a widely employed chemical and is associated with headache, fainting, vertigo, and mental disturbances [3]. As a raw industrial material, it is

widely used as a disinfectant, as an anti-septic, and in medicinal preparations such as mouthwash and sore throat lozenges. It is also a constituent of phenolic resins and employed in the manufacture of nylon and other synthetic fibers [4, 5].

PHE is absorbed into the human body following dermal, oral or inhalatory exposure and is extensively metabolized in the liver. Excretion is primarily through the urinary route, and therefore it can be found

in urine either in its unaltered form or as biotransformed substances. Conjugation with glucuronic acid or sulphation is the major detoxification pathways for phenolic compounds, and consequently glucuronide and sulphate conjugates can also be found in urine. Additionally, PHE and more polar phenolic compounds such as hydroquinone (HQ) and catechol (CAT) are benzene metabolites. These substances are substrates for peroxidases present in the bone marrow and are responsible for the immunological and hematological toxic effects of benzene [3, 6, 7, 8].

Quantitative methods for the determination of PHE and its derivatives in urine have been described in the scientific literature using high-performance liquid chromatography (HPLC) [9, 10, 11], capillary gas chromatography (GC) [12] and GC-MS [4, 13, 14]. For these applications, an extraction step is a necessary pre-requisite to isolate and pre-concentrate the analytes prior to chromatographic analysis.

Conventional liquid-liquid extraction (LLE) is the most frequently employed sample preparation technique used in the determination of phenolic compounds. However, it produces emulsions and sometimes requires an additional cleanup step which gives rise to further losses, as well as requiring large amounts of solvents and being a time-consuming and laborious technique as well [1]. Solid-phase extraction (SPE) has been extensively used for the analyses of organic compounds present in biological samples, which can be retained into the cartridges and eluted with an

Fig. 1. Full scan mode mass spectra of the trimethylsilylated derivatives of phenol (PHE-TMS), hydroquinone (HQ-2TMS) and catechol (CAT-2TMS)

adequate solvent. However, SPE has some important limitations, since it is a multi-step process and relatively high solvent volumes are necessary to clean up and elute the analytes [1, 4, 15].

Recently, a simple, rapid and solvent-free extraction technique has been developed. First introduced in 1990 by Arthur and Pawliszyn [16], solid-phase microextraction (SPME) does not require the use of an organic solvent for extraction. Nowadays, SPME has been widely used in a broad field of analyses, including the environmental, food and drug abuse areas [17]. The extraction efficiency is determined by the partitioning of analytes between the sample matrix and the solid-phase fiber coating. After a pre-determined extraction time, the fiber is placed into the injector port of gas chromatograph in order to strip the adsorbed substances [15, 18].

To our knowledge, SPME had never been employed for the determination of PHE and more polar metabolites in biological specimens. In the present work, an on-fiber derivatization method for the simultaneous analysis of PHE, HQ and CAT in urine was developed using SPME and GC-MS. The feasibility of applying this method was evaluated by investigating the limits of detection and quantification (LOD and LOQ), recovery values, linear dynamic ranges and reproducibility for the analytes.

Experimental

Reagents and Reference Standards

PHE (99.5% purity), HQ (99%) and catechol (99%) were purchased from

Sigma (St. Louis, USA). Their respective deuterated-labeled analogues, PHE-d5 (98%), CAT-d6 (98%) and HQ-d6 (98%) were purchased from Cambridge Isotope Laboratories (Andover, USA). The silylating reagent hexamethyldisilazane was obtained from Merck (Darmstadt, Germany). Other reagents of common use in the laboratory were of analytical grade purchased from Merck (Darmstadt, Germany).

Preparation of Standard Solutions

Working solutions of PHE, PHE-d5 (PHE-d5), HQ, HQ-d6 (HQ-d6), CAT and catechol-d6 (CAT-d6) at a concentration of 1000 µg mL⁻¹, and 100 µg mL⁻¹ were prepared with deionized water in volumetric glassware.

Instrumentation

Solid-phase microextraction (SPME) instrumentation were obtained from Supelco (Bellefonte, PA, USA) equipped with 85 µm polyacrylate coated fiber. GC-MS analyses for PHE, HQ and CAT were performed using a gas chromatograph model 6890 coupled with a mass selective detector (MSD) model 5972 (Agilent Technologies Inc., Palo Alto, CA, USA). Chromatographic separation was achieved on a HP5-MS fused-silica capillary column (30 m × 0.25 mm × 0.10 µm film thickness) using helium carrier gas at 0.6 mL min⁻¹ in a constant flow rate mode. The MSD was operated using electronic ionization (70 eV) in selected ion monitoring (SIM) mode. The injector port and interface temperature was 250°C. The injector was operated in splitless mode. The oven temperature was maintained at 50 °C for 2 min, programmed at 13 °C min⁻¹ to 250 °C with a hold at 250 °C for 5 min. The following ions were chosen for SIM analyses (quantification ions underlined): PHE: 151, 152, 166; PHE-d5: 156, 171; HQ: 239, 240, 254; HQ-d6: 243, 258; CAT: 239, 240, 254; CAT-d6: 243, 258.

Sample Preparation

Urinary conjugated phenolic derivatives were hydrolyzed by concentrated hydrochloric acid by adding 0.1 mL of

Fig. 2 A Chromatographic profiles obtained with the SPME and GC-MS analysis of a spiked urine sample containing $10 \mu\text{g mL}^{-1}$ of phenol (PHE), hydroquinone (HQ) and catechol (CAT) and B Blank sample. Chromatographic separation was achieved on a HP5-MS fused-silica capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.10 \mu\text{m}$ film thickness) using helium as carrier gas at 0.6 mL min^{-1} in a constant flow rate mode. The oven temperature was maintained at 50°C for 2 min, programmed at $13^\circ\text{C min}^{-1}$ to 250°C with a hold at 250°C for 5 min

concentrated HCl to 1.0 mL of urine sample. The solution was heated for 90 min at 95°C . After cooling, deuterated internal standards of PHE-d5 ($5 \mu\text{g}$), HQ-d6 ($10 \mu\text{g}$) and CAT-d6 ($10 \mu\text{g}$) were added. Afterwards, the solution was neutralized with approximately 0.27 mL of KOH 5M and the volume was completed to 2 mL with deionized water. The solution was acidified with 0.1 mL of glacial acetic acid to adjust pH to approximately 3.0. Analytes were extracted using SPME, dipping an 85 μm polyacrylate fiber into solution for 20 min with magnetic stirring to obtain an optimal exchange surface. Extraction was performed at room temperature ($20 \pm 3^\circ\text{C}$). After the extraction time, the fiber was immediately exposed to the headspace (vapor phase) over 100 μL of hexamethyldisilazane reagent (heated to 80°C) for 20 min and the SPME device was then directly inserted into the GC injector port at 250°C for a 20 min desorption. The derivatization step with hexamethyldisilazane converts PHE, HQ, CAT and their respective deuterated internal standards to corresponding trimethylsilyl-ethers prior to GC-MS analysis. Mass spectra in full scan mode of trimethylsilylated derivatives are shown in Fig. 1.

Validation of the Method

Validation of the method was carried out to establish recovery values, linearity, intra- and inter-assay precision, limits of detection (LOD) and quantification (LOQ). Negative urine samples, used in these studies, were prepared as follows: urine samples were added with 10% of concentrated hydrochloric acid and boiled until complete evaporation of water. Afterwards, the residue was resuspended with the same volume of deionized water. This procedure was performed to eliminate any PHE contents present in urine.

Recovery

The recovery studies for PHE, HQ and CAT were performed taking into consideration the possible loss of the analytes by thermal decomposition after the hydrolysis procedure. For this study, two sets of samples of different concentrations were analyzed. Set A consisted of three concentrations for all substances (5.0 ; 20 and $40 \mu\text{g mL}^{-1}$) and they were analyzed, according to the method described above, in six replicates for each concentration (processed). Set B also consisted of six replicates for each concentration (5.0 ; 20

and $40 \mu\text{g mL}^{-1}$) and the standard solutions (PHE, HQ and CAT) were spiked to the sample after the hydrolysis procedure (unprocessed). The absolute recovery was evaluated by comparison of the mean response obtained for set A (processed) and the response of set B (unprocessed). The unprocessed response represented 100 % recovery.

Linearity

The study of linearity was carried out by the analysis of urine samples in triplicate submitted to the method with the following concentrations for PHE, HQ and CAT: (LOQ; 8.0 ; 15 ; 30 ; 60 ; $100 \mu\text{g mL}^{-1}$).

Intra-Day and Inter-Day Precision

Precision, defined as the relative standard deviation or coefficient of variation (CV), was determined by intra- and inter-day repetitions. This was performed by analyzing urine samples (quality controls) on three different days at the following concentrations for PHE, HQ and CAT: 5.0 ; 20 and $40 \mu\text{g mL}^{-1}$. Six

Table 1. Confidence parameters of the validated method for the determination of phenol, hydroquinone and catechol in urine samples

	Phenol	Hydroquinone	Catechol
Recovery (%)			
QC1	85.10	92.50	89.70
QC2	76.40	77.20	81.30
QC3	86.70	85.60	80.30
LOD ($\mu\text{g mL}^{-1}$)	0.30	0.15	0.02
LOQ ($\mu\text{g mL}^{-1}$)	0.50	0.40	0.10
Intra-assay precision (CV %)			
QC1	3.30	7.20	6.60
QC2	3.90	4.20	5.70
QC3	3.90	7.20	7.80
Inter-assay precision (CV %)			
QC1	3.50	4.90	2.60
QC2	1.00	2.30	1.00
QC3	3.70	1.40	2.40

LOD = limit of detection; LOQ = limit of quantification; QC1 = $5.0 \mu\text{g mL}^{-1}$; QC2 = $20 \mu\text{g mL}^{-1}$; QC3 = $40 \mu\text{g mL}^{-1}$

replicates analyses were performed for each concentration.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD was considered to be the concentration that produced a signal-to-noise ratio of about 3:1 and the LOQ was considered to be the concentration that produced a signal-to-noise ratio of about 10:1. The LOD and LOQ should still satisfy the predetermined acceptance criteria of qualification (retention time within 1% of standards and ion ratios within 20%).

Results and Discussion

Several methods have previously been proposed to quantify phenols in urine, however, none of them employed solid phase microextraction (SPME) for the simultaneous determination of PHE and more polar phenolic metabolites, such as HQ and CAT. Data herein describes an analytical method using SPME and GC-MS for quantification of three phenolic compounds in urine.

LLE or SPE have been used for determination of PHE, HQ and CAT in urine [4, 12, 13]. Recently, solid-phase microextraction (SPME), a relatively new technique, has gained considerable interest in a broad field of analysis [15, 18]. All steps of the conventional liquid-liquid extraction (LLE) such as extraction, concentration, derivatization and transfer to the chromatograph are integrated into one device, considerably simplifying the

sample preparation technique. In the present method, over 20 urine samples could be extracted, injected and analyzed by one person in approximately 8 h. The polar polyacrylate fiber was shown to be suitable to extract phenolic compounds in urine. Here, more than a hundred injections could be performed with the same fiber.

Before the extraction procedure of the analytes, a hydrolysis step was necessary because PHE and its derivatives are excreted as glucuronide or sulphate conjugates in urine. In these cases, acidic and enzymatic hydrolyses are the chosen techniques. We favored acidic hydrolysis with the use of concentrated hydrochloric acid and incubation of samples at 95°C for 90 min because enzymatic hydrolysis, largely employed [9, 20], is hampered by the long time required for complete hydrolysis of phenolic compounds [21].

The acidic protons present on PHE, HQ and CAT require a chemical derivatization step before chromatographic analyses. Most derivatization procedures use acetylation, alkylation and silylation to block the active protons in phenolic hydroxyl group [13, 14, 22]. The formation and extraction of derivatives in the aqueous solution by a single-step procedure would be highly desirable. Nevertheless, the aqueous acetylation with acetic anhydride according to the method described by Llompert et al. [23] is not suitable for HQ and CAT since they are prone to oxidative degradation at a pH above eight [22]. Hence, a SPME step followed by an on-fiber derivatization procedure was necessary. Acetic anhydride was also used as a reagent to produce acetylated derivatives of PHE,

HQ and CAT in our initial experiments with on-fiber derivatization. The reaction was demonstrated to have a high success rate, however after only 4–6 derivatizations, the coating of the fiber was stripped off. Probably, the derivatizing reagent chemically attacked the epoxy that holds the fiber. This problem was solved with the use of hexamethyldisilazane, which promoted a rapid conversion of the phenolic compounds to trimethylsilyl derivatives, without damaging the fiber (Fig. 1).

Chromatograms obtained with the practical use of SPME and GC-MS analyses of urine samples are shown in Fig. 2. For this purpose, a blank sample was employed (PHE was excluded by adding 10% of concentrated hydrochloric acid and further boiled until complete evaporation of water) along with a sample spiked with PHE, HQ and CAT at the concentration of $10 \mu\text{g mL}^{-1}$ each.

The calibration curves were linear over the specified range for all analytes (PHE from 0.5 to $100 \mu\text{g mL}^{-1}$; HQ from 0.4 to $100 \mu\text{g mL}^{-1}$; CAT from 0.1 to $100 \mu\text{g mL}^{-1}$). The linear regression equations and coefficients of correlation were: $Y = 0.184X - 0.183$; $r^2 = 0.998$ (PHE); $Y = 0.123X + 0.099$; $r^2 = 0.999$ (HQ), and $Y = 0.129 - 0.377$; $r^2 = 0.987$ (CAT), where Y and X represents the relationship between the peak area ratio (compound/internal standard) and the corresponding calibration concentration, respectively.

The confidence parameters of the validated method (LOD, LOQ, intra- and inter-assay precision and recoveries) are shown in Table 1. The internal standards (IS's) employed were with deuterium-labeling in the aromatic ring. For this reason, the ions chosen were 5, 4 and 4 a.m.u. higher than the non-labeled PHE, HQ and CAT, respectively. The analytical technique developed for the determination of PHE, HQ and CAT in urine with the use of respective deuterated IS's provided better precision (CV < 8%) than previous published methods [11, 13] (CV < 17%). The sensitivity was equivalent for all analytes if compared with the method proposed by Marrubini et al. [11].

Conclusion

As far as we know, this is the first report dealing with an on-fiber derivatization

method for the determination of PHE, HQ and CAT in urine samples. It is demonstrated that these compounds could be isolated from urine samples by SPME and sensitively quantified using the SIM mode with GC-MS. Hence, this methodology could be an efficient tool to follow phenol kinetics in several toxicological studies.

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Toxicology xxx (2006) xxx–xxx

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Effect of *in vivo* phenol or hydroquinone exposure on events related to neutrophil delivery during an inflammatory response

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Abstract

Phenol (PHE) and hydroquinone (HQ) are metabolites of benzene that affect leukocytes after solvent intoxication. Hence, we investigated the effects of PHE or HQ exposure on neutrophil mobilization during an inflammatory response. Male Wistar rats received intraperitoneal injections of PHE, HQ or vehicle only and assays were performed 24 h after the last dose. Quantifications of bone marrow or circulating leukocytes showed that only HQ exposure induced neutrophilia, probably due to the accelerated mobilization from the bone marrow compartment, since reduced numbers of segmented cells in the last phase of maturation were detected there. Intravital microscopy showed that circulating leukocytes of HQ-exposed rats increased their rolling behavior and adherence to the mesenteric postcapillary venule wall *in vivo*. The enhanced leukocyte–endothelium interaction was not dependent on microvascular reactivity or perivascular mast cell degranulation. Instead, it was the result of neutrophil activation, demonstrated by a decrease in L-selectin and an increase in β_2 integrin expression on neutrophil membranes. This pattern of neutrophil activation may have contributed to the higher number of neutrophils in the subcutaneous inflammatory response of HQ-exposed rats after oyster glycogen injection. Taken together, our results indicate that HQ exposure alters neutrophil mobilization, which results in an exacerbated response after an injury. Although PHE is endogenously metabolized to HQ, PHE exposure only induced an increment in rolling behavior, which was not sufficient to alter the inflammatory response.

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Keywords: Phenol; Hydroquinone; Inflammation; Leukocyte–endothelial interaction; Adhesion molecules

1. Introduction

Human exposure to phenolic compounds can occur in the environment, occupationally, through diet, cosmetics, medicines and cigarette-smoke (Ong et al., 1994; Duarte-Davidson et al., 2001; Cheynier, 2005). These highly reactive compounds are substrates to various enzymes, including polyphenoloxidases, peroxidases,

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S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx–xxx*

glycosidases and esterases (Cheynier, 2005). For this reason, their pharmacological and toxic effects are varied and complex.

Two such compounds, phenol (PHE) and hydroquinone (HQ), are reactive metabolites from benzene biotransformation and are responsible for serious effects of solvent intoxication (Schrenk et al., 1996; Snyder, 2000, 2002, 2004). After pulmonary or dermal absorption, benzene is oxidized in the liver by cytochrome P450 2E1 to form benzene epoxide, which can be either conjugated to ring-opened metabolites or transformed into PHE and catechol. Moreover, CYP450 enzymes convert PHE and catechol to HQ and 1,2,4-benzenetriol, respectively (Goldstein et al., 1982; Snyder, 2002, 2004). The ability of these circulating phenolic compounds to easily penetrate into the bone marrow compartment and be locally metabolized and accumulated contributes in an important way to the myelotoxicity in benzene intoxication (Henderson, 1996; Snyder, 2002, 2004). In the bone marrow, these compounds are auto-oxidized or further oxidized via reactions mediated by peroxidases, such as myeloperoxidase, to their highly active quinone and semiquinone derivatives (Schlosser and Kalf, 1989; Schlosser et al., 1990).

The effects of PHE or HQ administration on mononuclear leukocytes are detected even in stem cells in the bone marrow microenvironment and in distinct phases of maturation and proliferation (Li et al., 1996; Pyatt et al., 1998, 2000; Stillman et al., 2000; Smith et al., 2000; Doecker et al., 2000; Kalf, 2000; Poirier et al., 2002; McCue et al., 2003). The consequent reduced number and activity of B and T lymphocytes contribute to leukemia and immunosuppressive effects (Snyder, 2002, 2004). Conversely, PHE and HQ may stimulate granulocyte production. Previous studies demonstrated an increase in the number of granulocyte-macrophage progenitor cells in the bone marrow of mice pre-treated with HQ and an increased proliferation of murine multipotent hematopoietic progenitor cells in vitro. Addition of PHE to HQ generated even larger effects (Hazel et al., 1996a; Henschler et al., 1996). Furthermore, it has been shown in vitro that low concentrations of HQ induce terminal granulocytic differentiation in murine myeloblasts and inhibit apoptosis at the myeloblasts/myelocyte stage of differentiation (Hazel and Kalf, 1996; Hazel et al., 1996b). Although the mechanisms involved are not totally understood, influence on growth-stimulating factors, such as granulocyte-macrophage colony-stimulating factor and IL-3, and leukotriene D4 receptor has been suggested (Hazel et al., 1996a, 1996b; Hazel and Kalf, 1996; Wiemels and Smith, 1999).

Effective mobilization of leukocytes to the site of injury is crucial for the host defense against infections. At the early stages of an acute inflammatory response, neutrophils are mobilized from the bone marrow or peripheral compartments to the blood and to the site of lesion, respectively. Circulating cells initially roll and adhere to the endothelium from postcapillary venules of the microcirculatory network and subsequently transigrate into the extravascular space and move directly to the site of inflammation. The rolling and adherence of leukocytes on endothelium of postcapillary venules is influenced by events that alter hemodynamic parameters, such as vascular reactivity, which can modify the blood flow at the site of inflammation, and by endogenous mediators, which trigger cellular interactions. In this context, constitutive mast cells neighboring postcapillary venules release starter chemicals that induce the rolling behavior (Salazar-Mather and Hokeness, 2003; Sallusto and Mackay, 2004). A coordinated interplay involving a spectrum of adhesion molecules successively expressed on cellular membranes mediates the interactions in the microvasculature and the extravascular tissue (Granger and Kubes, 1994; Steeber and Tedder, 2000; Bochner, 2004; Steeber et al., 2005).

It has been extensively demonstrated that the adhesion molecule L-selectin, constitutively expressed in leukocytes, mediates the rolling behavior through the interaction with sialylated carbohydrate determinants on the endothelial membrane (Cambi and Figdor, 2003; Rosen, 2004). After activation, L-selectin undergoes ectodomain cleavage, resulting in its intermittent expression. The significance of L-selectin endoproteolytic shedding was experimentally verified by an increased recruitment of neutrophils to the inflamed area (Venturi et al., 2003). The stronger cellular interaction during subsequent adhesion is mediated by β_2 integrin molecules on activated leukocytes, which bind to molecules of the immunoglobulin superfamily on the endothelium (Hubbard and Rothlein, 2000; Hogg et al., 2002). The sequential expression of adhesion molecules on leukocyte trafficking is linked to the ability of rolling leukocytes and endothelial cells to sense signals, which stimulate cellular events responsible for firm adherence to the vessel wall surface. Such signals can be given by endogenous mediators, which are secreted by immune competent cells on the vessel wall or on the adjacent tissue of the microcirculatory network of an inflamed area (Ebnet and Vestweber, 1999; Kim et al., 2004; Kuldo et al., 2005). Recent studies have suggested that adhesion molecule interactions induce a bidirectional signaling between leukocytes and the endothelium, which mediate events that facilitate the onward migration of

FOX 49319 1–10

+ Model

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx–xxx*

3

leukocytes through venule walls, as well as their responsiveness, behavior, differentiation state and survival at inflammation sites (Hubbard and Rothlein, 2000; Wang and Doerschuk, 2002; Kluger, 2004; Nourshargh and Marelli-Berg, 2005).

Despite abundant descriptions of the effect of PHE and/or HQ on neutrophil production or maturation *in vivo*, the effect of these substances on neutrophil recruitment to different compartments has not yet been reported. Therefore, in this study we evaluated the consequence of a 17-day exposure to PHE or HQ *in vivo* on cellular events involved in the phases of neutrophil mobilization. In addition, the outcome of the altered mechanisms detected in neutrophils was investigated after an acute inflammatory response. Our data also indicate that although PHE is endogenously transformed into HQ, PHE exposure did not alter the mechanisms involved in neutrophil distribution and the inflammatory process.

2. Materials and methods

2.1. Chemicals

PHE (Merck, Darmstadt, Germany), pentobarbital sodium (Cristália, São Paulo, Brazil), cellulose ester filter 0.25 μ m (Millipore, São Paulo, Brazil), FITC-labeled monoclonal anti-rat CD62L (L-selectin, LECAM-1) and FITC-labeled monoclonal anti-rat CD18 (β_2 integrin; BD Pharmingen, San Diego, CA, USA), heparin (Liquemine®, Roche), oyster glyco-gen (Type II), EDTA, HQ, noradrenaline (NA), acetylcholine (Ach), compound 48/80 and McCoy's 5A medium (Sigma, St. Louis, MO, USA).

2.2. Animals

Adult male Wistar rats weighing 180–220 g were used. Animals were kept under a light/dark cycle (12 h on/12 h off), allowed a standard pellet diet and water *ad libitum*. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. At the time of the experimental assays, animals were anaesthetized with pentobarbital sodium (65 mg/kg; intraperitoneally).

2.3. Protocols of exposure

PHE or HQ (50 mg/kg in a final volume of 1 ml) was intraperitoneally injected into the rats once a day. The animals received 13 injections, daily on Days 1–5, 8–12 and 15–17. Assays were carried out on Day 18, 24 h after the last injection with each phenolic agent. Control rats received the same amount of vehicle through the same route. PHE or HQ was dissolved in absolute ethanol (5% of the final solution) and the volume was completed with sterile saline solution. Body weight was determined before and after the exposure to assess tolerance. The gain of corporal mass was equivalent

in the three groups of animals studied (PHE = 107.8 ± 4.89 , HQ = 104.5 ± 10.14 and Vehicle = 105.6 ± 8.13 , $n = 36$ animals for each group).

2.4. Quantification of bone marrow and circulating leukocytes

Numbers of bone marrow and circulating leukocytes after chemical exposure were determined 24 h after the last injection. For circulating leukocytes, we obtained blood samples from the abdominal aorta using EDTA (100 mg/mL) as anti-coagulant. Bone marrow leukocytes were obtained by flushing the femoral cavity with McCoy's 5A medium and with EDTA (100 mg/mL). The total number of leukocytes was assessed using a Neubauer chamber. Differential counts were performed on the basis of 500 cells per slide in cytocentrifuge smears stained with May–Grünwald and Giemsa dyes.

2.5. Intravital microscopy assay

Rats were anesthetized intraperitoneally with pentobarbital sodium (65 mg/kg) and the mesentery was exteriorized. After surgery, the animals were kept on a special board thermostatically controlled at 37 °C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with a warmed Ringer-Locke solution (pH 7.2–7.4; NaCl 154 mM; KCl 5.6 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2 mM; NaHCO_3 6 mM and glucose 5 mM) containing 1% gelatine. The rate of out-flow of the solution onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of the liquid. Transilluminated images were obtained by optical microscopy (Axioplan II, Carl–Zeiss equipped with $\times 5.0/0.30$ plan-neofluar or $\times 10.0/0.25$ Achromplan longitudinal distance objectives/numeric aperture and $\times 1.0$, 1.25 or 1.60 optovar). The images were captured by a video camera (ZVS, 3C75DE, Carl–Zeiss) and were transmitted simultaneously to a TV monitor and a computer. Images obtained on the TV monitor were recorded on video-tape. Digitized images on the computer monitor were subsequently analyzed by an image-analyzing software (KS 300, Kontron).

2.5.1. Leukocyte–endothelial interaction

The interaction between leukocytes and vessel walls was evaluated by determining the number of rolling and adhered leukocytes on the postcapillary venule wall (20–30 μ m diameter, 200 μ m length) of the mesentery at 10-min intervals. Three fields from each animal were evaluated. Leukocytes moving in the peripheral of the axial stream, in contact with the endothelium, were considered to be rollers. These leukocytes moved sufficiently slowly to be individually visible and were counted as they rolled past a selected point on one side of the vessel. The number of leukocytes adhering to the endothelium (stopped at the vessel wall) was determined at the same vascular segment (Dahlen et al., 1981; Fortes et al., 1991; Farsky et al., 1995, 2004).

TOX 49319 1–10

+ Model

4

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx–xxx*

2.5.2. Microvascular reactivity

The constrictor response to NA (2.5 or 5 µg/mL) and the vasodilatation to Ach (300 µg/mL) were tested. Drugs dissolved in Hanks' balanced solution (HBSS) were topically applied to vessels (20–30 µm) of the mesentery in a standard volume of 10 µL and removed by washing with Ringer-Locke's solution. Rats from each group were tested for different stimuli. The latency time required for arteriole stasis in response to each dose of NA and the subsequent reflow in response to Ach were recorded. Results were obtained after evaluating five fields from each animal.

2.5.3. Mast cells: number and activity

Another set of experiments was designed to assess the influence of phenolic agents on the number and behavior of mast cells. The degranulation of mast cells under topical application of 10 µg of compound 48/80 was determined in the tissue adjacent to postcapillary venules of the mesentery. The number of constitutive degranulated mast cells in an area of 7200 µm² and the time of latency required to begin the process were recorded in 10 vessels from each animal. The number of constitutive mast cells was evaluated after 10 min of topical application of blue toluidin dye and 10 areas were evaluated.

2.6. Blood flow cytometry

In order to quantify L-selectin or β₂ integrin expression, we isolated leukocytes from blood collected from the abdominal aorta (EDTA, 100 mg/mL). Briefly, erythrocyte lysis was performed using an ammonium chloride solution (0.13 M) and leukocytes were recovered after washing with HBSS. Cells (1 × 10⁶) were incubated with or without *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 10^{−8} M for 10 or 30 min). After washing, leukocytes were further incubated for 30 min at 4 °C in the dark with 10 µL of monoclonal antibody against L-selectin or β₂ integrin. Immediately after incubation, cells were analyzed on a FACScalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data from 10,000 cells were obtained and only morphologically viable leukocytes were considered for analysis. The cytometer separated leukocytes by size and granularity. As circulating leukocytes in rats are represented by lymphocytes (about 80–85%), neutrophils (about 10–15%), monocytes (about 4–5%), eosinophils (about 1%), gates were selected as mononuclear (lymphocytes and monocytes) and polymorphonuclear (neutrophils and eosinophils) (Farsky et al., 2004). Fluorescence intensity was obtained by median of gated cells and results express the mean of four assays, performed in duplicate.

2.7. *In vivo* neutrophil migration: Air Pouch model

Animals were anaesthetized with pentobarbital sodium (65 mg/kg; intraperitoneally) and 20 mL of sterile air were subcutaneously injected on the 8th day of chemical treatment. After 7 days, the pouch was upheld with approximately 5 mL of air. This procedure was described by Edwards et al., 1981.

On the 10th day following the first injection of air, oyster glycogen (5% in saline solution, 1 mL) was injected into the pouch. Six hours later, the animals were again anesthetized and later sacrificed. The pouches were washed with 4 mL of PBS/heparin (5 UI/mL) and the total numbers of leukocytes in the collected exudate were determined in Neubauer chambers. Differential cell counts were performed using smears stained with Giemsa dye.

2.8. Statistical analysis

Means and standard errors of means (S.E.M.) of all data are presented and were compared using Student's *t*-test or ANOVA with a significance of less than 0.05. Individual comparisons were subsequently carried out with Bonferroni's *t*-test for unpaired values and with Tukey test.

3. Results

3.1. Numbers of circulating leukocytes

To investigate if chemical exposure resulted in altered leucogram, leukocyte numbers were quantified in blood collected from control, PHE- or HQ-exposed rats. As seen in Fig. 1, the total number of leukocytes in PHE- or HQ-exposed rats was equivalent to that from control animals. In addition, differential analysis demonstrated that HQ exposure did not modify the number of lymphocytes or monocytes, but resulted in an increase in the number of circulating neutrophils compared to control rats. PHE exposure did not modify the number of any type of peripheral white cells (Fig. 1).

3.2. Production and maturation of neutrophils in the bone marrow

The effects of chemical exposure on neutrophil production or maturation were evaluated by bone marrow cellularity 24 h after the last injection of HQ or PHE. Our data demonstrate that neither exposure altered leukocyte production, since the number of lineage precursor cells was equivalent in the three groups of animals studied (Fig. 2). HQ exposure did not modify the number of polymorphonuclear leukocytes (PMN) in the early phase of maturation, but induced a significant decrease in the number of segmented cells at the last stage of maturation (Fig. 2). PHE exposure did not alter any phase of neutrophil maturation (Fig. 2).

3.3. Leukocyte–endothelial interaction

The behavior of leukocytes in the peripheral blood was evaluated by direct observation of the microcir-

+ Model

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx–xxx*

5

Fig. 1. Bar graphs demonstrate the number of circulating leukocytes in rats exposed to vehicle, PHE, or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Blood samples were obtained from the abdominal aorta 24h after the last dose of exposures and quantifications were carried out in Neubauer chambers and cytocentrifuge smears stained with May–Grünwald and Giemsa dye. Results express mean $\pm$ S.E.M. of six animals in each group. * $P < 0.05$ in comparison with the respective value in vehicle-exposed animals.

culatory network. As shown in Fig. 3A, HQ or PHE exposure increased initial contact between white cells and endothelial membranes, as shown by the increased number of rolling leukocytes in postcapillary venules of the mesentery. In HQ-exposed rats, this early interaction progressed to firm leukocyte attachment to the vessel wall, given that the number of cells adhered to the endothelium was also higher in this group of animals than in the PHE- or vehicle-exposed groups (Fig. 3B).

3.4. Microvascular response to noradrenaline and acetylcholine

At resting conditions, the diameters of arterioles and venules used for the assays were equivalent and varied between 20 and 30 μ m. To investigate the vascular reactivity after chemical exposure, NA and Ach were topically applied to the microvascular network of the mesentery. Results presented in Fig. 4 show that chem-

Fig. 2. Number of bone marrow leukocytes in rats exposed to vehicle, PHE or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Cells were obtained 24h after the last injection by flushing the femoral cavity with McCoy's 5A medium with EDTA. The total number of cells was determined in Neubauer chambers and the differential number in cytocentrifuge smears stained with May–Grünwald and Giemsa dye. Results express mean $\pm$ S.E.M. of six animals in each group. * $P < 0.001$ in comparison with the respective value in vehicle-exposed animals.

TOX 49319 1–10

+ Model

6

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx-xxx*

Fig. 3. Number of rolling (Panel A) and adhered leukocytes (Panel B) in postcapillary venules (20–30 μ m diameter) of the mesentery of rats exposed to vehicle, PHE or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Results were obtained by intravital microscopy 24 h after the last dose of exposures. Data express mean $\pm$ S.E.M. of six animals in each group. * P < 0.01 or ** P < 0.001 in comparison with the respective values in vehicle-exposed animals.

ical exposure did not modify the arteriolar reactivity in response to NA or Ach. Constriction to NA and subsequent dilation to Ach were similar in the three groups of animals studied.

3.5. Mast cells: number and activity

In vivo images of the mesenteric network demonstrated that HQ or PHE exposure did not influence mast cell numbers or ability to degranulate. Topical application of compound 48/80 caused equivalent mast cell degranulation in the three groups of animals, as visualized by the number of cells adjacent to postcapillary venules (Fig. 5) and by the time required for degranulation (10–15 s for each animal).

3.6. Effects of PHE and HQ exposure on leukocyte expression of adhesion molecules

The expression of adhesion molecules on the membranes of circulating leukocytes was characterized by

Fig. 4. Microvascular reactivity after topical application of norepinephrine (NA) and acetylcholine (Ach) in rats exposed to vehicle, PHE or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Results were obtained by intravital microscopy of the mesentery microcirculatory network, 24 h after the last dose of exposures. Data represent the time required for the cessation of blood flow in response to NA and its recovery after Ach. Data are expressed as mean $\pm$ S.E.M. of six animals in each group.

blood flow cytometry. Data presented in Fig. 6A show that the expression of L-selectin on PMN cells collected from PHE- or HQ-exposed rats was lower than that found in cells from control rats. After fMLP *in vitro* stimulation, cells obtained from HQ-exposed rats lost their ability to increase L-selectin expression on the membrane (Fig. 6A). On the other hand, the increment on L-selectin expression on PMNs collected from PHE-exposed rats after fMLP stimulation was equivalent to that found in cells from control rats (Fig. 6A). Expressions of L-selectin on mononuclear cells (MN) were not altered by chemical exposures (data not shown).

Analysis of β_2 integrin expression on PMN cells showed that cells collected from PHE- or vehicle-exposed rats presented a similar pattern of molecule expression even before or after fMLP stimulation (Fig. 6B). In contrast, PMN cells obtained from HQ-exposed animals presented elevated levels of β_2 integrin in both before or after *in vitro* stimulated conditions (Fig. 6B). As detected to L-selectin expression, the chem-

Fig. 5. Degranulated mast cell number in the mesentery of rats exposed to vehicle PHE or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Results were obtained by intravital microscopy, 24 h after the last injection and 10 min after topical application of 48/80 compound. Data are expressed as mean $\pm$ S.E.M. of six animals in each group.

TOX 49319 1–10

+ Model

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx-xxx*

7

Fig. 6. Leukocyte adhesion molecule expression on peripheral PMN cells obtained from vehicle-, PHE- or HQ-exposed rats (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Leukocytes were obtained from blood samples collected from the abdominal aorta. 1-Selectin (Panel A) or β_2 integrin (Panel B) expression was quantified in quiescent (basal condition) neutrophils or after fMLP stimulation (10^{-8} M) by blood flow cytometry. Results are expressed as mean $\pm$ S.E.M. of samples collected from four animals in each group. Assays were performed in duplicate. * $P < 0.01$ vs. basal values from vehicle-exposed rats, ** $P < 0.05$ vs. respective basal values and *** $P < 0.05$ vs. values on fMLP stimulated cell from vehicle- or PHE-exposed rats.

ical exposures did not modify the β_2 integrin expression on MN cells (data not shown).

3.7. Local leukocyte recruitment during an inflammatory response

The leukocyte influx into inflamed subcutaneous tissue was quantified 6 h after the injection of oyster glyco-gen into the air pouch. As shown in Fig. 7A, leukocyte migration into the site of lesion in PHE-exposed rats was equivalent to the migration to the inflamed cavity in control animals. However, the total number of leukocytes present in the inflamed pouch of HQ-exposed rats was higher than that found in PHE- or vehicle-exposed rats (Fig. 7A). Differential analysis demonstrated that the higher numbers of leukocytes in inflammatory exudates of HQ-exposed rats represented a significant increase in the number of neutrophils accumulated in the pouch (Fig. 7B).

4. Discussion

Neutrophils are among the first leukocytes recruited from reservoir compartments into an injured area, and their interaction with different cells during the migration process triggers intracellular events whose steps advance the inflammatory response. Alterations in this complex process of delivery may reduce or increase the accumulation of white cells at the site of lesion; in both cases, the host defense is impaired. In the current study we proposed an experimental protocol involving intoxication with PHE or HQ to investigate their role on mechanisms responsible for neutrophil mobilization, as well as their effect during an acute inflammatory response.

Our data showed that HQ exposure induced neutrophilia, probably because of a more intense mobilization of segmented cells from the bone marrow compart-

Fig. 7. Number of total (Panel A) or differential (B) leukocytes in inflammatory exudates of rats exposed to vehicle, PHE, or HQ (50 mg/kg; i.p. route; 13 doses, daily on Days 1–5, 8–12 and 15–17). Inflammation was induced in the subcutaneous cavity 24 h after the last dose of exposures (oyster glycogen; 1 mL 5% solution). Exudates were collected 6 h later. Total leukocytes were quantified in a Neubauer chamber and differential cells in cytocentrifuge smears stained with May-Grünwald and Giemsa dye. Results are expressed as mean $\pm$ S.E.M. of six animals in each group. MN, mononuclear cells; PMN, polymorphonuclear cells. * $P < 0.001$ in comparison with the respective values in vehicle-exposed animals.

TOX 49319 1–10

+ Model

8

S.M.D. Macedo et al. / *Toxicology xxx (2006) xxx–xxx*

ment. While it has been previously suggested that HQ impairs production and/or maturation of granulocytes (Snyder, 2002, 2004), our data show that HQ does not affect precursor cell numbers, but induces earlier maturation of neutrophils. In HQ-exposed rats we detected a significant reduction in the number of PMN cells at the last phase of maturation in the bone marrow compartment and increased neutrophil numbers at the peripheral compartment. In contrast, PHE exposure did not alter any phase of cell maturation in the bone marrow compartment and did not modify the circulating number of leukocytes. These effects might be expected since PHE seems to act synergistically with HQ or other phenolic compounds on granulocytic lineage alterations (Kolachana et al., 1993; Moran et al., 1996; Henschler et al., 1996).

In the circulation, leukocytes move in the mainstream of blood. However, during activation states, white cells interact with the microcirculatory vessel wall for subsequent migration into the site of injury. Modifications in microvascular wall reactivity and in the activation of leukocytes and the endothelium – both effects mediated by endogenous substances secreted at the site of injury – promote leukocyte–endothelial interactions. Data herein suggest that HQ and PHE exposure induced rolling of leukocytes to postcapillary venules of the mesentery. However, only leukocytes from HQ-exposed rats were able to advance and adhere to the vessel wall.

It has been reported that HQ is a potent constrictor of small arterioles, mainly by blocking microvessel dilation dependent on nitric oxide (NO) (Lubbe et al., 1994; Zhao and Joshua, 1998). It is well-known that NO production is increased in response to Ach, and that NO diffuses from endothelial cells into the cytosol of adjacent vascular smooth muscle cells to activate the enzyme guanylyl cyclase, increasing GMPc levels, which result in vascular smooth cell relaxation (Russo et al., 2002). Thus, the possibility that the enhancement of leukocyte–endothelial interactions detected in rats exposed to phenolic compounds could result from modifications in the microvascular reactivity was investigated by measurements of arteriolar constriction and its subsequent dilation after topical application of NA and Ach on the mesenteric tissue, respectively. Our data showed that the times required for pharmacological responses were similar in all groups of animals studied, suggesting that the leukocyte–endothelial interactions are not dependent on changes in microvascular reactivity. Additionally, the mesenteric tissue has postcapillary venules surrounding mast cells, which secrete mediators that clearly induce *in vivo* rolling behavior, such as histamine (Kubes and Granger, 1996; Yamaki et al., 1998). We have also shown that local injections of phenolic compounds for a long

period of time did not alter the numbers of mesentery mast cells or their ability to degranulate in response to compound 48/80, suggesting that the elevated number of rolling leukocytes in the postcapillary venules of animals exposed to phenolic compounds does not involve substances secreted from an elevated mast cell activity.

The expression of adhesion molecules on both leukocytes and the endothelium mediates leukocyte–endothelial interactions and also indicates the stage of cellular activation (Ley, 2002; Ehrhardt et al., 2004). Neutrophils rapidly express L-selectin and subsequently upregulate β_2 integrin under stimulation with chemotactic factors. This pattern of adhesion molecule expression during leukocyte priming in several inflammatory diseases has been fully demonstrated (Fernandez-Segura et al., 1996; Walcheck et al., 1996; Williams and Solonkin, 1999; Kaba and Knauf, 2001; Noguera et al., 2004; Caimi et al., 2005). Our data suggest that HQ exposure induces neutrophil activation, as indicated by a downregulation of L-selectin and an upregulation of β_2 integrin expression, which may lead to cellular activities during an inflammatory process. Then, the importance of adhesion molecule expression in neutrophils from HQ-exposed rats was demonstrated by the higher influx of neutrophils into subcutaneous inflamed areas compared to PHE- or vehicle-exposed rats.

Taken together, our data support the notion that the benzene metabolite quinone plays a role in the toxicity of granulocytes and influences the host defense against non-specific agents. This is the first report of neutrophil activation by HQ *in vivo*, resulting in an excessive inflammatory response. Additionally, our data demonstrate the differential role of phenolic compounds in cellular reactivity, since unlike exposure to HQ, PHE administration did not promote alterations on neutrophil maturation and activation. Although PHE is endogenously transformed into HQ, endogenous transformation of PHE into HQ may not be sufficient to generate the same level of neutrophil priming as direct HQ exposure. It is important to emphasize that rats are able to metabolize benzene, and that they have been used as a model for human exposure because of close similarities in the biotransformation of this solvent (Sammett et al., 1979; Orzechowski et al., 1995; Seaton et al., 1995; Henderson, 1996).

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TOX 49319 1-10

+ Model

S.M.D. Macedo et al. / Toxicology xxx (2006) xxx–xxx

9

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TOX 49319 1–10

Model

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S.M.D. Macedo et al. / Toxicology xxx (2006) xxx–xxx

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TOX 49319 1–10

IN VIVO HYDROQUINONE EXPOSURE INHIBITS LEUKOCYTE MIGRATION DURING AN ALLERGIC LUNG INFLAMMATION IN RATS

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Abstract

We investigated the effects of hydroquinone (HQ) exposure on the host defense during a lung allergic inflammatory response. Male Wistar rats were intraperitoneally exposed to vehicle or HQ once a day, for 22 days with a two-day interval every five days. On day 10, the animals were intraperitoneally sensitized with ovalbumin (OVA). Thirteen days after sensitization and 24 hours after the last injection of HQ or vehicle, rats were challenged with OVA by inhalation. The numbers of leukocytes in the bronchoalveolar fluid were reduced in HQ-exposed rats. However, numbers of polymorphonuclear cells in the pulmonary tissue were equivalent in HQ or vehicle-exposed rats, as measured by myeloperoxidase activity. The inability of leukocyte migration into inflamed tissue was not influenced by changes in the number of circulating leukocytes or the expression of adhesion molecules on circulating leukocytes or pulmonary endothelium. Nevertheless, the effect is dependent on impairment on IgE-mediated mast cell degranulation, since tracheal contraction *in vitro* in response to OVA was reduced in tissues obtained from HQ-exposed rats, and the capacity to mount passive cutaneous anaphylaxis was impaired in normal rats previously sensitized with serum from HQ-exposed rats. Equivalent proliferation of spleen lymphocytes in response to OVA or Concanavalin A in cells collected from vehicle- or HQ- exposed rats excluded possible alterations on antigen recognize and on intracellular signaling of proliferation. Together, our data show that HQ exposure diminishes leukocyte recruitment during a host allergic response, primary depending on inhibition of IgE-mediated mast cell degranulation.

Keywords: hydroquinone, leukocyte migration, pulmonary allergic inflammation, ovalbumin, mast cell degranulation, IgE.

1. Introduction

Asthma is an inflammatory disease of the lungs characterized by local mast cell activation, influx of leukocytes and reversible episodes of bronchospasm (Lazaar and Panettieri, 2005). Briefly, the early phase of asthma is characterized by airway smooth muscle contraction, mucus secretion and increase of microvascular permeability caused by mast cell-derived mediators (Pascual and Peters, 2005; Lazaar and Panettieri, 2005), which in turn leads to cellular influx and airway epithelial injury and bronchial hyper responsiveness (Pascual and Peters, 2005). All of these hallmarks of asthma are dependent on the synthesis of immunoglobulin E (IgE) antibodies and their fixation on high-affinity surface receptors (Fc ϵ RI) of mast cells in sensitized subjects (Miyasaka et al., 1999; Bochner et al., 2004). The first step in this process is the antigen presentation by professional antigen-presenting cells (APC) to B lymphocytes, and the subsequent enhancement of immunoglobulin E (IgE) antibody production. During a challenge, Th2 helper T cells are activated and proliferate by action of Interleukins-4 (IL-4), 5 (IL-5) and 13 (IL13). Subsequently, these activated T cells secrete cytokines, such as IL-4 and IL-13, which drive B-lymphocytes to produce specific antibodies (Murphy and Reiner, 2002; Careau et al., 2002; Ansel et al., 2003).

In terms of cellular traffic, the mechanisms underlying involve tethering, rolling and adherence of leukocytes to the endothelium of the lung microvasculature under influence of chemical mediators secreted by different resident cells, including mast cells, fibroblasts and macrophages (Kubes and Kerfoot, 2001; Bochner, 2004). The initial contact of rolling leukocytes with the endothelium is mediated by expressions of L-selectin and integrins (very late antigen-4 (VLA-4) and α d β 2) on leukocytes, which bind to specific carbohydrate ligands and to vascular cell-adhesion molecule 1 (VCAM-1) on the endothelium, respectively (Grayson et al., 1999; Kikuchi et al., 2003). As the response progresses, this initial binding is followed by the induction of firm adhesion, mediated primarily by the expression of β 1 (CD19) and β 2 (CD18) integrins on

respectively (Grayson et al., 1999; Kikuchi et al., 2003). As the response progresses, this initial binding is followed by the induction of firm adhesion, mediated primarily by the expression of $\beta 1$ (CD19) and $\beta 2$ (CD18) integrins on leukocyte membranes and their interaction with intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 (von Andrian and Mackay, 2000; Tachimoto et al., 2002). Subsequently, leukocytes undergo diapedesis into the tissue by expression of ICAM-1, platelet-endothelial cell-adhesion molecule 1 (PECAM-1) and migrate at the extravascular tissue by expression of $\beta 1$ and $\beta 7$ integrins (Vaporciyan et al., 1993; Miller et al., 2001).

Various experimental models of asthma have been developed for studying the inflammatory mechanisms of the airways (Bice et al, 2000). Challenged rats previously sensitized to an antigen associated to the adequate adjuvant, induces the secretion of the complex network of chemical mediators, migration and activation of leukocytes and resident cells, which are responsible for the beginning and improvement of the process (Tavares de Lima and Silva, 1998; Kim et al., 2004; Leung et al., 2004). In a IgE-mediated response, mast cells degranulation trigger the process (Tavares de Lima and Silva, 1998; Guo et al., 2001).

Benzene, an organic compound with well-known immunotoxic properties, is widely used as an industrial solvent. It is a component of cigarette smoke and a common environmental pollutant generated by the processing, combustion and evaporation of gasoline. The immunosuppressive effects of benzene have been associated to its phenolic bio-products (Hoffmann et al., 1983; Li et al., 1996; 1997; McCue et al., 2000; Snyder, 2002,2004).

Hydroquinone (HQ) is a reactive metabolite from benzene biotransformation that can cause serious solvent intoxication (Schrenk et al., 1996; Snyder, 2000; 2002; 2004). It is produced via oxidation reactions catalized by liver cytochrome P450 enzymes (Goldstein et al., 1982; Snyder 2002; 2004). The ability of circulating HQ to easily penetrate into the bone marrow compartment, to be locally metabolized and to accumulate there contributes in an important way to the myelotoxicity in benzene intoxication

(Schlosser and Kalf, 1989; Schlosser et al., 1990). The complex immunosuppressive effects of HQ and its quinone derivatives have been demonstrated and their underlying mechanisms are likely associated with higher concentrations of HQ in different body compartments, which affect cell maturation and function.

In vitro assays have demonstrated that HQ has potential immunosuppressive effects on lymphocytes, mainly blocking their production and their ability of proliferation (Li et al., 1996; 1997; Pyatt et al., 1998; 2000; McCue et al., 2003). Regarding to granulocyte lineage, *in vivo* and *in vitro* exposures have shown that HQ promotes alterations on phases of neutrophil maturation at the bone marrow cavity, with consequent neutrophilia (Kolachana et al., 1993; Henschler et al., 1996; De Caprio, 1999; Macedo et al., 2006).

Despite the broad actions of HQ on white cells, the role of this phenolic compound on leukocyte recruitment during an allergic response *in vivo* has not been shown. Using ovalbumin (OVA)-sensitized rats as an experimental model, we herein demonstrated for the first time that HQ exposure for an extended period of time inhibits leukocyte infiltration into the lung during an allergic inflammatory response. The mechanisms involved are not related to leukocyte production, expression of adhesion molecules involved in leukocyte recruitment neither or ability of lymphocyte proliferation. However, we verified that inhibition on immunoglobulin synthesis is an important mechanism responsible for the subsequent impaired mast cell degranulation and development of the response.

2. Materials and methods

2.1. Chemicals

Pentobarbital sodium (Cristália, Brazil); Chloral hydrate (Rhodia-Farma, Brazil); Ketamine (Vetaset®, Lab. Font Dodte, USA); Xylazine (Vensol, Lab. Konie); FITC-labeled anti-rat CD62L (L-selectin, LECAM-1), FITC-labeled anti-rat CD18 ($\beta 2$ integrin), R-PE-labeled anti-rat CD31 (PECAM-1), R-PE-labeled anti-rat CD106 (VCAM-1) were purchased from BD Pharmingen; FITC-labeled anti-rat CD54 (ICAM-1), (Southern Biotechnology Associates); Heparin (Liquemine®, Roche); Ovalbumin (chicken egg albumin crude, grade II), Concanavalin A from *Canavalia ensiformis* (type IV), RPMI 1640 medium, θ -dianisidine, Hexadecyltrimethyl ammonium bromide, EDTA and Hydroquinone compound were purchased from Sigma (St. Louis, MO, USA); O.C.T. Tissue Tek™ mounting medium (Raymond A Lamb, London, UK); Block solution (Pierce CO., Rockford, Illinois); H₂O₂ and Ethanol (Merck, USA); [³H] thymidine (Amershan Pharmacia Biotech UK Limited, UK); Fetal calf serum (Gibco-BRL, MD, USA).

2.2. Rats

Adult male Wistar rats weighing 180-220 g were employed. Animals were kept under light/dark cycle (12 h on, 12 h off), allowed a standard pellet diet and water *ad libitum*. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

2.3. Protocol of exposure

HQ (50mg/Kg in a final volume of 1mL) was intraperitoneally injected, once a day. Animals used as control received the same volume of vehicle by

the same route. HQ was dissolved in absolute ethanol (5% of the final solution) and the volume was completed with sterile saline solution. The animals received 16 daily doses of treatments, with 2-day intervals every 5 doses. Assays were carried out 24 hours after the last dose of exposure.

2.4. Induction of hypersensitivity reaction

Rats were sensitized by a single intraperitoneal injection of ovalbumin (OVA, 10 µg) mixed with aluminum hydroxide (10 mg) as adjuvant on the 10th day of HQ or vehicle exposure. Thirteen days later, rats were challenged with OVA administered by inhalation (1% in PBS; 15 min application using an ultrasonic inhaler). The time frame was chosen based on Coleman et al. (1983), who demonstrated that the levels of circulating immunoglobulin E (IgE) antibodies in rats increases rapidly 7-14 days after intraperitoneal injection with OVA.

2.5 Bronchoalveolar lavage (BAL) and cell counting

Animals were anesthetized with ketamine and xilazine and exsanguinated. A cannula was then inserted into the trachea and 20 mL (2 × 10 mL) phosphate-buffered saline (PBS) was injected. Immediately following PBS injection, BAL was collected and the fluid was centrifuged at 500 × g for 10 min at 4°C. The cell pellet was re-suspended in 1 mL of PBS. The number of cells was determined using a Neubauer chamber and stained cell suspensions (0,5% crystal violet dissolved in 30% acetic acid). Differential cell counts were performed from cytopspin preparations and then stained with May-Grünwald and Giemsa dyes.

2.6 Myeloperoxidase activity

In order to quantify neutrophil infiltration in the lung tissue, we assayed myeloperoxidase activity in lung homogenates. The thorax was cut open and the lungs perfused with saline containing heparine (5 IU/mL) with PBS (pH 7.0) via the pulmonary artery. The whole lung was then homogenized using a

Brinkmann Tissue Homogenizer (Polytron®) (2 × 30s) in 3 mL/g of 0.1M phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethyl ammonium bromide and 5 mM EDTA. The samples were then sonicated (Vibra Cell-Sonics Materials®) (3 × 2 min, 40 Hz) and centrifuged (37,000 g, 15 min) at 4°C. Myeloperoxidase activity was assayed in 40 µL aliquots of the supernatants by measuring changes in absorbance at 460 nm resulting from decomposition of H₂O₂ in the presence of θ -dianisidine in a final volume of 1.5 mL as previously described (Henson et al., 1978).

2.7 Circulating leukocyte number: total and differential

The number of circulating leukocytes was determined on day 10 of chemical exposure immediately before sensitization, on day13 after sensitization and before challenge, and 24 hours after challenge. The leukocytes were obtained from blood samples from the orbital plexus using heparin (5UI/mL) as anticoagulant. The total number of leukocytes was assessed using a Neubauer chamber. Differential counts were performed on the basis of 100 cells per blood extension stained with May-Grünwald and Giemsa dyes.

2.8 Endothelial immunohistochemistry

The lungs were rapidly removed and insufflated with O.C.T. Tissue TekTM mounting medium diluted 2:1 with PBS. Regions of the left and right lung lobes were frozen in nitrogen-hexan solution, cryosectioned (8 µm thickness) and fixed with cold acetone for 10 min. For direct immunohistochemistry, sections were incubated with block solution to block unspecific sites for 24 h in a humidified atmosphere at 4°C, followed by an overnight incubation (humidified box, 4°C) with FITC-labeled rat anti-mouse ICAM-1 (1:50) monoclonal antibody, R-PE-labeled mouse anti-rat PECAM-1 (1:500) monoclonal antibody, and R-PE labeled mouse anti-rat VCAM-1 (1:100) monoclonal antibody. The preparations were analysed using an image analysing software (KS 300, Kontron) and

fluorescence microscopy. To evaluate background reactions, procedures were also performed in sections incubated without antibodies.

2.9 Blood flow cytometry

Leukocytes were isolated from blood collected from the orbital plexus (EDTA, 100 mg/mL) before and after challenge to quantify L-selectin or β_2 -integrin expression. Briefly, erythrocyte lysis was performed using ammonium chloride solution (0.13M) and leukocytes were recovered after washing with HBSS. After washing, leukocytes were incubated for 30 minutes at 4°C in the dark with 10 μ L of monoclonal antibody against L-selectin or β_2 integrin. Immediately after incubation, cells were analysed on a FACScalibur flow cytometer (Becton & Dickinson - San Jose, CA). Data from 10,000 cells were obtained and only the morphologically viable leukocytes were considered for analysis.

2.10 Anaphylactic reaction *in vitro*

Thirteen days after sensitization, the anaphylactic response in isolated distal tracheal segments was tested *in vitro*. Rats were killed under deep chloral hydrate anesthesia (>400mg/Kg, i.p.) and exsanguinated via the abdominal aorta. The trachea was removed and dissected free of connective tissue. Tracheal rings corresponding to the last 3-5 cartilaginous rings closest to the carine, designated the distal trachea were set up for the measurement of isometric contractions by suspending them with two steel hooks in 8 mL organ baths containing Krebs-Henseleit solution with the following composition (mM): NaCl 115.0, KCl 4.6, CaCl_2 2, H_2O 2.5, KH_2PO_4 1.2, MgSO_4 7, H_2O 2.5, NaHCO_3 25.0 and glucose 11.0. The solution was gassed with 95% O_2 and 5% CO_2 and maintained at 36°C. The tissue was allowed to equilibrate for 60 min under an initial tension of 500 mg. During this period, the tension was adjusted to 1.0 g, and the contractile activity of the tissue was then assessed by the addition of iso-osmolar Krebs-bicarbonate solution containing 60 mM KCl. The

tissue was subsequently challenged by the addition of AO at final concentration of 100 µg/mL.

2.11 *Passive cutaneous anaphylaxis (PCA) reaction*

An IgE-dependent cutaneous reaction was generated by sensitizing the skin of non-manipulated rats with an intradermal injection of diluted serum from vehicle- or HQ-exposed rats and sensitised with OVA. 24 h later animals received an intravenous injection of 1 mL of solution containing 500 µg OVA and 2,5 mg Evans blue. After 30 min, the rats were sacrificed; the dorsal skin was removed for measurement of the pigmented area. Passive cutaneous anaphylaxis was expressed by PCA title, which represents the greater dilution of sera those induced an tissue reaction with area greater than 5 mm in diameter.

2.12 *Cell proliferation assays*

Thirteen days after sensitization, the spleen was harvested and 2×10^5 cells per well were cultured in 96-well flat bottom tissue culture plates with 25 to 125 µg/mL of OVA in complete RPMI with 5% FCS or with concanavalin A (ConA; 7µg/mL). During the last 18 h of the 4-day culture period, [³H]thymidine was added (1 µCi/well). At the end of this period of incubation, cells were collected with an automated cell harvester, and incorporated radioactivity was measured by liquid scintillation spectrometry. Data are expressed as mean ± standard deviation counts per minute of [³H]thymidine incorporation.

2.13 *Statistical analysis*

Means and standard errors of means (SEM) of all data are presented and were compared by Student's *t*-test or ANOVA with a significance probability of less than 0.05. Individual comparisons were subsequently performed with Bonferroni's test for unpaired values and Tukey test.

3. Results

3.1 HQ exposure inhibits leukocyte migration to the allergic inflammatory focus

There were significantly fewer leukocytes present in the bronchoalveolar lavage (BAL) fluid 24 h after OVA challenge in HQ-exposed rats compared to vehicle-exposed rats (Figure 1A), and differential analysis showed that chemical exposure reduced the influx of both polymorphonuclear (PMN) cells and mononuclear (MN) cells into the bronchoalveolar cavity (Figure 1A). Figure 1B shows the activity of myeloperoxidase in the pulmonary tissue, as an indicative of PMN number in the tissue. A comparison of results from HQ- and vehicle-exposed rats shows that the decreased number of PMN in the BAL fluid from HQ-exposed rats is not dependent on its accumulation in the pulmonary tissue (Figure 1B).

3.2 HQ exposure does not alter the number of circulating leukocytes

The number of circulating leukocytes was not modified by HQ exposure, since the amount of total white cells and their subtypes MN and PMN cells of HQ exposure were not different from the values detected in vehicle-exposed rats 10 days after the exposures (Table 1). Similar numbers of cells were also detected after OVA sensitization and challenge in both groups of animals (Table 1).

3.3 HQ exposure does not alter the expression of adhesion molecules involved in pulmonary leukocyte recruitment

Characterization of L-selectin and $\beta 2$ integrin expression on circulating leukocytes showed that HQ exposure did not change the basal pattern of expression of both molecules on PMN and MN membranes (Figure 2). Twelve hours after OVA challenge, there was a comparable decrease in the expression of L-selectin on PMN and MN from HQ- and vehicle-exposed rats, and $\beta 2$ integrin expression was not significantly altered on the two types of leukocytes from both groups of animals (Figure 2). Also, the expression of counter-

receptors for $\beta 2$ integrin on pulmonary endothelial cells was not modified by HQ exposure even at basal conditions or 12 h after OVA challenge. The expressions of PECAM-1, ICAM-1 and VCAM-1 were comparable on cells from both groups of animals at basal or OVA-challenge conditions (Figure 3). Figures 3A and B show similar expression of PECAM-1 on the vessel walls of vehicle- or HQ-exposed rats, and also confirm the inhibitory effect of HQ on impairment of leukocyte migration to the pulmonary tissue, since the amount of PECAM-1-labeled leukocytes in this tissue is much lower in HQ- exposed rats than in vehicle-exposed animals.

3.4 HQ exposure reduces tracheal contraction *in vitro*

Tracheas obtained from HQ- or vehicle-exposed rats were assayed to investigate their ability of contraction after *in vitro* OVA challenge. As shown in Figure 4, the tension developed by tracheas from HQ-exposed rats was significantly lower than that developed by tracheas from vehicle-exposed rats.

3.5 HQ exposure impairs the production of IgE

Results depicted in Figure 5 show the ability of sera obtained from HQ- or vehicle-exposed rats to induce an increase in cutaneous permeability in non-manipulated rats. HQ-exposed rats required higher concentrations of sera than vehicle exposed rats to evoke a pattern of PCA similar to that of the latter, indicating that vehicle-exposed rats presented lower amounts of IgE in their sera.

3.6 HQ exposure does not alter the proliferation of lymphocytes

In vitro lymphocyte proliferation in response to OVA or ConA challenge was not altered by HQ exposure as demonstrated in Figure 6. Lymphocyte proliferation was equivalent in spleen cells collected from HQ- or vehicle-exposed rats. HQ exposure also did not modify the total number of spleen leukocytes in vehicle-exposed rats (HQ= $18,6 \pm 1,7 \times 10^6$ cell/mL; Vehicle $18,6 \pm 3,1 \times 10^6$ cell/mL; n= 4 for each group).

4. Discussion

We have shown that *in vivo* HQ exposure impairs leukocyte influx into the lungs during an allergic inflammatory reaction. The mechanism involved is dependent on reduced antibody production, which may decrease mast cell degranulation and the beginning of the process.

Allergic asthma is a form of chronic inflammatory response characterized by reversible obstruction of the airways, secretion of inflammatory mediators and airway hyper responsiveness (Rankin et al., 2000; Taube et al., 2003; Bochner, 2004). After exposure to allergens, the airway is infiltrated with a variety of inflammatory cells, including lymphocytes, macrophages, neutrophils and eosinophils, each with a different kinetics of migration. Neutrophils are the first inflammatory cells to accumulate in the airways (Fahy et al., 1995; Koh et al., 1993). This kinetic of leukocyte migration was detected in an experimental model of asthma similar to the one employed (Tomkinson et al. 2001). Our data showed that HQ exposure is capable of impairing the recruitment of both PMN and MN cells to the bronchoalveolar cavity in rats sensitized and challenged to OVA. In addition, the reduced PMN migration did not reflect the accumulation of neutrophils, the main PMN cell in rats, in the pulmonary tissue. It was detected comparable low myeloperoxidase activity in pulmonary tissues of both groups of animals studied, indicating that in HQ-exposed rats neutrophils do not even arrive at lung tissues.

In vivo exposure to benzene alters the production and maturation of leukocytes in the bone marrow compartment, which may impair the delivery of white cells to the circulation and/or may alter their specific functions (MacEachern et al., 1992; Farris et al., 1997; Yoon et al., 2001). The importance of HQ in these effects has been demonstrated after its *in vitro* addition to cultured precursor cells (King et al., 1987; 1989; Li et al., 1996; 1997; 1998; Pyatt et al., 1998; 2000; McCue et al., 2000; 2003) and *in vivo* by reducing the number of B cell progenitors in the spleen and bone marrow of HQ- or catechol-treated mice (Wierda and Irons, 1982). Here we showed that

HQ- or catechol-treated mice (Wierda and Irons, 1982). Here we showed that our experimental procedure of HQ exposure did not modify the number of circulating leukocytes or the number of spleen lymphocytes after sensitization, suggesting that the impaired leukocyte migration to inflamed area does not reflect effects of HQ on leukocytes production and/or their maturation phases.

The possibility that HQ alters the specific functions involved in the initial phases of leukocyte migration was excluded by the observation that the expression of both L-selectin and $\beta 2$ integrin adhesion molecules was not modified in circulating PMN and MN cells. The reduction in L-selectin expression after OVA challenge was equivalent in cells from both groups of animals studied, and may represent its shedding from cell membranes after the initial rolling to postcapillary venules during the early stages of inflammation (Fors et al., 2001; Venturi et al., 2003; Smalley and Ley, 2005). The expected upregulation of $\beta 2$ integrin expression after OVA challenge was not also detected in cells from both groups of animals. After 12 hours of inflammation other integrins, such as $\beta 1$ and $\beta 7$, may mediate leukocyte migration in pulmonary tissue (Bochner, 2004). However, the similar pattern of expression of selectin and integrin in both groups of animals indicates that HQ exposure does not induce a different state of leukocyte activation. As observed in leukocytes, HQ exposure also did not modify the expression of endothelial counter-ligand molecules ICAM-1, VCAM-1 and PECAM-1. Interestingly, our data indicate that PECAM-1 can be an important molecule expressed by leukocytes after OVA challenge, given that its expression was detected on white cells present in the pulmonary tissue. In this case, the number of PECAM-1-labeled leukocytes was higher in vehicle-exposed rats than HQ-exposed animals, confirming that HQ exposure reduces neutrophil numbers in the pulmonary tissue.

In allergic asthma, IgE-activated mast cells secrete endogenous mediators, which are capable of producing smooth muscle contraction in the airways, including arachidonic acid metabolites, histamine and 5-hydroxytryptamine (Shin et al., 2000; Bochner, 2004). Here we showed that isolated tracheal segments from HQ-exposed rats did not respond to the *in vitro*

of mast cells to degranulate in response to OVA-IgE. Data from previous study, where we employed similar experimental protocol, but in absence of immunological sensitization, may exclude these possibilities. HQ exposure did not alter the number of mast cells or their ability to degranulate in response to 48/80 compound (Macedo et al., 2006). Additional data here presented suggest that the inhibitory effect of HQ is dependent on IgE production, since serum from HQ-exposed rats produced an ineffective response to OVA in non-manipulated rats. This was demonstrated by inducing the IgE-dependent cutaneous reaction, a typical model to measure IgE levels (Shin et al., 2000; Hong et al., 2003). The reduced production of IgE seems to be independent on antigen presentation or the ability of lymphocytes to proliferate, given that our *in vitro* assays showed that the proliferation of spleen lymphocytes in response to OVA was equivalent in cells from vehicle- or HQ-exposed rats. Additionally, a similar response to Con A indicates that spleen lymphocytes maintain intact the intracellular mechanisms of proliferation. In contrast to these data, results from other groups obtained in *in vitro* assays have demonstrated that HQ impairs lymphocyte proliferation and activation by different mechanisms. For instance, it has been proposed that HQ inhibits progression of the cell cycle (Li et al, 1996, 1997; McCue et al., 2003) and the activity of ribonucleotide reductase (Li et al., 1998; McCue et al., 2000), consequently blocking lymphocyte proliferation. It has also been shown that HQ reduces IL-2 and TNF production by T and B cells, respectively, in a mechanism dependent on NF-kappa B in response to various stimuli (Pyatt et al., 1998, 2000).

HQ may differently affect the chemical mediation of B lineage production and functions, depending on its phases of maturation and cell activation state. *In vitro* assay using murine bone marrow cell suspension showed that HQ blocks the final maturation stages of B cells differentiation, generating reduced number of mature cells. The mechanism proposed is toxicity of regulatory cells involved in the process (King et al., 1987). Complementary assays demonstrated that the effect was dependent, at least in part, on inhibition of IL-4 production by fibroblast stromal cells and by IL-1 secretion by macrophages

production by fibroblast stromal cells and by IL-1 secretion by macrophages (King et al., 1989). Differently, Lee et al. (2002) showed that HQ significantly increased IL-4 production in murine keyhole limpet haemocyanin (KLH)-primed CD4⁺T cells *in vitro*. The effect was *in vivo* corroborated by elevated levels of IgE after HQ exposure in sensitized mice.

Despite the complexity of controlling IgE B-cell differentiation, down regulating IgE levels can be achieved by bypassing the cytokine regulatory circuits, such as IL-4, IL-13, and CD40 (Chen et al., 2005). Therefore, we expect that the *in vivo* HQ exposure here employed may impair the cell signalling for B-cell differentiation and IgE production, with consequent immunosuppressive activity. This possibility will be further investigated to elucidate the mechanisms involved on the inhibitory effect of HQ on IgE production here proposed.

Together, our data clearly show that *in vivo* HQ exposure, for an extended period of time, which can be addressed in an occupational or environmental exposure, may lead to an inability of allergic response, as measured here by impairment on leukocyte mobilization.

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Legends for figures

Figure 1: Number of leukocytes in the BAL fluid (A) from rats exposed to vehicle or HQ (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses). The BAL fluid was obtained 24 hours after OVA challenge and quantification of cells was carried out in a Neubauer chamber with stained cell suspensions (0.5% crystal violet dissolved in 30% acetic acid). Differential cell counts (MN= mononuclear; PMN=polymorphonuclear) were performed from cytopspin preparation and then stained with May-Grünwald. Myeloperoxidase activity (B) was assessed in lung homogenates from both groups of rats. The supernatants of homogenates were assayed with H₂O₂ in the presence of θ -dianisidine and absorbance was assessed at 460nm. Results are expressed as mean $\pm$ s.e.m. of samples collected from 4 animals in each group. Assays were performed in duplicate. *p<0.001 vs values obtained on vehicle-exposed rats.

Figure 2: Leukocyte adhesion molecule expression on peripheral polymorphonuclear (PMN) and mononuclear (MN) cells obtained from vehicle- and HQ-exposed rats (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses). Leukocytes were obtained from blood samples collected from the orbital plexus. L-selectin or β_2 integrin expression was quantified before and after 12 hours OVA challenge by blood flow cytometry. Results are expressed as mean $\pm$ s.e.m. of samples collected from 4 animals in each group. Assays were performed in duplicate. *p<0.001 vs values obtained on before challenge.

Figure 3: Endothelium adhesion molecule expression in lungs obtained from vehicle- and HQ-exposed rats (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses). PECAM-1, ICAM-1 or VCAM-1 expressions were quantified before and after 12 hours of OVA challenge by immunohistochemistry. Results are expressed as mean $\pm$ s.e.m. of tissues collected from 3 animals in each group. PECAM-1 expression on migrated leukocytes and postcapillary vessels from vehicle- (A) or HQ-exposed rats (B) are represented. Arrows show an individual leukocyte interacted to vessel wall and migrated to the tissue. Assays were performed in duplicate.

Figure 4: *In vitro* anaphylactic response in isolated distal tracheal segments from vehicle- and HQ-exposed rats (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses). The tracheal segment was set up for the measurement of isometric contractions by suspending them with two steel hooks in Krebs-Henseleit solution (gassed with 95% O₂ and 5% CO₂; maintained at 36°C). The contractile activity of the tissue was assessed by the addition of iso-osmolar Krebs-bicarbonate solution containing 60 mM KCl and subsequently challenged by addition of OVA at final concentration of 100 μ g/mL. Results are expressed as mean $\pm$ s.e.m. of tracheas collected from 4 animals in each group. Assays were performed in duplicate. * $p < 0.001$ vs values obtained on vehicle-exposed rats.

Figure 5: Passive cutaneous anaphylaxis (PCA) reaction on skin of non-manipulated animals sensitised with an intradermal injection of diluted serum obtained from vehicle or HQ-exposed rats (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses) sensitised with OVA. 24 after i.d. injections, animals were intravenous challenged with OVA solution. Evans blue intravenous injection was carried out as a dye. 30 min later, the dorsal skin was removed for measurement of the pigment area. Passive cutaneous anaphylaxis was expressed by PCA title that represents the greater dilution of sera those induced an area greater than 5 mm in diameter. Results

are expressed as mean $\pm$ s.e.m. of samples collected from 4 animals in each group. Assays were performed in duplicate. * $p < 0.001$ vs values obtained on vehicle-exposed rats.

Figure 6: *In vitro* proliferation of spleen lymphocytes from vehicle- and HQ-exposed rats (50mg/Kg; i.p. route; once a day; 16 doses with a two-day interval after every five doses). 2×10^5 cells were cultured per well with 25 to 125 $\mu\text{g/mL}$ of OVA or with Concanavalin A (ConA; 7 $\mu\text{g/mL}$). During the last 18 h of the 4-day culture period, [^3H]thymidine was added (1 $\mu\text{Ci/well}$). At the end of this period of incubation, cells were collected with an automated cell harvester, and incorporated radioactivity was measured by liquid scintillation spectrometry. Data are expressed as mean $\pm$ s.e.m. counts per minute of [^3H]thymidine incorporation in cells obtained from 3 animals in each group. Assays were performed in triplicate. * $p < 0.001$ vs basal values.

Figure 1

L-selectin

β_2 Integrin

Figure 2

Figure 4

Figure 5

Figure 6

Table 1. Number of circulating leukocytes in rats exposed to HQ or vehicle. Blood was collected from orbital plexus on day 10 of exposures, before and after OVA challenge.

	10 th day of vehicle or HQ exposure and before OVA sensitization		13 th day after OVA sensitization and before OVA challenge		24h after OVA challenge	
	Vehicle	HQ	Vehicle	HQ	Vehicle	HQ
Total	5686±293	5557±452	6186±331	6240±559	5610±451	5433±485
MN	4893±307	4678±429	5045±301	5063±480	4646±398	4379±400
PMN	793±112	879±87	1141±83	1177±83	964±154	1054±185

Data represent mean ± s.e.m. of 5 animals in each group.