日本臨牀　65巻 増刊号9（2007年11月28日発行）別刷

新時代の骨粗鬆症学

—骨折予防を見据えて—

XVII. 骨折予防

転倒ハイリスク者の早期発見における‘転倒スコア’の有用性

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転倒ハイリスク者の早期発見における'転倒スコア'の有用性

A simple screening test for risk of falls in the elderly

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Key words：転倒，危険因子，内的要因，環境要因，実用性

はじめに

転倒・骨折は高齢者における寝たきり要因の第3位に位置づけられ，骨粗鬆症性骨折の中で最も重い骨折である大腿骨頸部骨折は，その90％以上が転倒によって生ずるとされている[1]．転倒は骨折を合併しなくても，数度の転倒を経験すると，意欲や日常生活動作能力(ADL)を低下させる[2]．地域住民におけるADL依存の危険因子として，転倒は約2倍のリスクであり[2]，転倒予防は寝たきり予防に極めて重要である．

従来，転倒危険因子は，特定のフィールドでの横断的，あるいは縦断的解析によってなされているが，抽出された危険因子は，身体的脆弱性，歩行機能の低下など共通の危険因子がある一方，めまいや痴呆などは成績が一致していない[2]．転倒は，内的要因である身体的側面と，外的要因である環境要因による複合的症候群ととらえられるが，後者は地域や文化的，生活習慣的側面により大きく異なる可能性もある．

従来の転倒危険因子は，病歴，現症，血液検査，生活能力などの簡便な検査，専門調査員による測定検査，特殊な機器を用いた検査などが統一性なく調査され，一般健康診断に適応できるかどうかの観点に著しく欠けていた(表1)．本研究では，内外の文献的レビューを基に，転倒ハイリスク者の早期発見の評価方法作成ワーキンググループの研究班によって簡易な'転倒リスク予測表'を作成し，その評価表の妥当性，有効性を検証した．

表1　測定方法の難易度で分けた転倒の危険因子

特殊機器，医師の問診などが必要な専門検査
歩行運動系(関節症，ミオパチーなど)
歩行速度遅延
バランス低下
下肢筋力低下
心血管系障害(不整脈，起立性低血圧など)
神経系障害(痴呆，パーキンソニズムなど)
薬剤(鎮静薬，睡眠薬など)
問診票などで可能な簡易な方法
老研式活動能力指標低下
(手段的ADL，知的能動性，社会的役割の13項目で構成)
過去の転倒歴
環境要因：照明不良，障害物，段差，不適切な履物など

1. 方　法

平成14年度厚生労働省科学研究効果的医療技術の確立推進，転倒骨折班の合同討議，内外のレビュー[1,3]から，筋力低下，バランス欠如，歩行障害，視力障害，移動障害，認知機能障害，

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0047-1852/07/¥40/頁/JCLS

ADL障害，起立性低血圧，加齢，転倒の既往，慢性疾患，薬剤，段差が必須項目としてあげられた．これらの項目を具体的に質問票のみで被験者が内容を理解し，かつ因子のもつ意味が変容しないよう議論を重ね，問診票を完成した(表2)．繰り返し再現性，季節変動などの基本的検討は既になされ，良好な結果を得ている[3]

2. 調査対象

全国7地域(浦臼町，仙台市，塩尻市，多摩地区，香北町，相良村，水上町)の住民2,439人(男性932人，女性1,507人：76.3±7.4歳)．問診票の意味を説明し調査の同意を得た後，自記式にて回答，自記不可能な場合は調査員が聞き取り調査を行った．

3. 解　　析

(1) 過去の転倒歴を従属変数として，多変量解析を行った．

(2) 観察期間中の転倒歴を従属変数として，過去の転倒歴を含む，調査票の項目を独立変数とし重回帰分析を行った．年齢，性は強制注入した．

p値が0.05未満を統計学的に有意とした．なお有意な傾向として，$p<0.1$の項目も記載した．

4. 結　　果

各項目の出現頻度：過去1年の転倒歴は708人(男性229人，女性479人，平均年齢77.5±7.4歳)，転倒率は29.0%，観察期間中は25%であった．骨折は1.8%にみられた．問診票と出現頻度を**表2**に示す．

転倒スコアと転倒率を**図1**に示す．スコアが大きくなるほど転倒率が高くなることが示された．

観察期間中の転倒を評価し得た376症例で，過去の転倒歴を従属変数として，重回帰分析を行った．

独立した有意な危険因子として，つまずく($p<0.0001$)，階段昇降に手すりが必要($p<0.01$)，歩く速度が遅くなってきた($p<0.01$)，片足立ち($p=0.06$)，家の中に障害物がある($p<0.05$)，家の中の段差($p=0.09$)，階段の使用($p<0.05$)，坂道の使用($p<0.05$)が抽出された．ロジスティック回帰分析においては，つまずくが3.8倍の転倒危険率，階段昇降に手すりが必要，歩く速度が遅くなってきたの身体要因が2倍の危険率だったのに対し，環境要因は2倍以下だった．階段の使用は0.6倍で，転倒に対して転倒率を減少させる方向の因子であった．年齢，性は有意ではなかった．

同一の症例で，観察期間中の転倒を従属変数として行った多変量解析では，加齢($p<0.0001$)，過去の転倒($p<0.0001$)，背が丸くなってきた($p<0.05$)，物忘れの自覚($p<0.05$)，めまい($p<0.05$)，膝の痛み($p=0.09$)が抽出された．ロジスティック回帰分析においては，過去の転倒が5.8倍と最もオッズ比が高く，次いで加齢(10歳)は2.9倍，物忘れの自覚は2.1倍であり，背が丸くなってきた，と膝の痛みは2倍未満であった．めまいは0.56倍で転倒率を減少させる方向の因子であった．性差は有意ではなかった．

表2　質問項目と出現頻度

1) 転倒：回答数2,439人で708例(4.7±1.0回/年)	28.8%
2) つまずくことがある	56.5%
3) 手すりにつかまらず階段の昇り降りができない	50.6%
4) 歩く速度が遅くなってきた	65.2%
5) 横断歩道を青のうちに渡りきれない	17.0%
6) 1kmくらい続けて歩けない	35.8%
7) 片足で5秒くらい立てない	38.6%
8) 杖を使っている	28.3%
9) タオルを固く絞れない	16.8%
10) めまい，ふらつきがある	32.4%
11) 背中が丸くなってきた	44.9%
12) 膝が痛む	47.3%
13) 目が見えにくい	53.1%
14) 耳が聞こえにくい	42.5%
15) 物忘れが気になる	63.7%
16) 転ばないかと不安になる	45.8%
17) 毎日薬を5種類以上飲んでいる	31.2%
18) 家の中で歩くとき暗く感ずる	11.4%
19) 廊下，居間，玄関に障害物がある	20.8%
20) 家の中に段差がある	69.1%
21) 階段を使わなくてはならない	27.7%
22) 生活上，家の近くの急な坂道を歩く	33.3%

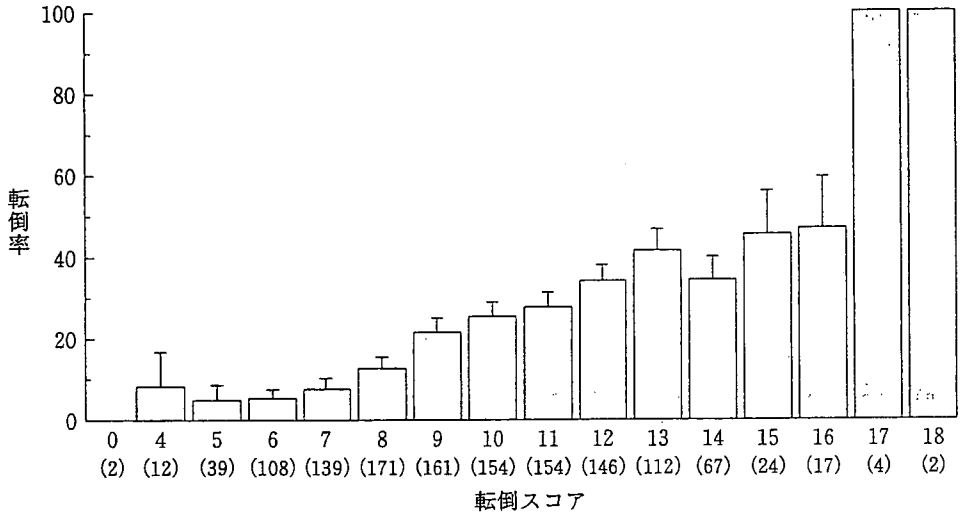

図1 転倒スコアと転倒率(過去1年)
21項目(21点満点)

5. 考　　察

転倒は多数の内的要因，外的要因による，多危険因子の重層的な症候群(multiple risk factor syndrome)の一つである[1]．

Rubensteinは，転倒に関する大規模研究のレビューを行い，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害はほとんどすべての研究で一致した危険因子であるが，視力障害，認知機能障害は半数の研究では危険因子として有意でなく，起立性低血圧は7研究中2つのみ有意であったと報告している[4]．このように，比較的人種や地域の差異が大きくないと予測される内的要因でも，危険因子としての重みは，対象によって異なる成績である．転倒の危険評価表の開発は，主として，介護施設[5]や病院[6-8]で行われ，その要因として過去の転倒，認知機能，感覚機能，運動・歩行機能，薬剤，立ちくらみ，慢性疾患があげられている．転倒の大部分は家庭内で起き，居間など室内で過半数が起きるとされているが，外的要因に関しては，危険因子を標準化する試みはほとんどない．地域における転倒危険因子の抽出は多く行われているが[9-13]，機能評価は質問紙票のみで完了せず，測定に人手を要するものがほとんどである．また，内的要因と外的要因を公平に並べて，転倒の危険因子としての意味を調査した研究はなく，外的要因を加えた地域での簡易な危険因子評価表は見当たらない．

本研究では，過去の成績[1,2,4]および，転倒評価表ワーキンググループの研究成績から，内的要因の選択を行った．外的要因に関しては，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害と関連する外的因子に焦点を絞り，バリアフリーの観点から，障害物，段差，階段，坂道など多様な因子を下位項目にあげた．視力障害と関連して，'部屋が暗く感ずるか'も加えた．

本研究では，過去の転倒と将来の転倒を従属変数として，各因子が独立した危険因子であるかを検定した．過去の転倒に対しては，独立した有意な危険因子として，内的要因では'つまずく'，'階段昇降に手すりが必要'，'歩く速度が遅くなってきた'，'片足立ち5秒ができない'が抽出され，外的要因では'家の中に障害物がある'，'家の中の段差'，'階段の使用'，'坂道の使用'が抽出された．これらは，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害[3]と関連する内的因子を具体的記述によって因子としてとらえたものと評価されよう．最近の転倒の前向きコホート研究のメタアナリシスでは，下肢筋力低下が上肢筋力低下よりオッズ比が高い

ことが示されている[14]. 今回の下位項目でも, 下肢筋力に関係がある項目のみが抽出されたことと一致した成績と考える.

外的要因では, '家の中に歩行上の障害物がある', '家の中の段差'という, 比較的改善可能な因子が関連していたことは, 転倒予防に関連しても興味深い. また, 階段の使用が転倒予防の方向に働いていたことは, 転倒予防体操, 筋力訓練の可能性を示唆するものとして興味深い.

過去の成績では, 転倒の既往は, 転倒危険因子として最も重要で, 内外研究で3.8倍平均である[1]. 今回の前向き研究でも, 過去の転倒が5.8倍と最もオッズ比が高く, 加齢(2.9倍), 骨粗鬆症と関連する'円背'(1.8倍), 痴呆と関連する'物忘れの自覚'(2.1倍), 変形性膝関節症と関連する'膝関節痛'(1.6倍)などの身体要因のみが有意であり, 下肢筋力や環境要因は有意ではなかった.

下肢筋力や環境要因関連の要素が有意でなかった原因は, これらの要素を強くもつ群は過去の転倒者に多いため, '過去の転倒'を独立変数とした場合に, 独立した危険因子としてのパワーを失ったと考えるのが妥当であろう.

他の検査方法との比較としては, 本スコアの実用性に関し, 松林らが, この問診票を用い, 北海道浦臼町で施行した調査がある. 過去の転倒を従属変数として, 転倒危険を察知するカットオフポイントは, 転倒スコア10点以上で, 感度, 特異度とも70%以上の結果であった. また, 従来のUp & Goテストや, 歩行速度, functional reachより, 転倒予測の感度, 特異度に優れている結果を得ている(松林公蔵:長寿科学転倒介入研究班(班長鳥羽研二), 2006年度報告書).

おわりに

転倒予防事業で, 今後の転倒危険者を抽出する検査を考える場合, 従来のように, 環境因子の問診票と下肢筋力検査(歩行速度, 片足立ち時間)などに時間を費やすより, 過去の転倒回数を十分聴取し, 身体的側面(骨粗鬆症, 痴呆, 膝関節症)の情報を得るため, '転倒スコア'を活用することが, 簡易で有用であると示唆された.

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本研究は, 厚生労働省科学研究, 長寿科学総合研究事業の助成によって行われた.

■文 献

1) 鈴木隆雄:転倒の疫学. 日老医誌 40: 85-94, 2002.
2) 鳥羽研二ほか:効果的医療技術の確立推進研究, 2003年度班研究報告書, 2003.
3) 鳥羽研二ほか:転倒リスク予測のための「転倒スコア」の開発と妥当性の検証. 日老医誌 42: 346-362, 2005.
4) Rubenstein LZ: Falls. In: Ambulatory Geriatric Care (ed by Yoshikawa TT), McGraw Hill, New York, 1993.
5) Tinetti ME, et al: Fall risk index for elderly patients based on number of chronic disabilities. Am J Med 80(3): 429-434, 1986.
6) Nyberg L, Gustafson Y: Using the Downton index to predict those prone to falls in stroke rehabilitation. Stroke 27(10): 1821-1824, 1996.
7) Morse JM, et al: Development of a scale to identify the fall-prone patients. Can J Aging 8: 366-377, 1989.
8) Brians LK, et al: The development of the RISK tool for fall prevention. Rehabil Nurs 16: 67-69, 1991.
9) Tinetti ME, et al: Risk factors for falls among elderly persons living in the community. N Engl J Med 319: 1701-1707, 1988.
10) O'Loughlin JL, et al: Incidence of and risk factors for falls and injurious falls among the community-dwelling elderly. Am J Epidemiol 137: 342-354, 1993.

11) Davis JW, et al: Risk factors for falls and for serious injuries on falling among older Japanese women in Hawaii. J Am Geriatr Soc 47: 792–798, 1999.
12) Campbell AJ, et al: Risk factors for falls in a community-based prospective study of people 70 years and older. J Gerontol 44: M112–M117, 1989.
13) Tromp AM, et al: Fall-risk screening test: a prospective study on predictors for falls in community-dwelling elderly. J Clin Epidemiol 54: 837–844, 2001.
14) Moreland JD, et al: Muscle weakness and falls in older adults: a systematic review and meta-analysis. J Am Geriatr Soc 52: 1121–1129, 2004.

α_2-Adrenergic receptor regulates TLR4-induced NF-αB activation through α-arrestin 2

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Short title: Cross-talk between α_2AR and TLR pathways

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Key words: Monocytes/Macrophages, Nitric Oxide , α_2-Adrenergic Receptor, Toll-like Receptor, NF-αB,

Summary

Toll-like receptors (TLRs) play an important role in innate immunity. Meanwhile, α_2-adrenergic receptors (α_2AR) provide the key linkages for the sympathetic nervous system to regulate the immune system; however, its role in macrophages remains uncertain. Here, we demonstrated the cross-talk between α_2AR and TLR signaling pathways. α_2AR expression was down-regulated by TLR4 ligand lipopolysaccharide (LPS) stimulation. To investigate the physiological consequence of the down-regulation, the macrohage cell line, RAW264 cells were transfected with α_2AR expression vector (RAWar). LPS-stimulated inducible nitric oxide synthase (NOS II) expression and NO production were markedly suppressed in RAWar cells. The activation of nuclear factor αB (NF-αB) and degradation of the inhibitor of NF-αB (IαBα) in response to LPS were markedly decreased in these cells. The level of α-arrestin 2, which regulates α_2AR signaling, was also reduced in RAW264 cells after stimulation with LPS, but not in RAWar cells. Overexpression of α-arrestin 2 (RAWarr2) also inhibited NO production and NOS II expression. Further, we demonstrated that α-arrestin 2 interacted with cytosolic IαBα and that the level of IαBα co-immunoprecipitated by anti-α-arrestin 2 antibodies was decreased in RAW264 cells but not in RAWar or RAWarr2 cells. These findings suggest that LPS-stimulated signals suppress α_2AR expression, leading to down-regulation of α-arrestin 2 expression, which stabilizes cytosolic IαBα and inhibits the NF-αB activation essential for NOS II expression, probably to ensure rapid and sufficient production of NO in response to microbial attack.

Introduction

The ability of the innate immune system to recognize and respond to microbial components has been chiefly attributed to a family of type I transmembrane receptors termed Toll-like receptors (TLRs), which are expressed abundantly on antigen-presenting cells such as macrophages and dendritic cells and can discriminate among the distinct molecular patterns associated with microbial components [1, 2]. TLR-initiated activation of NF-αB is essential for the regulation of inducible nitric oxide synthase (NOS II) and several proinflammatory cytokines in response to invading pathogens. NO produced by NOS II has a number of important biological functions, including roles in host defense against intracellular pathogens and tumor-cell killing. Although this basic definition is still accepted, over the past decade, NO has been shown to play a much more diverse role in the immune system as well as in other organ systems, including not only beneficial but also detrimental effects [3, 4]. For example, the systemic inflammatory response syndrome, which includes severe septic shock and multiple organ system failure, remains a leading cause of death in critically ill patients. Therefore, it is necessary to clarify the molecular mechanisms of TLR-initiated signaling that leads to NO production in response to microbial components.

NF-αB is found predominantly in the cytoplasm complexed with members of the inhibitor of NF-αB (IαB) family. The release of NF-αB from IαB proteins is an essential step in the generation of transcriptionally competent NF-αB. The consensus is that IαB proteins mask the nuclear localization signals of NF-αB proteins, thereby regulating NF-αB activity, primarily by limiting their nuclear translocation. Recent studies, however, have indicated that IαBα is detected in both the nucleus and cytoplasm and that although the NF-αB complexes shuttle between the nucleus and cytoplasm under all conditions, they are unable to bind DNA due to their association with proteins of the IαB family [5-7]. Nuclear IαBα is not sensitive to signal-induced degradation. Therefore, following stimulation, NF-αB activities are dependent on the level of cytoplasmic NF-αB/IαBα complexes.

Recently, we demonstrated that the level of α_2 adrenergic receptor (α_2AR) expression influences TLR4 signaling [8]. α_2AR is a member of a family of G protein-coupled receptors (GPCRs) and is the key link involved in immune system regulation via the sympathetic nervous system [9, 10]. Primary and secondary lymphoid organs, such as the thymus, spleen, and lymph nodes, receive extensive sympathetic/noradrenergic innervation, and lymphocytes, macrophages, and many other immune cells bear functional α_2AR. Therefore, α_2AR stimulation regulates proinflammatory cytokine production, lymphocyte traffic and proliferation, and antibody secretion through cAMP generation and protein kinase A (PKA) activation [10, 11]. However, the role of α_2AR in the TLR signaling pathway in macrophages remains vague. On the other hand, we others showed that arrestins are cytosolic proteins that play a critical role in the regulation of GPCR signaling [12, 13].

Recent studies have shown that they also interact with their partner molecules in a variety of signaling pathways, including NF-αB signaling [14-16]. In the present study, we investigated the physiological consequence of the down-regulation of α_2AR expression in macrophages and analyzed the cross-talk between the signaling of α_2AR and TLRs.

Materials and Methods

Cell Culture

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described in our previous study [17]. The cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) from *E. coli* 055 (Sigma-Aldrich, St. Louis, MO). Cell viability was assessed by using the trypan blue dye exclusion test. Cell size was measured by flow cytometric analysis of forward light scatter characteristics (FSC) using a FACSCalibur flow cytometer (Becton Dichinson, Mountain View, CA).

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described [18]. The NF-αB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG-3′) was purchased from Promega (Madison, WI) and labeled with biotin at its 3′ end. The nuclear protein (2 μg) and excess amount of labeled oligonucleotide probes were incubated in 20 μl EMSA buffer [20 mM HEPES, pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 1 mM EDTA, 0.2% Tween, 30 mM KCl, 1 μg poly (dI-dC), 1 μg poly L-lysine] at room temperature for 15 min, electrophoresed in 7% polyacrylamide gels, transferred onto the Biodyne Plus Membane (Pall BioSupport Division, port Washington, NY), and UV cross-linked. To detect signals, the blots were incubated with streptavidin-horseradish peroxidase conjugate in a blocking reagent for 15 min and with a chemiluminescent reagent for 5 min. The blots were then exposed to Kodak X Omat AR film (GE Healthcare Bio-Science, Piscataway, NJ).

Western Blotting Analysis

Cell membrane proteins were prepared using the Plasma Membrane Protein Extraction Kit (Bio Vision, Mountain View, CA). Cytoplasmic protein extracts were prepared as described (30). The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA), and equal amounts of membrane proteins or cytoplasmic proteins were loaded. The samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA). The membranes were blocked with 10% non-fat dried milk in Tris-buffered saline (TBS) and incubated with goat polyclonal antibodies (Abs) against α_2AR, goat polyclonal Abs against

α-arrestin 2, or rabbit polyclonal Abs against IαBα′ and NOS II (Santa Cruz Biotechnology, Santa Cruz, CA); this was followed by incubation with appropriate secondary Abs (horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit IgG; DAKO, Kyoto, Japan). To ensure equal protein loading, the membranes were incubated with rabbit anti-actin or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz) for detection of cytoplasmic or cell surface GAPDH [19] after stripping. Immunoreactivity was visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare Bio-Science).

Immunoprecipitation

The cells were lysed with lysis buffer (20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors). The samples were clarified by centrifugation at 21,000 *g* at 4°C for 30 min. The protein concentration was determined using the Bradford reagent (Bio-Rad). α-Arrestin 2 was immunoprecipitated with anti-α-arrestin 2 monoclonal Abs (Santa Cruz Biotechnology) from equal amount of samples, followed by treatment with 10 μl of protein G-Sepharose beads (GE Healthcare Bio-Science). After extensive washing, the complexes were analyzed by SDS-PAGE and Western blotting by using rabbit polyclonal Abs against IαBα′.

Determination of Nitrite Concentration

Nitrite in the cell culture supernatants was measured using the assay system of Ding *et al.* [20]. The nitrite concentration was calculated by comparing with sodium nitrite that was used as a standard. In some experiments, 200 μM pyrrolidine dithiocarbamate (PDTC, Sigma) was added to the cultures.

Determination of Intracellular cAMP Concentration

Cells were cultured with or without LPS for 6 h and were stimulated with Salbutamol (1×10^{-6} M) for final 30 min. Cell supernatants were then removed and cells were lyzed. Intracellular cAMP was determined with a commercially available enzyme immunoassay (GE Healthcare Bio-Science).

Real-time PCR

Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Qiagen), and aliquots of 2 μg were reverse-transcribed with ReverScript I (Wako Pure Chemical Industries, Osaka, Japan) and an oligo(dT)15 primer (Roche Diagnostics, Indianapolis, IN) at 42°C for 50 min. cDNAs were amplified by PCR under the following conditions by using the oligonucleotide primers and cycles listed in Table 1: 94 °C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for NOS II and 18S rRNA, and 94 °C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec for total and transfected α_2AR and α-arrestin 2. The quantity of the cDNA template included in these reactions and the number of

amplification cycles were optimized to ensure that the reactions were stopped during the linear phase of product amplification, thus permitting semiquantitative comparisons of mRNA abundance between different RNA preparations.

κ_2AR and κ-arrestin 2 Plasmid Constructs and Stable Transfection

Full-length murine α_2AR (*$\kappa_2$ar*) and α-arrestin 2 (*κarrestin2*) cDNAs were obtained by PCR using the primers 5′-GCTGAATGAAGCTTCCAGGA-3′(sense) and 5′-GCCTGTATTACAGTGGCGAG-3′ (antisense) for α_2AR and 5′-GGCGGGCGGAGGGCGGCGAG-3′(sense) and 5′-CGTCCTAGCAGAACTGGTCA-3′ (antisense) for α-arrestin 2. The amplified α_2AR and α-arrestin 2 fragments were subcloned into the pGEM-T Easy vector (Promega) and then into *Not*I-digested pcDNA4 (Invitrogen, Carlsbad, CA). The amplified PCR products were sequenced using an automatic DNA sequencer (Applied Biosystems). The plasmid DNA used for transfection was prepared using the EndoFree Plasmid Kit (Qiagen). RAW264 cells were transfected with the pcDNA4 vector, pcDNA4-$\alpha_2$ar, or pcDNA4-αarrestin2 using LipofectAMINE Reagent (Invitrogen). Selection was initiated in a medium containing 500 μg/ml Zeocine (Invitrogen).

Luciferase Assays

The full-length murine NOS II promoter fragment was cloned into the pGL3-enhancer luciferase reporter gene vector (Promega) (pGL3-NOS II) as described [21]. RAW264 cells were transfected using the LipofectAMINE Reagent with constructs containing the luciferase reporter gene, and luciferase activity was determined using the Dual Luciferase Assay System Kit (Promega) as described [21]. Activity was normalized relative to an internal co-transfected constitutive control (*Renilla* luciferase expression vector, pRL-TK; Promega). In some experiments, RAW264 cells were transiently co-transfected with NF-αB-responsive promoter reporter-luciferase construct pNF-αB-Luc (Clontech, Palo Alto, CA) or pGL3-NOS II and pcDNA4-$\alpha_2$ar or IαBα dominant-negative vector pCMV-IαBαM (Clontech).

Statistical Analysis

The Student's t test for unpaired samples was used to compare two means. For more than two groups, statistical significance of the data was assessed by ANOVA. Where significant differences were found, individual comparisons were made between groups using the t statistic and adjusting the critical value according to the Bonferroni method. Differences were considered significant at $P < 0.05$. Data in the text and figures are expressed as means ± SEM.

Results

Preventing the Down-regulation of κ_2AR Inhibits LPS-stimulated NOS II Expression

α_2AR protein and mRNA levels were markedly decreased in RAW264 cells following LPS stimulation (Fig. 1A). To investigate the role of α_2AR down-regulation in response to LPS, a stable α_2AR transfectant (RAWar) and a vector control (RAWvec) were established. Although the levels of both α_2AR protein and mRNA expression were notably decreased in RAWvec cells following LPS stimulation, the down-regulation of α_2AR expression was prevented in the RAWar cells (Fig. 1B). Because the transfected α_2AR protein did not have a tag sequence capable of modifying α_2AR function, the protein levels of only transfected α_2AR could not be analyzed. The mRNA levels of transfected α_2AR were low in unstimulated RAWar cells but markedly increased in the cells following LPS stimulation (Fig. 1C). In our previous study, we showed that the levels of both protein and mRNA of transfected cDNA cloned into the pcDNA4 vector were low in unstimulated RAW264 cells but markedly increased in the cells following LPS stimulation [17]. Therefore, it appears that the levels of total α_2AR expression in unstimulated RAWar cells were not so higher than those in RAWvec cells and that decrease in the level of intrinsic α_2AR expression in the LPS-stimulated RAWar cells was masked by the increased expression of transfected α_2AR due to the LPS stimulation. Although, the intracellular cAMP concentration in RAWar cells stimulated with salbutamol was similar to those in RAWvec cells, LPS stimulation decreased an accumulation of intracellular cAMP in RAWvec cells but increased it in RAWar cells (Fig. 1D), suggesting that the transfected α_2AR is functionally active. The similar histograms of the distribution of FSC were observed in RAWvec and RAWar cells, suggesting that the α_2AR transfection did not alter the cell size (Fig. 1E). Also, cell viabilities were more than 98 % in both cells.

The effects of forced α_2AR expression on NO production were examined. The nitrite concentration in the culture supernatants of the LPS-stimulated RAWar cells was considerably lower than in the culture supernatants of the RAWvec cells (Fig. 2A). After stimulation with LPS for 6 h, a distinct 130-Kd NOS II protein band was observed in the RAWvec cells but not in the RAWar cells (Fig. 2B). Although a protein band corresponding to NOS II was observed in the RAWar cells after stimulation with LPS for 24 h, the expression level was apparently lower than in the RAWvec cells. Similar results were obtained on RT-PCR analysis of NOS II mRNA expression (Fig. 2B).

Preventing the Down-regulation of κ_2AR Inhibits LPS-stimulated NF-κB Activation.

Next, the effects of forced α_2AR expression on NF-αB activation in response to LPS were analyzed. As illustrated in Fig. 3A, marked NF-αB activation was observed in the RAWvec cells stimulated with LPS for 3 and 6 h but not in the RAWar cells. The level of cytoplasmic IαBα was definitely

decreased in the RAWvec cells after LPS stimulation for 6 h; however, this level was not decreased in the RAWar cells (Fig. 3B). To further confirm the role of α_2AR in LPS-stimulated NF-αB activation, the effects of forced α_2AR expression on NF-αB-dependent gene transcription were analyzed. NF-αB-mediated-luciferase reporter activity (Fig. 3C) and NOS II promoter activity (Fig. 3D) after stimulation with LPS were inhibited in cells co-transfected with the pcDNA4-$\alpha_2$ar construct (AR) as well as in cells co-transfected with pCMV-IαBαM (DN-αB). These findings suggested that α_2AR functions as a negative regulator of NF-αB activation by inhibiting IαBα degradation in LPS-stimulated macrophages. Previously, it has been shown that PDTC blocks NF-αB activation by inhibiting IαBα degradation and subsequently the translocation of NF-αB subunits to the nucleus [22]. Thus, to elucidate the effects of NF-αB activation on the expression of the responsive gene, *Nos2*, PDTC was added to the RAW264 cell cultures at several time points after the addition of LPS, and NO accumulation in the supernatants was analyzed after LPS stimulation for 24 h. As illustrated in Fig. 3E, when PDTC was added to cultures at 0~9 h after the addition of LPS, the NO concentrations in these cultures were markedly lower than those in cultures stimulated with LPS for 24 h without PDTC (right column), indicating that continuous NF-αB activation is essential for adequate NOS II induction.

κ_2AR Regulates NF-κB Activation through κ-arrestins.

As α-arrestin 2 has been reported to interact with IαBα [15, 16], we examined whether α-arrestin 2 participates in the α_2AR-mediated regulation of IαBα degradation and NF-αB activation in response to LPS. α-Arrestin 2 expression was also down-regulated in the LPS-stimulated RAW264 cells (Fig. 4, left panels). Forced α_2AR expression abolished the down-regulation of α-arrestin 2 expression (middle panels), suggesting that α-arrestin 2 expression was regulated by α_2AR. Deletion of α_2AR by siRNA decreased α-arrestin 2 expression (data not shown), supporting that α-arrestin 2 expression is regulated by α_2AR. To investigate the role of α-arrestin 2 down-regulation in response to LPS, a stable α-arrestin 2 transfectant (RAWarr2) was established (right panels). Since transfection with the vector did not influence NO production (Fig. 1C), cells transfected with α-arrestin 2 were compared with RAW264 cells. As shown in the RAWar cells (Fig. 2), NO production (Fig. 5A) and NOS II protein and mRNA expressions (Fig. 5B) were definitely decreased in the RAWarr2 cells.

Anti-α-arrestin 2 Abs co-immunoprecipitated IαBα in RAW264 cells before but not after LPS stimulation for 6 h (Fig. 6). On the other hand, the amount of IαBα co-precipitated by anti-α-arrestin 2 Abs was not reduced but rather increased in the RAWar and RAWarr2 cells after LPS stimulation, indicating that the LPS-stimulated down-regulation of α_2AR and α-arrestin 2 is essential for IαBα degradation.

Discussion

In this study, we investigated the role played by α_2AR in the anti-microbial responses of macrophages was investigated. First, we demonstrated that α_2AR expression is decreased by LPS stimulation. To directly investigate the role of α_2AR down-regulation in response to LPS, we established a macrophage cell line, RAWar. Prevention of the down-regulation of α_2AR expression in RAWar cells resulted in reduced NO production, suggesting that the LPS-associated down-regulation of α_2AR expression plays an important role in NO production in macrophages.

Decreases in NOS II mRNA expression were observed in the RAWar cells, indicating that NOS II expression was transcriptionally down-regulated by forced α_2AR expression. Prevention of the down-regulation of α_2AR expression in the RAWar cells resulted in a marked decrease in NF-αB activation and inhibited cytosolic IαBα degradation, indicating that the forced α_2AR expression inhibited LPS-induced NF-αB activation by IαBα stabilization.

On the other hand, α-arrestins, which are universally expressed members of the arrestin family, are the major regulators of GPCR signaling that bind to activated GPCRs causing receptor desensitization and internalization [14]. Recently, α-arrestins have been shown to play functional roles in the regulation of a variety of signaling pathways and in the mediation of cross-talk between signaling pathways. Moreover, there is accumulating evidence that α-arrestin 2, which is expressed abundantly in the spleen, is functionally involved in some important immune responses [23-26]. We have demonstrated that α-arrestin 2 is down-regulated in LPS-stimulated RAW264 cells. α-Arrestin 2 down-regulation was abolished in RAWar cells, suggesting that α-arrestin 2 expression is regulated by α_2AR. These findings suggest that α_2AR participates in signal transduction pathways from TLR4 by regulating the level of α-arrestin 2 expression. Meanwhile, the amount of IαBα co-immunoprecipitated by anti-α-arrestin 2 Abs was decreased in the RAW 264 cells after their stimulation with LPS but not in the RAWar or RAWarr2 cells, suggesting that α_2AR inhibited LPS-induced NF-αB activation by stabilizing IαBα through α-arrestin 2. The release of NF-αB following the degradation of IαBα proteins is an essential step in the generation of transcriptionally competent NF-αB. In addition, NF-αB activity following stimulation is dependent on the level of cytoplasmic NF-αB/IαBα complexes free from stabilizing factors. Therefore, the following appear likely: (1) LPS-stimulated signals suppress α_2AR expression, (2) the reduction of α_2AR results in the down-regulation of α-arrestin 2 expression, (3) α-arrestin 2 stabilizes cytoplasmic IαBα and inhibits NF-αB activation; thus, reduction in the level of α-arrestin 2 accelerates IαBα degradation and NF-αB activation in LPS-stimulated cells, and (4) nuclear translocation of NF-αB enhances NOS II expression.

The cross-talk between α_2AR and TLR signaling pathways is schematically summarized in Fig. 7. Catecholamines increase cAMP *via* α_2AR activation, and PKA activation inhibits NF-αB-induced

transcription by phosphorylating cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (CBP) (Fig. 7A(a)) [27]. However, α_2AR agonists did not suppress NO production (unpublished observation). In the present study, we demonstrated that LPS stimulation suppressed the cAMP accumulation in RAWvec cells stimulated with α_2AR agonist. In addition, we showed that prevention of the down-regulation of α_2AR inhibits the degradation of IαBα through α-arrestin 2, which stabilizes IαBα in the steady state (Fig. 7A (b)). Therefore, the down-regulation of α_2AR and α-arrestin 2 expressions by the TLR4-dependent pathway might provide a mechanism for "escaping" anti-proinflammatory signals, such as the α_2AR-cAMP-PKA pathway [27] or the α_2AR-α-arrestin 2-IαBα pathway. As the levels of α_2AR ligands vary under different conditions, understanding the cross-talk between TLRs and α_2AR pathways may have both physiological and pathophysiological importance. Taken together, the observations of the present study regarding the regulation of TLR4 signaling through α_2AR appear to provide another therapeutic target for the regulation of inflammatory disease conditions.

Acknowledgments

We thank Dr. T. Seya (Hokkaido University, Sapporo. Japan) for providing helpful comments.

Grants: This study was supported in part by Grants-in-Aid for Scientific Research (including the Academic Frontier Project) from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by a Grant-in-Aid for Promotion and Mutual Aid Corporation for Private Schools of Japan (to T.K. and H.O.).

References

1. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-76.

2. Janeway CA,Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002; 20:197-216.

3. Hierholzer C, Harbrecht B, Menezes JM, et al. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J Exp Med 1998; 187:917-28.

4. Bogdan C. Nitric oxide and the immune response. Nat Immunol 2001; 2:907-16.

5. Lain de Lera T, Folgueira L, Martin AG, et al. Expression of IkappaBalpha in the nucleus of human peripheral blood T lymphocytes. Oncogene 1999; 18:1581-8.

6. Tergaonkar V, Correa RG, Ikawa M, Verma IM. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. Nat Cell Biol 2005; 7:921-3.

7. Rodriguez MS, Thompson J, Hay RT, Dargemont C. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem 1999; 274:9108-15.

8. Itoh CE, Kizaki T, Hitomi Y, et al. Down-regulation of beta2-adrenergic receptor expression by exercise training increases IL-12 production by macrophages following LPS stimulation. Biochem Biophys Res Commun 2004; 322:979-84.

9. Downing JE, Miyan JA. Neural immunoregulation: Emerging roles for nerves in immune homeostasis and disease. Immunol Today 2000; 21:281-9.

10. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: The brain and the immune system. Pharmacol Rev 2000; 52:595-638.

11. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. Pharmacol Rev 2001; 53:487-525.

12. Ferguson SS, Downey WE,3rd, Colapietro AM, Barak LS, Menard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 1996; 271:363-6.

13. Ogasawara J, Sanpei M, Rahman N, Sakurai T, Kizaki T, Hitomi Y, Ohno H, Izawa T.

Beta-adrenergic receptor trafficking by exercise in rat adipocytes: Roles of G-protein-coupled receptor kinase-2, α-arrestin-2, and the ubiquitin-proteasome pathway. FASEB J 2006; 20:350-2.

14. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002; 115:455-65.

15. Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ. Beta-arrestin inhibits NF-kappaB activity by means of its interaction with the NF-αB inhibitor IαBα. Proc Natl Acad Sci U S A 2004; 101:8603-7.

16. Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, Pei G. Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-αB pathways. Mol Cell 2004; 14:303-17.

17. Kizaki T, Suzuki K, Hitomi Y, et al. Uncoupling protein 2 plays an important role in nitric oxide production of lipopolysaccharide-stimulated macrophages. Proc Natl Acad Sci U S A 2002; 99:9392-7.

18. Kizaki T, Ookawara T, Iwabuchi K, et al. Age-associated increase of basal corticosterone levels decreases $ED2^{high}$, $NF\text{-}kappaB^{high}$ activated macrophages. J Leukoc Biol 2000; 68:21-30.

19. Raje CI, Kumar S, Harle A, Nanda JS, Raje M. The macrophage cell surface glyceraldehyde-3-phosphate dehydrogenase is a novel transferrin receptor. J Biol Chem 2007; 282:3252-61.

20. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. comparison of activating cytokines and evidence for independent production. J Immunol 1988; 141:2407-12.

21. Kizaki T, Suzuki K, Hitomi Y, et al. Negative regulation of LPS-stimulated expression of inducible nitric oxide synthase by AP-1 in macrophage cell line J774A.1. Biochem Biophys Res Commun 2001; 289:1031-8.

22. Staal FJ, Roederer M, Herzenberg LA, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci U S A 1990; 87:9943-7.

23. Sun Y, Cheng Z, Ma L, Pei G. α-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. J Biol Chem 2002; 277:49212-9.

24. Barlic J, Andrews JD, Kelvin AA, et al. Regulation of tyrosine kinase activation and granule release through α-arrestin by CXCRI. Nat Immunol 2000; 1:227-33.

25. Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD. Defective lymphocyte chemotaxis in α-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci U S A 2002; 99:7478-83.

26. Walker JK, Fong AM, Lawson BL, Savov JD, Patel DD, Schwartz DA, Lefkowitz RJ. α-arrestin-2 regulates the development of allergic asthma. J Clin Invest 2003; 112:566-74.

27. Parry GC, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-αB-mediated transcription. J Immunol 1997; 159:5450-6.

Legends for figures

Fig. 1. LPS stimulation down-regulates α_2AR expression. (A) RAW264 cells were stimulated with LPS. The protein levels of α_2AR and GAPDH (loading control) in the plasma membrane were analyzed by Western blotting (left panel). The α_2AR mRNA and 18S rRNA (loading control) were analyzed by RT-PCR (right upper panel). Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. 0 h. (B) RAW264 cells were transfected with the *$\kappa_2$ar* construct or vector alone. The protein levels of α_2AR and GAPDH (left panel) and mRNA expressions of α_2AR and 18S rRNA (right upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. 0 h. (C) mRNA expressions of α_2AR and 18S rRNA (upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (lower panel). *P < 0.01 vs. 0 h. (D) Cells were cultured with or without LPS for 6 h and were stimulated with Salbutamol (1×10^{-6} M) for final 30 min. Then, intracellular cAMP concentrations were analyzed. *P < 0.05 vs. without LPS. (E) Cell size was measured by flow cytometric analysis of forward light scatter characteristics (FSC).

Fig. 2. Forced α_2AR expression suppresses NO production and NOS II expression. (A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. *P < 0.001 vs. LPS-stimulated RAW264 or RAWvec cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA were analyzed as in A (right upper panel). Bar graphs show the relative intensity of the PCR bands from four separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. corresponding RAWvec cells. Data shown are representative of three–four separate experiments.

Fig. 3. Forced α_2AR expression suppresses NF-αB activation.
(A) The vector control cells and α_2AR transfectant were stimulated with LPS, and NF-αB activation was analyzed by EMSA. (B) The vector control cells and α_2AR transfectant were stimulated with LPS, and cytoplasmic IαBα and GAPDH (loading control) was analyzed by Western blotting. (C, D) RAW264 cells were co-transfected with the pNF-αB-Luc vector (C) or NOS II promoter-luciferase construct (D) and vector (Vec), pcDNA4-α_2AR (AR) or pCMV-IαBαM (DN-IαB). The cells were cultured with LPS for 24 h, and luciferase activities were determined. The results are expressed as means ± SEM from six-well cultures. *P < 0.001 vs. cells co-transfected with Vec. (E) PDTC was