Capsaicin promotes the development of burst-forming units-erythroid (BFU-E) from mouse bone marrow cells

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Abbreviations: BFU-E, burst-forming units-erythroid; CFU-GM, colony forming units-granulocyte/macrophage; EpoR, erythropoietin receptor; GPA, surface markers glycoprotein A; HSC, hematopoietic stem cell

Abstract

Capsaicin, the pungent component of chilli peppers, is known to induce mediators of hematopoiesis. We investigated the effect of capsaicin on hematopoiesis in mouse progenitor cells. Treatment of mouse bone marrow cells with capsaicin induced the formation of colony of burst-forming units-erythroid (BFU-E). We also found that the number of erythropoietin receptor (EpoR)-positive cells was increased by capsaicin. To clarify the effect of capsaicin on erythroid lineage, BFU-E colonies were separated from non-BFU-E colonies by colony-picking after *in vitro* culture of mouse bone marrow cells. Quantitative RT-PCR analysis revealed that capsaicin stimulated the expression of the erythroid-specific genes encoding EpoR, glycoprotein A (GPA), β -globin (Hbb-b1), GATA-1, PU.1, nuclear factor erythroid-derived 2 (NF-E2), and Krüppel-like factor 1 (KLF1) in the BFU-E colonies. Furthermore, capsaicin could effectively stimulate the transfected GATA-1 promoter in K562 cells. GATA-1 is known as an essential transcription factor for the development of erythroid cells. Our results show that development of the erythroid lineage from bone marrow cells can be induced by treatment with capsaicin, and that GATA-1 seems to play a role in this induced erythroid maturation.

Keywords: capsaicin; erythroid progenitor cells; erythropoiesis; GATA1 transcription factor; receptors, erythropoietin

Introduction

Hematopoiesis is a paradigmatic development system, representing the progressive restriction of the cell fate potential of initially multipotent hematopoietic stem cells (HSCs). The developmental pathways from HSCs to mature cells contain many lineage branch points, and once lineage choices are made in response to physiological needs, progenitors display increasingly limited lineage potentials. Lineage commitment of HSCs involves the upregulation of particular transcription programs and the concomitant suppression of multipotentiality and transcriptional programs associated with other lineages. During hematopoiesis, cytokines act as developmental signals that orchestrate the commitment of progenitor cells into and through particular lineages. The differentiation of HSCs to erythroid cells is controlled by a complex array of extrinsic and intrinsic factors, but the detailed mechanisms involved remain poorly defined; GATA-1, erythropoietin receptor (EpoR), surface markers glycoprotein A (GPA), and β -globin mRNA are up-regulated, whereas GATA-2 mRNA is down-regulated (Edvardsson *et al.*, 2004).

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is the major phenolic pungent principle of hot peppers of the genus *Capsicum*. It has been used against neuropathic pain and inflammation, and as an anti-carcinogen (Winter *et al.*, 1995; Kim *et al.*, 2003; Lee and Park, 2003), suggesting that it may be an important dietary phytochemical with potential therapeutic activities (Surh *et al.*, 1998). A recent study revealed that capsaicin attenuated the transcription of cytokines, GM-CSF, IFN- γ , and IL-2 (Gertsch *et al.*, 2002). In addition, capsaicin administration enhanced the release of calcitonin gene-related peptide (CGRP) and substance P (Katagiri *et al.*, 2005; Mizuguchi *et al.*, 2005), which stimulates colony forming units-granulocyte/macrophage (CFU-GM) colony formation.

The goal of the present study was to explore the effect of capsaicin on the development of the erythroid lineage in bone marrow HSCs *in vitro*.

Materials and Methods

Cells and cell culture

Bone marrow cells were obtained from 5-week-old male C57BL/6 mice and separated from dead cells and RBCs using Lympholyte M (Cedarlane Laboratories Ltd. Mornby, Canada). Cells were incubated in Iscove's modified Dulbecco's medium (IMDM) containing 15% FBS, 1% BSA, 10 mg/ml transferrin, 100 μ M 2-mercaptoethanol, 2 mM L-glutamine, and 3 U/ml of erythropoietin (M3334; StemCell Technologies, Vancouver, Canada). Cells were plated in 35-mm culture dishes. K562 (chronic myelogenous leukemia) cells were cultured in RPMI medium containing 10% (v/v) FBS (Yang *et al.*, 2006). Before transfection and treatment, the cells were kept in medium supplemented with 0.1% FBS for 24 h.

Colony-forming cell assays

In vitro colony-forming assays were carried out in triplicate by plating 3×10^5 cell/ml in M3334 with capsaicin (1, 10, and 100 μ M) and counting on 8 day culture. Capsaicin (Sigma, St. Louis, MO) was dissolved in DMSO (Sigma) and diluted in culture medium before treatment. Colonies were counted under an inverted microscope as previously described (Han *et al.*, 2003).

Flow cytometry

FACS analysis was performed using a Becton Dickinson FACS Calibur (Oxnard, CA). Colonies were harvested after 8 d, and blocked with 2.4G2 anti-Fc γ R antibody (BD PharMingen, San Diego, CA) for 10 min at 4°C. The cells were washed twice with FACS buffer (0.1% BSA, 0.02% NaN₃ in 1 $\times$ PBS) and stained with anti-EpoR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by FITC-conjugated anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA).

RT-PCR analysis

Burst-forming units-erythroid (BFU-E) and non BFU-E cells (day 8) were picked with Pasteur pipettes. The cells were washed with 1 $\times$ PBS and total RNA was prepared using Tri-reagent (Sigma). Total RNA was reverse-transcribed with oligo (dT)₁₈ primer (Invitrogen, Carlsbad, CA) using RevertAidTM M-MuLV Reverse Transcriptase (Fermentas Life Sciences, Hanover, MD) and PCR reactions were performed. The amount of cDNA was normalized using the housekeeping gene, GAPDH. Cycling parameters were denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The primer sets were as follows: GAPDH, sense primer 5'-GCA-

TTGTGGAAGGGCTCATG-3' and reverse primer 5'-GATGCCTGCTTCACCACCTT-3'; EpoR, sense primer 5'-TCCTGGAGCACCTATGACCA-3' and reverse primer 5'-ACCGTCAGCAACAGCGAGAT-3'; GPA, sense primer 5'-CTGTGGTGGCTTCAACTGTA-3' and reverse primer 5'-CGATAATCCCTGCCATCACG-3'; GATA-1, sense primer 5'-CACTTCCCCAAA-TGCAGCCA-3' and reverse primer 5'-GAAAGGACTGGGAAAGTCAG-3'; GATA-2, sense primer 5'-GCCGTATTGAATGCGCAGCA-3' and reverse primer 5'-AGCCCCCTGGGTAAACAGACA-3'; PU.1, sense primer 5'-TGACTACTACTCCTTCGTGG-3' and reverse primer 5'-GATAAGGGAAGCACATCCGG-3'; nuclear factor erythroid-derived 2 (NF-E2), sense primer 5'-TGTTGGGGAGCTTGGAGAGA-3' and reverse primer 5'-TGAGCAGGCCTGAAAGGGTA-3'; Krüppel-like factor 1 (KLF1), sense primer 5'-CCAT-CAGTACACTAACCACC-3' and reverse primer 5'-ACAGGTCACGTCCCTCTCAT-3'; β -globin (Hbb-b1), sense primer 5'-AAGGCACCTTTGCCAGCCTCA-3' and reverse primer 5'-CGTTAGTGGTACTTGTGAGCC-3'.

Real-time PCR

Real-time PCR was performed using SYBR green I *Taq* polymerase (Qiagen, Hilden, Germany) according to the manufacturer's protocol with an MJ Research OpticonTM Continuous Fluorescence Detector. All assays were carried out twice as independent PCR runs for each cDNA sample. Threshold cycles (CT) were determined with Opticon MonitorTM analysis software, and used to calculate the relative amount of each transcript.

Transient transfection

Murine genomic DNA was extracted from C57BL/6 mouse splenocytes by standard procedures. Reporter assay plasmids were constructed with upstream fragments of 1041 bp and 912 bp of mouse GATA-1 (mGATA-1) and GATA-2 (mGATA-2) genes, respectively, based on the sequences in GenBank (Accession Nos X57530 and NT039353). Transfection of the mGATA-1 or mGATA-2 reporter constructs in pGL3-basic (Promega, Madison, WI), or of empty pGL3-basic, into K562 cells was performed with LipofectamineTM (Invitrogen). For each experiment, exponential K562 cells (4×10^4 cells/well) were cotransfected with 0.8 μ g of pGL3-GATA-1 or pGL3-GATA-2 and 40 ng of pCMV- β Gal per well. After 24 h, capsaicin (0.1 and 1 μ M) was added and the cells were cultured for an additional 24 h. Cell lysates were prepared in lysis buffer (Promega) and luciferase activity was measured using a Wallac Multichannel Counter C (Perkin Elmer Inc., Gathisburg, MD). Ratios of arbitrary

luciferase light units to β -galactosidase activities were calculated and compared to those of cells transfected with empty pGL3-basic. All results are presented as fold increases or decreases over the standardized luciferase activity in the absence of capsaicin.

Results

Capsaicin increases erythroid lineage development in mouse bone marrow cells cultured *in vitro*

To investigate the effects of capsaicin on lineage development in mouse HSC, bone marrow cells were exposed to a range of capsaicin concentrations, and colony formation was monitored. As

Figure 1. Effect of capsaicin on erythroid lineage development from mouse bone marrow cells. Mouse (C57BL/6 male 5 weeks) bone marrow cells were cultured in M3334 methylcellulose medium in the presence of the indicated concentrations of capsaicin. Morphology of colonies on day 8 was assessed by the hematopoietic progenitor cell colony-forming assay. Results of triplicate experiments are shown as means $\pm$ SE of the number of colony formation.

shown in Figure 1, treatment with 100 pM capsaicin enhanced the number of BFU-E colonies 1.5-fold. We carried out FACS analysis of the bone marrow cells stained with anti-EpoR antibody to determine whether capsaicin affected expression of this erythroid lineage marker. Treatment with capsaicin increased erythroid differentiation, as measured by the fraction of cells expressing EpoR (Figure 2).

Capsaicin induces the expression of erythropoiesis-associated genes in BFU-E colonies

Since whole cells cultured from bone marrow include several lineages, we focused on the influence of capsaicin on the development of erythroid progenitor cells. After *in-vitro* culture of mouse bone marrow cells on methylcellulose medium, BFU-E colonies and non BFU-E colonies were picked separately under a microscope and total RNAs were obtained from the two cell types. We performed three independent experiments evaluating the expression of erythropoiesis-related genes in these cells by RT-PCR. When colonies were harvested on 8 day culture, capsaicin increased EpoR and GPA mRNAs in the BFU-E cells and there was no significant change in the non-BFU-E cells (Figure 3). EpoR mRNA, which was barely expressed in quiescent hematopoietic progenitor cells (HPCs), is slowly induced in the erythropoietic pathway, whereas it is hardly expressed in the granulopoietic pathway (Labbaye *et al.*, 1995). GATA-1 expression was increased 4-fold by capsaicin in the BFU-E cells, whereas capsaicin did not affect GATA-2 expression in either the BFU-E or non-BFU-E cells. In addition, PU.1 and β -globin transcripts increased in the BFU-E cells with no increase in the non-BFU-E cells. NF-E2 and KLF1 mRNAs, which are constantly expressed in the erythroid pathway, were highly induced in the BFU-E cells.

To examine whether capsaicin can stimulate the expression of erythropoiesis-related genes in the

Figure 2. Flow cytometric analysis of EpoR-expression in mouse bone marrow cells. Mouse bone marrow cells were cultured in the presence of capsaicin for 8 d and colonies were harvested for flow cytometric analysis. The graphs show cell numbers versus highly fluorescent FITC EpoR-expressing cells. (A) mock; (B) 10 pM capsaicin.

Figure 3. Expression of EpoR, GPA, GATA-1, PU.1, Hbb-b1, NF-E2, KLF1 and GATA-2 in BFU-E cells treated with capsaicin. RNA isolated from the BFU-E cells after culture on M3334 medium was subjected to reverse transcription. (A) RT-PCR and (B) Quantitative real-time PCR were performed and the data were normalized to GAPDH. (C) Capsaicin solution (1 μ M of 10 pM/colony) was dropped directly onto the colonies and after 2 d, expression of genes in the colonies was analyzed by RT-PCR.

Figure 4. Induction of luciferase activity driven by the GATA-1 promoter by capsaicin. K562 cells were transfected with constructs (pGL3-GATA-1, pGL3-GATA-2 or empty pGL3-basic as a control). After transfection, cells were incubated with or without capsaicin (0, 0.1, 1 μ M of final concentration) for 24 h and luciferase and β -galactosidase activities were measured. Results represent luciferase/ β -galactosidase activities. Triplicates were used for each experiment and results are shown as means $\pm$ SE.

differentiated BFU-E cells, we administrated capsaicin directly to the BFU-E colonies on day 6 and assessed expression of erythropoiesis-associated genes in individual colonies by RT-PCR analysis on day 8 (Figure 3C). The expression pattern of genes was consistent with the one shown in Figure 3A.

Activation of the GATA-1 promoter by capsaicin

To ascertain the effect of capsaicin on GATA-1 promoter activity, K562 cells were transiently transfected with various reporter constructs. Our results showed that the luciferase activity driven by the GATA-1 promoter was increased by capsaicin, whereas GATA-2 promoter activity was not affected (Figure 4).

Discussion

Red blood cell development starts with the commitment of pluripotent hematopoietic stem cells into self-renewing lineage specific progenitors, including BFU-E and CFU-E. A key issue in HSC studies has focused on how their commitment to a particular lineage and maturation is regulated at the cellular and molecular levels. This issue is of great clinical relevance (Goodnough and Marcus, 1998; Beauchemin *et al.*, 2004; Kostaridou *et al.*, 2004; Chung *et al.*, 2005).

We have shown here that capsaicin can act as an inducer of bone marrow erythropoiesis. Capsaicin treatment of mouse bone marrow cells resulted in an increase in formation of BFU-E colonies and up-regulation of an erythroid marker, EpoR. However, bone marrow cells are fairly heterogeneous in lineage and development stage. Therefore, it was necessary to further separate the cells before

analyzing the erythroid-associated gene expression pattern. BFU-E colonies were collected for RT-PCR analysis, and the induction of erythroid-specific gene transcription by capsaicin was detected both in the BFU-E colonies and in the whole bone marrow experiments. The induction of erythroid-specific gene transcription by capsaicin was also observed by an experiment in which capsaicin solution was dropped onto BFU-E colonies and transcripts levels were analyzed. This result indicates that capsaicin can stimulate expression of erythroid-specific gene even after commitment to erythroid progenitor cells. We found that transcription of the erythroid markers, GPA and EpoR, was strongly induced by capsaicin. GPA has been used to characterize erythroid differentiation because its expression is observed exclusively in erythroid cultures and parallels the expression of β -globin. Signaling through EpoR regulates the proliferation, differentiation, and survival of erythroid progenitor cells (Lu *et al.*, 2000). During erythrocyte differentiation cells became progressively more sensitive to erythropoietin due to the production of EpoR in these cells. Therefore, our results underscore the likelihood that capsaicin promotes the erythroid pathway by acting upstream of the Epo/EpoR-mediated regulation of erythroid differentiation or proliferation. The different differentiation potentials of cells at the progenitor stage may reflect their differential expression of specific combinations of lineage-related transcription factors. Our data support the notion that capsaicin may promote erythroid lineage commitment by regulating the expression of transcription factors such as GATA-1 and NF-E2. In addition, transient transfection assays showed that capsaicin stimulated the expression of a GATA-1 reporter in K562 cells, but not of a GATA-2 reporter. These results support the hypothesis that capsaicin enhances erythroid lineage development by up-regulating GATA-1.

GATA-1 stimulates megakaryocyte/erythroid lineage commitment and simultaneously inhibits granulocyte/monocyte (GM) development (Iwasaki *et al.*, 2003). Studies in mice and in cell lines have pointed to a major role of GATA-1 in erythroid commitment and in the subsequent erythroid differentiation (Cantor and Orkin, 2002). GATA-1 is barely expressed in quiescent erythroid progenitors, but is rapidly induced when these cells are stimulated towards erythroid differentiation by Epo. It has been shown using conditional knockout models that the expression of GATA-1 in embryonic stem cells strongly induces expression of Bcl-XL, but not Bcl-2, in erythroid cells, and this may be the basis of one of the anti-apoptotic effects of GATA-1 in erythroid cells (Gregory *et al.*, 1999).

We have noted that capsaicin did not change

GATA-2 expression. GATA-2 is highly expressed in HSCs and declines with blood cell maturation, implying that it is a key factor in the maintenance of stem cell function, but not in provoking cell fate decisions in the HSCs. An experiment using haplo-insufficiency of GATA-2 in mice indicated that the level of GATA-2 selectively affected GM colony formation (Rodrigues *et al.*, 2005). Down regulation of GATA-2 expression was required for stem cells to contribute to normal hematopoiesis *in vivo*. Capsaicin may not affect the GATA-2 regulation, which is associated with normal development of the CFU-GM lineage.

The expression of PU.1 is tightly controlled in hematopoietic cells (Rosenbauer *et al.*, 2004). We observed that capsaicin increased PU.1 expression in non-BFU-E colonies, which were mainly myeloid-lineage cells, but not in BFU-E colonies. This suggests that capsaicin can stimulate HSC commitment to the myeloid lineage by regulating the PU.1 transcription factor. This is supported by previous evidence that PU.1 prevents differentiation and maintains self-renewal in the erythroid lineage, whereas it is a positive regulator of myeloid-lineage differentiation (Back *et al.*, 2004).

We cannot be certain which signaling pathway(s) is involved in the promotion of the BFU-E lineage by capsaicin. The inhibitory effect of TNF- α on erythroid maturation seems to involve NF- κ B induction (Xiao *et al.*, 2002). Later in differentiation, a decline in the levels of the NF- κ B family of factors leads to derepression and a consequent increase of NF-E2, which in turn activates a subset of erythroid-specific genes (Liu *et al.*, 2003). Capsaicin can block NF- κ B activation by suppressing the degradation of I κ B α (Singh *et al.*, 1996). These results together suggest that the enhanced erythroid-specific gene expression in response to capsaicin is mediated via the NF- κ B signaling pathway. This hypothesis is supported by our observation that the level of NF-E2 mRNA is also increased by capsaicin.

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