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Cyclic ADP-Ribose-Mediated Expansion and Stimulation of Human Mesenchymal Stem Cells by the Plant Hormone Abscisic Acid

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ABSTRACT

Abscisic acid (ABA) is a phytohormone involved in fundamental processes in higher plants. Endogenous ABA biosynthesis occurs also in lower Metazoa, in which ABA regulates several physiological functions by activating ADP-ribosyl cyclase (ADPRC) and causing overproduction of the Ca^{2+}-mobilizing second messenger cyclic ADP-ribose (cADPR), thereby enhancing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Recently, production and release of ABA have been demonstrated to take place also in human granulocytes, where ABA behaves as a proinflammatory hormone through the same cADPR/$[Ca^{2+}]_i$ signaling pathway described in plants and in lower Metazoa. On the basis of the fact that human mesenchymal stem cells (MSC) express ADPRC activity, we investigated the effects of ABA and of its second messenger, cADPR, on purified human MSC. Both ABA and cADPR stimulate the in vitro expansion of MSC without affecting differentiation. The underlying mechanism involves a signaling cascade triggered by ABA binding to a plasma membrane receptor and consequent cyclic AMP-mediated activation of ADPRC and of the cADPR/$[Ca^{2+}]_i$ system. Moreover, ABA stimulates the following functional activities of MSC: cyclooxygenase 2-catalyzed production of prostaglandin E_2 (PGE_2), release of several cytokines known to mediate the trophic and immunomodulatory properties of MSC, and chemokinesis. Remarkably, ABA proved to be produced and released by MSC stimulated by specific growth factors (e.g., bone morphogenetic protein-7), by inflammatory cytokines, and by lymphocyte-conditioned medium. These data demonstrate that ABA is an autocrine stimulator of MSC function and suggest that it may participate in the paracrine signaling among MSC, inflammatory/immune cells, and hemopoietic progenitors. STEM CELLS *2008;26:2855–2864*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSC), the precursors of hemopoietic stroma, can be purified from bone marrow (BM), expanded in vitro, and induced to differentiate toward osteoblasts, chondrocytes, and adipocytes [1, 2]. The hemopoietic stroma plays an essential role in the BM microenvironment by providing hemopoietic progenitors (HP) with soluble factors essential to their proliferation and differentiation and by preventing lymphocyte activation. Several clinical trials are exploring the use of MSC as trophic support in BM transplant, as immunomodulators in the treatment of the graft versus host disease, and as osteoblast precursors for bone regeneration [3, 4].

The increasingly recognized role of MSC in the regulation of hemopoiesis through the production of both growth-promoting and cytotoxic factors [5] justifies the current interest in identifying the biochemical signals that are produced by MSC and active on hemopoietic stem cells (HSC). These studies, along with the characterization of the biomolecular heterogeneity of MSC populations [6, 7], will enable researchers to define the role of MSC in tissue repair and to address the clinical issues related to defective HSC engraftment and consequent BM failure.

We have demonstrated that cyclic ADP-ribose (cADPR), a universal mobilizer of intracellular Ca^{2+} [8, 9], behaves as a hemopoietic growth factor [10, 11]. Ectocellular ADP-ribosyl cyclase (ADPRC) activity, responsible for cADPR synthesis from NAD^+, is expressed in the BM microenvironment both on MSC and on HP [12]. Indeed, nanomolar concentrations of cADPR, such as those produced by an ADPRC-positive feeder, significantly increase the in vitro [10] and in vivo [11] proliferation of human HSC through an intracellular Ca^{2+} concentra-

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tion ($[Ca^{2+}]_i$)-dependent mechanism. Release of NAD^+ by MSC, across connexin-43 hemichannels [13], enables the ectocellular production of cADPR in the BM environment. Following internalization across concentrative nucleoside transporters [13], cADPR releases Ca^{2+} from ryanodine receptor (RyR) channels present in the endoplasmic reticulum (ER) of human HP.

The cADPR/$[Ca^{2+}]_i$ signaling pathway has been shown to be activated by the plant hormone abscisic acid (ABA) in higher plants [14], where ABA is involved in fundamental functions such as response to abiotic stress, regulation of seed dormancy and germination, and regulation of gene transcription [15]. Endogenous ABA synthesis has recently been demonstrated to also occur in Porifera and Hydrozoa (lower Metazoa), where it is stimulated by a temperature rise and by light exposure, respectively [16, 17]. In sponges and hydroids, ABA activates ADPRC through a protein kinase A-mediated phosphorylation, leading to an increase in the intracellular cADPR concentration and in the $[Ca^{2+}]_i$, which stimulates oxygen consumption in sponges and stem cell-mediated tissue regeneration in hydroids.

Recently, production and release of ABA have been demonstrated to occur also in human granulocytes, where ABA behaves as a proinflammatory hormone [18]. The signaling pathway of ABA in human granulocytes is strikingly similar to the one described in lower Metazoa and leads to the functional stimulation of these phagocytes.

The facts that (a) the cADPR/Ca^{2+} signaling pathway is involved in the functional stimulation of different cell types, including stem cells, from lower Metazoa to humans, and (b) MSC express activity of ADPRC, the target enzyme of ABA in Metaphyta and Metazoa, prompted us to explore the functional effects of ABA, and of its second messenger cADPR, on human MSC and to investigate the possible endogenous synthesis of ABA by these cells.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (Milan, Italy, http://www.sigmaaldrich.com) unless otherwise stated.

MSC Isolation and Cell Culture

MSC were purified from mononuclear cells (MNC) isolated from bone marrow samples obtained from the acetabulus of patients undergoing hip arthroplasty. At least 50 ml per sample was collected from six patients, ages 59–73 (two males, four females). MNC were isolated by centrifugation on Ficoll Paque Plus (Amersham Biosciences, Milan, Italy, http://www.amersham.com) and then plated on 175-cm^2 flasks at 2×10^5 cells per cm^2 in McCoy's medium (Lonza, Milan, Italy, http://www.lonza.com) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Milan, Italy, http://www.invitrogen.com) and with 100 U/ml penicillin plus 100 μg/ml streptomycin. After 48 hours of culture, the medium was removed, adherent cells were washed once with phosphate-buffered saline, and fresh medium was added to each flask. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and half of the medium was changed every 3 days. Cell confluence usually was reached in 2 weeks, this being considered passage 0: cells were then expanded (1:4) and cultured as described. Cells from passages 2–4 were used for all experiments.

Proliferation and Clonogenic Assays

The clonogenic assays (colony forming units fibroblasts [CFU-F]) were set up with freshly isolated BM-MNC, as previously described [2]. The cells were stimulated with the following compounds for the 1st week of culture: 10 or 100 nM cADPR or 1 or 10 μM ABA, the latter without or with 50 μM 8-bromo-cADPR (8-Br-cADPR). After 2 weeks, cells were stained with crystal violet, and the number of colonies was counted.

For the proliferation assay, cells were plated at a density of 3×10^3 cells per well in 96-well plates. After 24 hours, the same compounds used in the CFU-F assay were added, in quadruplicate. After 3 days of culture, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test was performed to evaluate the extent of cell proliferation [19]. To explore possible changes in cell phenotype, fluorescence-activated cell sorting analyses were performed on MSC cultured for 1 week in the presence or absence of 10 μM ABA or 10 μM cADPR. The surface antigens, listed in supplemental online Methods, and their percentage of expression in the untreated cells are displayed in supplemental online Table 1. No changes of antigen expression were observed upon ABA or cADPR treatment.

Reverse Transcription Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction Analyses

Total RNA was extracted from MSC (1.5×10^5) using the RNeasy Micro Kit (Qiagen, Milan, Italy, http://www1.qiagen.com) according to the manufacturer's instructions. Quality and quantity of RNA were analyzed using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, http://www.nanodrop.com). The cDNA was synthesized by using the iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy, http://www.bio-rad.com). The polymerase chain reaction (PCR) primers were designed through Beacon Designer 2.0 software (Bio-Rad) and are listed in supplemental online Table 2.

Real-time quantitative polymerase chain reaction (qPCR) was used to quantify the level of mRNA expression of target genes. PCRs were performed in an MJ Research Chromo4 detector (Bio-Rad) using 2× iQ Custom SYBR Green Supermix (Bio-Rad). All PCR products were checked by a melting curve analysis to rule out the formation of multiple or incorrectly sized products. Values were normalized to the mRNA expression of *GAPDH* and TATA binding protein (*TBP*) (reference genes). Statistical analyses of the qPCR were obtained using the iQ5 Optical System software, version 1.0 (Bio-Rad), on the basis of the $2^{\Delta\Delta Ct}$ method [20, 21]. Amplification efficiencies of target and reference genes were determined by generating standard curves.

Assays of ADP-Ribosyl Cyclase Activity

Ectocellular ADPRC activity was measured on 1.6×10^5 MSC seeded onto 35 × 10-mm tissue culture dishes. The cells (wild-type, scramble [SCR] short interfering RNA [siRNA], or CD38 siRNA) were pretreated in the presence or absence (control) of 10 μM ABA for 5 minutes or 24 hours and then incubated at 37°C in Hanks' balanced saline solution medium with 0.4 mM β-NAD^+ as substrate. At various times of incubation (5, 15, 30, and 60 minutes) 300-μl aliquots were withdrawn, and enzymatic reactions were stopped by addition of perchloric acid (PCA) (final concentration, 0.6 M). The concentration of the product cADPR was measured by a highly sensitive enzymatic cycling assay [22]. Protein determination was performed on an aliquot of each incubation [23].

Fluorimetric Ca^{2+} Measurements

MSC (2×10^4) were seeded onto 20-mm-diameter glass coverslips in complete medium and allowed to adhere overnight. 1-[2-(5-carboxyoxazol 2-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methyl-phenoxy)ethane-N,N,N′,N′-tetraacetic acid, pentaaceto-xymethyl ester (FURA 2-AM) 10 μM was added, and the cells were further incubated for 60 minutes. Real-time analyses of Ca^{2+} movements induced by ABA and cADPR on MSC, preincubated or not with the various inhibitors, were performed under continuous fluorescence recording at 25°C, and the $[Ca^{2+}]_i$ was determined as described [24].

Western Blots

Expression of cyclooxygenase (COX)-1 and COX-2 in human MSC after 6 hours of incubation without (controls) or with 10 μM ABA or cADPR was measured by Western blot analysis of total cell lysates. Briefly, 1.5×10^5 cells were seeded onto 60 × 15-mm tissue culture dishes and cultured for 18 hours. ABA or cADPR were then added to the culture medium, and cells were further incubated for 6 hours at 37°C. Western blot analyses were performed as previously described [25].

Assays of Prostaglandin E_2

The prostaglandin E_2 (PGE_2) concentration in the culture medium from MSC (1.5×10^5), incubated for 6 hours with various concentrations of ABA or cADPR and in the presence or absence of the specific COX-2 inhibitor NS398 (5 μM), the specific COX-1 inhibitor indomethacin (5 μM), 8-Br-cADPR (50 μM); or in CD38-silenced MSC, or in scramble-silenced MSC, was quantified on 10-fold diluted medium using the PGE_2 Monoclonal EIA Kit (Cayman Chemicals, Ann Arbor, MI, http://www.caymanchem.com) according to the manufacturer's instructions.

Chemotaxis and Chemokinesis

MSC were resuspended at 7×10^5 cells per milliliter in McCoy's medium with 5% FBS (chemotaxis medium). Chemotaxis and chemokinesis assays were performed using 96-well ChemoTx system microplates (Neuro Probe, Gaithersburg, MD, http://www.neuroprobe.com) with a 8-μm pore size polycarbonate filter. The filter was precoated with superfibronectin (1 μg/ml). For chemotaxis assays, ABA (1, 10, or 100 nM) and cADPR (0.01, 0.1, or 1 μM) were added to the bottom wells, whereas for chemokinesis, cells were preincubated for 20 minutes with or without the same compounds at the mentioned concentrations. Chemokinesis with 100 nM ABA was also evaluated in the presence of 50 mM 8-Br-cADPR and in CD38-silenced as well as scramble-silenced MSC. Cell suspensions (25 μl) were then placed on top of the filter and allowed to migrate for 15 hours at 37°C. Counting of transmigrated cells was performed as described previously [18]. The results were expressed as chemotaxis index (number of cells that migrated toward chemoattractant/number of cells that migrated toward medium) and chemokinesis index (number of ABA- or cADPR-incubated cells that migrated toward medium/number of untreated cells that migrated toward medium).

ABA Content in MSC

MSC (1×10^6) were seeded in 75-cm^2 flasks in complete medium. After 24 hours, the supernatant was removed, and cells were incubated for 48 hours in 7 ml of fresh medium in the absence (control) or in the presence of the following stimuli: 1 ng/ml human recombinant (h)-tumor necrosis factor (TNF)-α, 25 ng/ml h-M-CSF, 0.2 μg/ml h-bone morphogenetic protein (BMP)-7 (Stryker Italia, Rome, http://www.italia.stryker.com), 50 ng/ml regulated upon activation, normal T-cell expressed, and secreted (RANTES), 50 ng/ml h-interleukin (IL)-8, or a twofold dilution (in complete medium) of the medium conditioned for 24 hours by peripheral blood mononuclear cells (PBMNC; 1×10^6/ml) isolated by centrifugation of blood samples from healthy donors on Ficoll Paque Plus (Amersham Biosciences). After 6, 24, and 48 hours, ABA in the centrifuged culture supernatant and in the trypsin-detached, washed cells was determined by enzyme-linked immunosorbent assay (ELISA) and by high-performance liquid chromatography (HPLC)-coupled mass spectrometry, as previously described [18]. Additional methods are described in supplemental online data.

RESULTS

ABA and cADPR Enhance Colony Output and Proliferation of Human MSC

The effects of ABA and cADPR were first investigated on CFU-F output. As shown in Figure 1A, cADPR significantly stimulated colony output compared with untreated controls at concentrations ranging from 100 nM to 10 μM (Fig. 1A, gray bars; 1.3 ± 0.15-, 1.9 ± 0.3-, and 2.0 ± 0.18-fold increase for 100 nM, one and 10 μM cADPR, respectively). The stimulatory effect of ABA was somewhat lower compared with cADPR, but still significant over controls (Fig. 1A, black bars; 1.3 ± 0.2-, 1.7 ± 0.21-, and 1.4 ± 0.2-fold increase for 0.1, 1, and 10 μM ABA). 8-Br-cADPR, a membrane-permeant cADPR antagonist [26], abrogated the stimulatory effect of both 1 μM cADPR and 10 μM ABA on colony output and reduced colony growth below control values, suggesting that basal levels of endogenous cADPR are necessary for normal MSC proliferation, as already observed in $CD34^+$ cells [10].

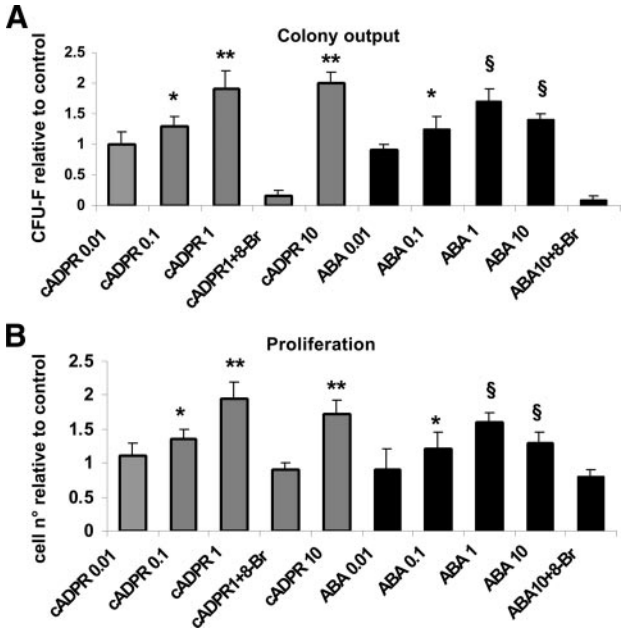

Figure 1. Effect of cADPR and ABA on mesenchymal stem cell (MSC) proliferation. **(A):** CFU-F assay in the presence of micromolar cADPR (gray bars) or ABA (black bars), without or with 50 μM 8-Br. Histograms represent the means ± SD from three experiments performed in triplicate. **(B):** MSC proliferation in the presence of micromolar cADPR or ABA for 3 days. Histograms represent the means ± SD from three independent experiments performed in quadruplicate. *, $p < .05$; **, $p < .0005$; §, $p < .0025$ compared with control. Abbreviations: ABA, abscisic acid; 8-Br, 8-bromo-cyclic ADP-ribose; cADPR, cyclic ADP-ribose; CFU-F, colony forming units-fibroblasts; n°, number.

Next, we investigated the effect of ABA and cADPR (Fig. 1B) on the MSC proliferation rate: cADPR again showed a higher stimulatory effect compared with ABA-treated cells (Fig. 1B, gray bars: 1.35 ± 0.15-, 1.95 ± 0.25-, and 1.72 ± 0.21-fold increase relative to untreated, control cells for 0.1, 1, and 10 μM cADPR, respectively; black bars: 1.25 ± 0.17-, 1.6 ± 0.15-, and 1.3 ± 0.15-fold increase relative to control for 0.1, 1, and 10 μM ABA, respectively). Also in these experiments, the increase in proliferation determined by cADPR and by ABA was prevented by 8-Br-cADPR.

Table 1. ADPRC activity in human MSC

Sample	ADPRC activity (pmol cADPR/min/mg)
Control	0.12 ± 0.03
10 μM ABA (5 min)	0.18 ± 0.03[b]
10 μM ABA (24 h)	0.15 ± 0.02[a]
CD38 siRNA	0.03 ± 0.03
SCR siRNA	0.10 ± 0.02

Comparison of ectocellular ADPRC activities of control and ABA-stimulated MSC, and of MSC transfected with CD38 siRNA or with scrambled (SCR) siRNA. Results are expressed as the mean ± SD from 6 experiments performed in duplicate.
[a] $p < .05$.
[b] $p < .0025$ compared to control.

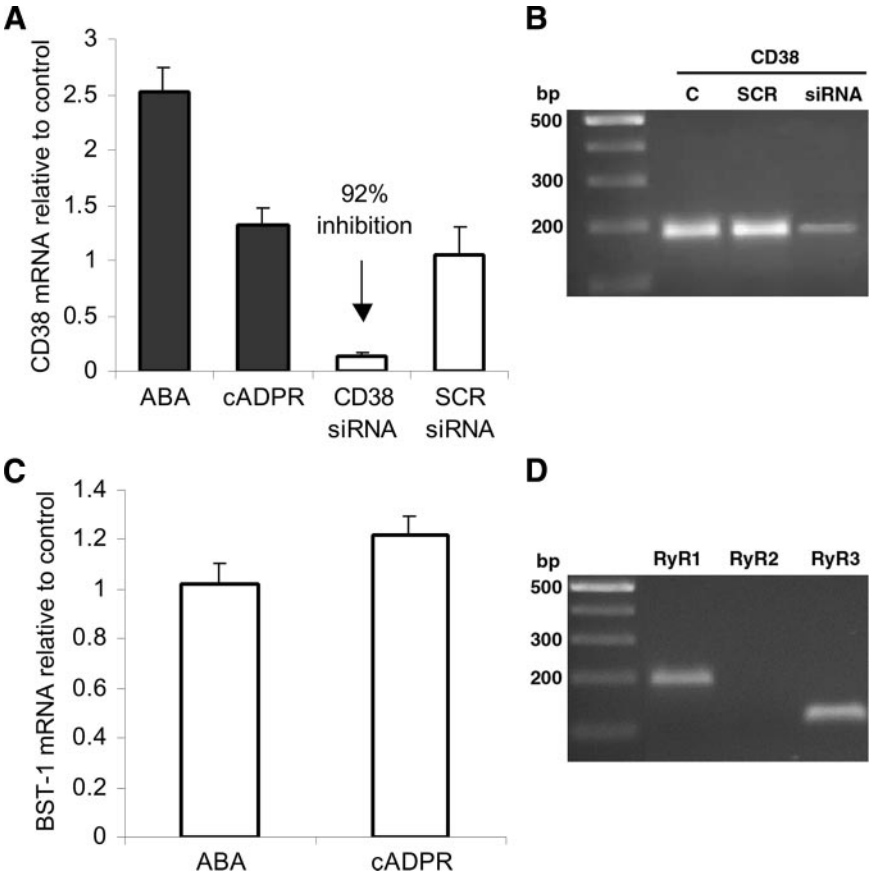

Figure 2. Expression of CD38, BST-1, and RyRs in human mesenchymal stem cells (MSC). **(A):** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of CD38 mRNA in MSC stimulated for 24 hours with 10 μM ABA or cADPR, or after 24 hours of CD38 RNA silencing (CD38 siRNA) or of SCR siRNA silencing, compared with control MSC. Results are expressed as means ± SD of three independent experiments performed in triplicate. **(B):** CD38 amplification bands on agarose gel in control MSC or after 24 hours of transduction with CD38 siRNA or SCR siRNA. **(C):** RT-qPCR of BST-1 mRNA in MSC stimulated for 24 hours with 10 μM ABA or cADPR compared with control MSC. Results are expressed as means ± SD of three experiments, performed in triplicate. **(D):** Visualization of the three RyRs amplification bands on agarose gel. Abbreviations: ABA, abscisic acid; bp, base pairs; BST-1, bone marrow stromal cell antigen-1; cADPR, cyclic ADP-ribose; RyR, ryanodine receptor; SCR, scramble; siRNA, short interfering RNA.

ABA Upregulates ADPRC Activity and CD38 Expression in Human MSC

In lower Metazoa [16, 17] and human granulocytes [18], ABA stimulates ADPRC activity, with consequent increases of the $[cADPR]_i$ and of the $[Ca^{2+}]_i$, which mediates the functional effects triggered by the hormone. Likewise, addition of 10 μM ABA to MSC cells stimulated ectocellular ADPRC activity. Specifically, the rate of cADPR formation was 1.5 times higher ($n = 6$; $p < .0025$) than in control, unstimulated cells at 5 minutes of incubation (Table 1). ADPRC activation was maintained for at least 24 hours following cell exposure to ABA, with a cyclase activity 1.3 times higher than that measured in ABA-treated cells compared with controls ($n = 6$; $p < .05$). Ectocellular ADPRC activity in MSC proved to be mostly expressed by CD38, as demonstrated by a significant loss (75%) of ADPRC activity in MSC transfected with CD38 siRNA but not in cells transfected with SCR siRNA (supplemental online Table 1). CD38 siRNA, but not SCR siRNA, indeed strongly inhibited CD38 mRNA synthesis (92%) in MSC as measured 24 hours after transfection (Fig. 2A).

CD38 expression in MSC was measured by qPCR analysis (Fig. 2A, 2B). Compared with untreated cells, the level of CD38 mRNA was upregulated in MSC incubated in the presence of 10 μM ABA or cADPR, with increases of 2.5-fold and 1.4-fold ($n = 9$; $p < .005$), respectively, being measured after 24 hours. Unlike expression of CD38, expression of BST-1 (the other ADPRC present in MSC) remained almost unchanged upon both ABA and cADPR stimulation (Fig. 2C).

Expression of Ryanodine Receptors in Human MSC

The cADPR receptor channels identified so far are the RyRs, which are expressed on ER membranes. Three types of RyRs are known, the most sensitive to cADPR being RyR2 and RyR3 [9, 27]. As detected both by reverse transcription qPCR and traditional PCR techniques, MSC were found to express the RyR1 and RyR3, whereas RyR2 mRNA was undetectable (Fig. 2D).

$[Ca^{2+}]_i$ Increase in ABA- and cADPR-Stimulated Human MSC

Since MSC were found to express RyR3, we investigated the effect of cADPR on the $[Ca^{2+}]_i$ in FURA-loaded cells. Indeed, addition of extracellular cADPR (0.1, 1, 10, and 100 μM) determined a slow, sustained increase of basal $[Ca^{2+}]_i$ (36.5 ± 3.9 nM), starting 10 minutes after cADPR addition and reaching 80.2 ± 6.0 nM after 20 minutes at the highest cADPR concentration (Fig. 3A). The 10-minute time lapse observed between cADPR addition and the onset of $[Ca^{2+}]_i$ increase suggests the presence of a membrane transporter that seems to reach saturation between 1 and 10 μM cADPR, since at 10 and 100 μM cADPR (traces 3 and 4, respectively), the $[Ca^{2+}]_i$ increase was similar, indicating a saturation, whereas in the presence of 0.1 and 1 μM cADPR (traces 1–2), the $[Ca^{2+}]_i$ increase was lower. To test this hypothesis, MSC were preincubated for 5 minutes in the presence of 8 μM digitonin, thereby determining a mild permeabilization of the plasma membrane, prior to addition of cADPR (Fig. 3B). In permeabilized cells, and in the absence of extracellular Ca^{2+}, addition of cADPR (Fig. 3B, arrow) induced an immediate $[Ca^{2+}]_i$ increase (Fig. 3B, trace 3). In cells preincubated with 8-Br-cADPR, the addition of cADPR was without effect (trace 2). Permeabilization per se did not modify the $[Ca^{2+}]_i$ over the time span explored (trace 1). The fact that 8-Br-cADPR abrogated the cADPR-induced $[Ca^{2+}]_i$ rise in permeabilized MSC indicates that this Ca^{2+} response is due to intracellular Ca^{2+} release.

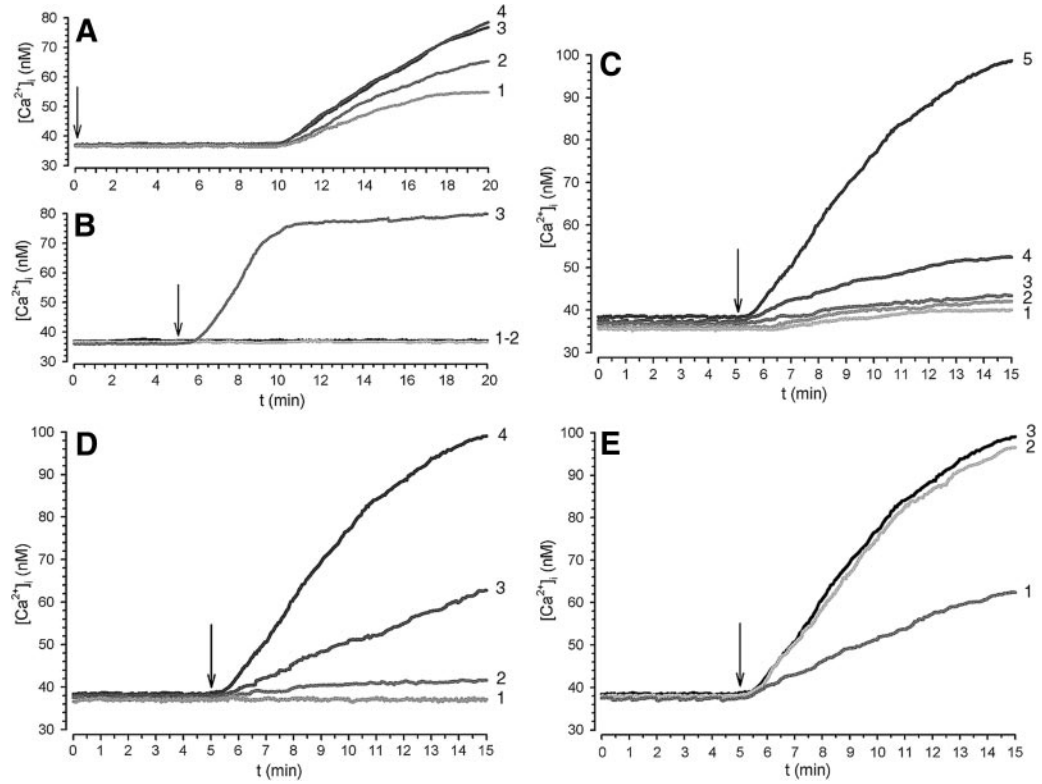

Figure 3. Effect of abscisic acid (ABA) and cyclic ADP-ribose (cADPR) on the intracellular calcium of mesenchymal stem cells (MSC). The $[Ca^{2+}]_i$ of FURA-2-loaded MSC was continuously recorded following addition (indicated by an arrow) of various concentrations of cADPR **(A, B)** or of ABA, either alone **(C)** or in the presence of various inhibitors **(D)**, and after CD38 silencing by short interfering RNA (siRNA) **(E)**. **(A):** $[Ca^{2+}]_i$ increased in MSC after addition of 100 nM (trace 1), 1 μM (trace 2), 10 μM (trace 3), or 100 μM (trace 4) cADPR in Hanks' balanced saline solution (HBSS). **(B):** $[Ca^{2+}]_i$ increased in MSC incubated with 8 μM digitonin for 20 minutes in Ca^{2+}-free HBSS without (trace 1) or with (traces 2 and 3) addition of 100 μM cADPR (arrow). MSC were preincubated (trace 2) or not (trace 3) for 45 minutes with 50 μM 8-bromo-cADPR (8-Br-cADPR). During the time span of the calcium measurements shown in traces 1 and 2, emissions at 340 and 380 nm remained constant, indicating that permeabilization per se did not affect the $[Ca^{2+}]_i$ or the FURA-2 content in the cells. **(C):** $[Ca^{2+}]_i$ increased in MSC after the addition of 2 nM, 20 nM, and 200 nM (traces 1–3, respectively) or 2 μM and 20 μM (traces 4 and 5, respectively) ABA. Traces were overlaid so as not to cross each other prior to ABA addition, to make comparison easier. **(D):** $[Ca^{2+}]_i$ increased in MSC after addition of 20 μM ABA alone (trace 4) and in the presence of 50 μM 8-Br-cADPR (trace 3), 0.1 mM EDTA (trace 2), or pertussis toxin (2 μg/ml; trace 1). **(E):** $[Ca^{2+}]_i$ increased in MSC after addition of 20 μM ABA, in wild-type cells (trace 3), in CD38 siRNA cells (trace 1), and in SCR siRNA cells (trace 2). Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; min, minutes; t, time.

We also explored the effect of ABA on the $[Ca^{2+}]_i$. As shown in Figure 3C, addition of ABA to FURA-loaded MSC induced a slow, sustained, dose-dependent $[Ca^{2+}]_i$ increase. The ABA-induced $[Ca^{2+}]_i$ rise was markedly inhibited (66%) by preincubation of the cells with 8-Br-cADPR for 60 minutes (Fig. 3D, trace 3), indicating a significant role of cADPR in mediating the effect of ABA on the $[Ca^{2+}]_i$ rise. Involvement of cADPR in the ABA-induced Ca^{2+} response was confirmed by the increase in its intracellular concentration (from 0.05 to 0.09 pmol per 10^6 cells; $n = 3$) after a 15-minute incubation of MSC with 20 μM ABA. The presence of extracellular EDTA inhibited the ABA-induced $[Ca^{2+}]_i$ increase almost completely (Fig. 3D, trace 2), demonstrating that the influx of extracellular Ca^{2+} was primarily responsible for the ABA-induced Ca^{2+} response in MSC, similar to that observed on human granulocytes [18]. ABA signaling in human granulocytes has been shown to involve a pertussis toxin (PTX)-sensitive G-protein [18]. Preincubation of MSC with 2 μg/ml PTX for 40 minutes abrogated the ABA-triggered $[Ca^{2+}]_i$ increase (Fig. 3D, trace 1).

CD38 phosphorylation and activation has been demonstrated to be responsible for the ABA-induced increase of ADPRC activity in human granulocytes [18]. Since upregulation of ADPRC activity and of CD38 expression by ABA proved to occur in MSC also (supplemental online Table 1; Fig. 2), the $[Ca^{2+}]_i$ levels were investigated on CD38-silenced MSC challenged with 20 μM ABA. As shown in Figure 3E, MSC transfected with SCR siRNA (trace 2) showed a Ca^{2+} response to ABA superimposable on that of untransfected cells (trace 3). Conversely, in MSC transfected with CD38 siRNA (Fig. 3E, trace 1), the $[Ca^{2+}]_i$ increase was significantly downregulated (65% of control values as measured 10 minutes after addition of ABA).

Effect of ABA and cADPR on Cyclooxygenase-2 Expression, PGE_2 Production, and Chemotaxis in Human MSC

It has previously been reported that PGE_2 released by MSC has a strong immunomodulatory effect on allogeneic PBMNC, inhibiting lymphocyte proliferation elicited by phytohemagglutinin (PHA) [28]. Thus, we decided to investigate whether ABA and cADPR could affect PGE_2 synthesis and release in MSC. As shown in Figure 4, MSC express both isoforms of COX, the constitutive COX-1 and the inducible COX-2. Incubation of MSC for 6 hours with ABA or cADPR induced a significant increase of COX-2 mRNA transcription (Fig. 4A), COX-2 expression (Fig. 4B), and PGE_2 release (Fig. 4C). Conversely, MSC incubation with ABA or cADPR did not modify COX-1 mRNA synthesis and expression (Fig. 4A, 4B). To confirm

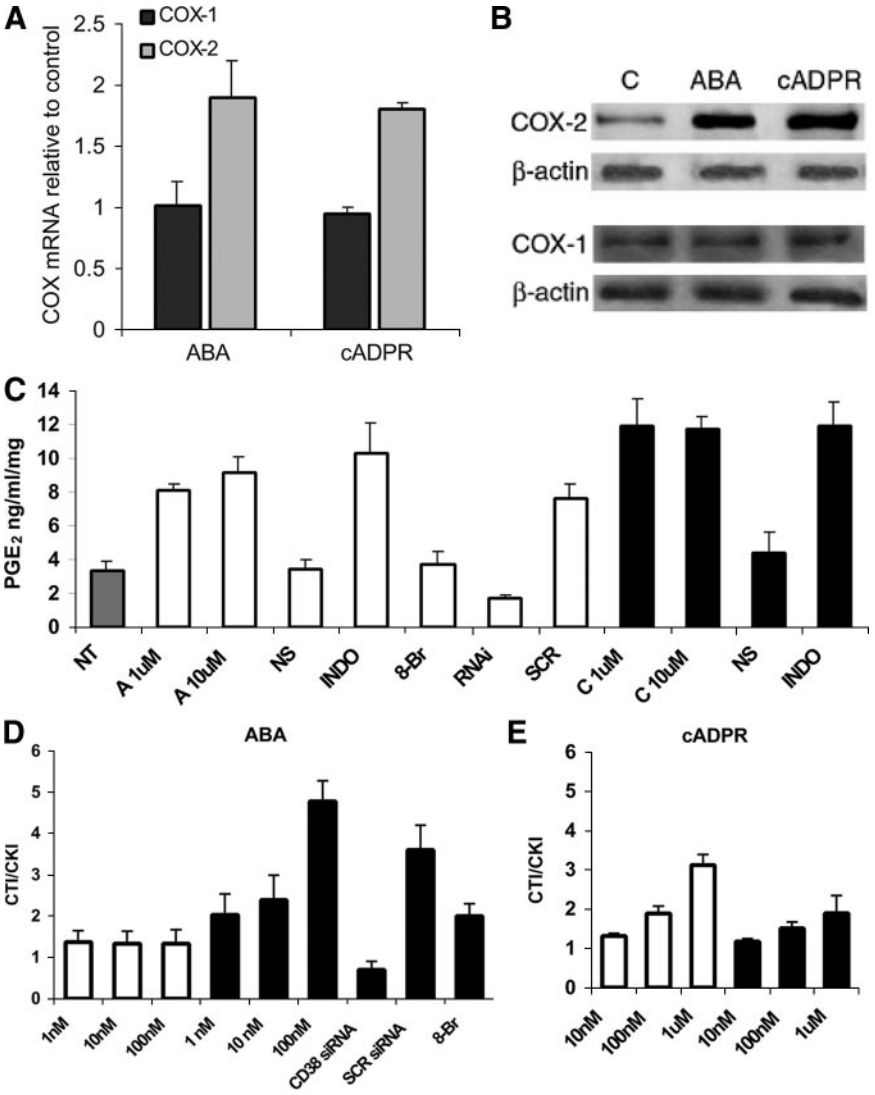

Figure 4. COX-1 and COX-2 mRNA expression, PGE_2 production, and cell migration in human mesenchymal stem cells (MSC) after ABA and cADPR treatment. **(A):** Reverse transcription-quantitative polymerase chain reaction of COX-1 and COX-2 in MSC after incubation for 6 h with 10 μM ABA or cADPR compared with untreated, control cells. Results are the means ± SD of three experiments, performed in triplicate. **(B):** Western blots of COX-1 and COX-2 protein expression in MSC incubated or not with ABA or cADPR for 6 h compared with the respective β-actin bands. The picture shows the result of one experiment representative of three, performed in duplicate, with similar outcomes. **(C):** PGE_2 release in the culture medium of untreated MSC (NT) or in cells incubated for 6 h with 1 or 10 μM ABA (A, white bars) or cADPR (C, black bars), with 10 μM ABA or cADPR in the presence of 5 μM NS398 (NS) or INDO, with 10 μM ABA in the presence of 50 μM 8-Br, or with 10 μM ABA in CD38 RNA-silenced MSC (RNAi) or SCR. Results are the means ± SD of three experiments performed in triplicate. **(D, E):** Chemotaxis index (CTI, white bars) and chemokinesis index (CKI, black bars) (CI) of human MSC incubated for 15 h in the presence or absence of increasing concentrations of ABA **(D)** or of cADPR **(E)**. In **(D)**, chemokinesis with 100 nM ABA-challenged cells was also performed in the presence of 50 μM 8-Br, in CD38-silenced MSC (RNAi), or in SCR. Results show the means ± SD of three independent experiments, performed in quadruplicate. Abbreviations: ABA, abscisic acid; 8-Br, 8-bromo-cyclic ADP-ribose; cADPR, cyclic ADP-ribose; COX, cyclooxygenase; h, hours; INDO, indomethacin; PGE_2, prostaglandin E_2; SCR, scramble-silenced mesenchymal stem cell; siRNA, short interfering RNA; uM, micromolar.

that the PGE_2 increase observed in ABA- and cADPR-treated MSC was due to COX-2 upregulation, MSC were incubated with ABA or cADPR in the presence of the specific COX-1 and COX-2 inhibitors indomethacin and NS398, respectively [28]. In both ABA- and cADPR-challenged cells, PGE_2 production was found to be inhibited by NS398 but not by indomethacin (Fig. 4C). Preincubation of MSC with 50 μM 8-Br-cADPR for 45 minutes prior to addition of 10 μM ABA, as well as transfection of cells with CD38 siRNA, but not with SCR siRNA, abrogated the ABA-induced PGE_2 increase (Fig. 4C), confirming CD38 and cADPR as key components of the signaling pathway triggered by ABA.

ABA has been demonstrated to behave as a chemoattractant and also to stimulate untargeted cell movement (chemokinesis) in human granulocytes [18]. At concentrations ranging between 1 and 100 nM, ABA stimulated MSC chemokinesis in a concentration-dependent manner (Fig. 4D, black bars), whereas chemotaxis was only slightly induced (white bars; $n = 12$; $p < .0025$). The chemokinetic effect of 100 nM ABA was abrogated in MSC transfected with CD38 siRNA and significantly reduced (by 50%) in cells preincubated with 8-Br-cADPR. cADPR stimulated chemotaxis at concentrations ranging between 10 nM and 1 μM and also stimulated chemokinesis, although to a lesser extent than ABA (Fig. 4E).

ABA Binding on Human MSC

Biotinylated abscisic acid (bio-ABA) has already been used to demonstrate the presence of ABA-binding sites on the plasma membrane of ABA-sensitive stomatal guard cells in plants [29], as well as in human granulocytes [18]. Live MSC were stained with the cytoplasmic marker calcein green and then incubated with bio-ABA. Confocal microscopy showed the presence of cell surface fluorescence (Fig. 5A, red staining), easily distinguished from that of the cell-permeant cytoplasmic marker calcein green (green staining), suggesting the presence of plasma membrane ABA-binding sites. Furthermore, as shown in Figure 5B, excess unconjugated ABA abrogated the red staining, indicating a specific competition between ABA and bio-ABA for the same membrane-binding sites.

ABA Production in Human MSC

The fact that ABA induced several biochemical and functional effects on MSC prompted us to explore the possibility that human MSC produced ABA. ABA was indeed detectable in human BM-derived MSC at a basal extracellular concentration of 1.34 ± 0.3 pmol/ml per 10^6 cells and intracellularly at 2.17 ± 0.4 pmol/mg protein ($n = 8$), as estimated both by HLPC-coupled mass spectrometry and by ELISA analyses of medium and cell extracts.

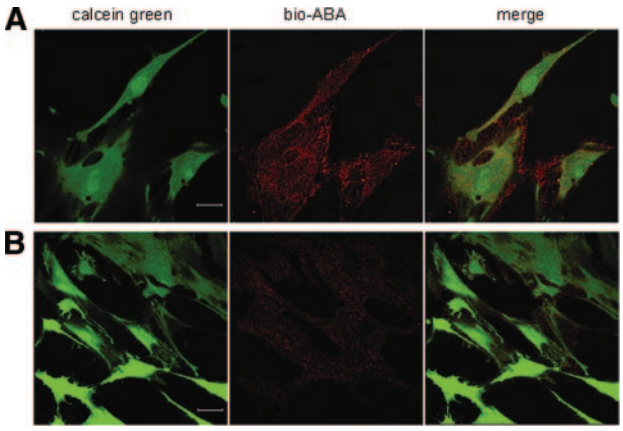

Figure 5. Bio-ABA staining in human mesenchymal stem cells (MSC). Images of bio-ABA labeling on live MSC were obtained using a Leica TCS SL confocal microscope (×63 oil objective, ×2 digital zoom; Leica, Heerbrugg, Switzerland, http://www.leica.com). The cell cytoplasm was stained with calcein green (green fluorescence); bio-ABA binding, revealed by Strp-Alexa 633 (red fluorescence), was performed in the absence **(A)** or in the presence **(B)** of unlabeled ABA. Scale bars = 20 μm. Abbreviation: bio-ABA, biotinylated abscisic acid.

Moreover, the $[ABA]_i$ increased in MSC cultured in the presence of the MSC-specific growth factor BMP-7 (at 0.2 μg/ml) by 1.6-, 6.0-, and 2.3-fold relative to untreated controls after 6, 24, and 48 hours of exposure, respectively (Fig. 6A).

The well-known immunomodulatory functions of MSC in the BM environment suggested exploration of whether allogeneic PBMNC could stimulate ABA synthesis in MSC. Indeed, culture of MSC in PBMNC-conditioned medium stimulated ABA release by MSC in a time-dependent manner. As shown in Figure 6B, a marked increase of the ABA concentration in the medium ($[ABA]_m$) was observed after 24 hours (4.7-fold compared with levels at the onset of culture, i.e., time = 0), followed by a decrease of the $[ABA]_m$ to near time = 0 values after 48 hours. Conversely, the $[ABA]_i$ was not modified throughout the culture (not shown). Thus, MSC stimulation by PBMNC induces release of ABA from MSC. A cytokine array performed on the PBMNC-conditioned medium revealed that these cells secrete TNF-α, M-CSF, IL-8, and RANTES (not shown). The effect of each of these cytokines on ABA release by MSC was investigated. M-CSF did not stimulate ABA production and release compared with untreated MSC at all time points tested (6, 24, and 48 hours; not shown). Conversely, TNF-α, RANTES, and IL-8 enhanced the $[ABA]_m$ as early as 6 hours of incubation (2.8-, 2.7-, and 2.8-fold, respectively, relative to control cells; Fig. 6C). At 24 hours, a further $[ABA]_m$ increase was observed in all samples (4.1-, 3.7-, and 5.5-fold increase in TNF-α-, RANTES-, and IL-8-stimulated cells, respectively), compared with ABA release in untreated cells at the same time point. Finally, at 48 hours all $[ABA]_m$ values were similar to those measured in unstimulated cells.

DISCUSSION

ABA is a phytohormone involved in the control of several physiological and developmental processes in higher plants [30–32]. In 1986, two papers raised the possibility that ABA might be a universal signaling molecule [33, 34]. Indeed, we recently demonstrated that ABA is produced and released by activated human granulocytes, which also respond to this hormone with several functional activities triggered by the $[Ca^{2+}]_i$ increase [18].

Results presented here indicate that the role of ABA in humans is not limited to the autocrine activation of mature granulocytes. Proinflammatory stimuli also induce ABA production in human MSC (Fig. 6); moreover, ABA stimulates MSC proliferation, without affecting the differentiation properties of MSC (Fig. 1 and supplemental online Fig. 1, respectively) and without affecting several functional activities of MSC, including release of PGE_2 and of a number of cytokines and stimulation of cell migration. It has been recently reported that exogenous ABA improves glucose tolerance in a mouse model of type II diabetes and activates the peroxisome proliferator-activated receptor γ transcription factor in murine white adipose tissue (WAT), promoting the differentiation of new adipocytes [35]. In our experiments, no increase of adipogenic differentiation was observed in ABA-treated MSC compared with controls (supplemental online Fig. 1B vs. 1A); the discrepancy could be due to a different reactivity of BM-resident MSC compared with WAT-resident MSC, or the enhanced adipogenic differentiation observed in ABA-fed mice could affect a population of progenitors more committed toward the adipocytic lineage than MSC [7].

The signaling pathway triggered by ABA in MSC is similar to the one described in granulocytes: the $[Ca^{2+}]_i$ increase induced by ABA on MSC (Fig. 3) is largely prevented by PTX, by removal of extracellular Ca^{2+}, by the cADPR antagonist 8-Br-cADPR, and by specific CD38 siRNA. 8-Br-cADPR also prevents the proliferative effect of ABA on MSC (Fig. 1), demonstrating a causal relationship between cADPR-induced $[Ca^{2+}]_i$ increase and proliferative effects of ABA. Inhibition of the ABA-induced $[Ca^{2+}]_i$ increase by preincubation with PTX indicates the involvement of a G protein-coupled receptor on the plasma membrane of MSC. The expression of a membrane receptor on MSC is supported by the presence of specific ABA-binding sites on the plasma membrane of these cells (Fig. 5). Through activation of a PTX-inhibitable G-protein, ABA triggers a rapid intracellular cAMP rise in MSC (supplemental online Fig. 2), which is apparently responsible for the subsequent increase of ADPRC (supplemental online Table 1), as already observed in human granulocytes [18]. The following results support the conclusion that cADPR is the second messenger of ABA in MSC: (a) 8-Br-cADPR and/or CD38 silencing strongly inhibits or abrogates the functional effects induced by ABA (Figs. 1, 4), and (b) extracellularly added cADPR induces functional effects similar to those triggered by ABA (Figs. 1, 4).

Extracellular cADPR elicits a delayed $[Ca^{2+}]_i$ increase in MSC, probably by means of equilibrative and concentrative transport systems widely expressed on most human cell types [12, 13, 36, 37], which enable cADPR to target and activate the cADPR-sensitive intracellular RyR channels. Indeed, qPCR experiments demonstrated for the first time expression of RyR1 and RyR3 in human MSC (Fig. 2D), in contrast with a previous study that reported the absence of RyRs in MSC, although on the basis of the less sensitive PCR technique [38]. Since the established targets of cADPR on the ER are the RyR2 and RyR3 isoforms [27], the latter is likely to be the one activated in human MSC by the ABA-triggered $[cADPR]_i$ rise.

ADPRC activity, which is responsible for autocrine cADPR production, was enhanced after both a brief (5-minute) and a long-term (24-hour) ABA stimulation (supplemental online Table 1). This enzymatic activity was previously held to be expressed only by the BST-1 (CD157) ectocyclase on the surface of MSC [12]. Here we demonstrated that almost all of the ADPRC activity in human MSC is in fact due to low-level expression of the CD38 ectocyclase (Fig. 2A, 2B), whose specific inhibition, by means of

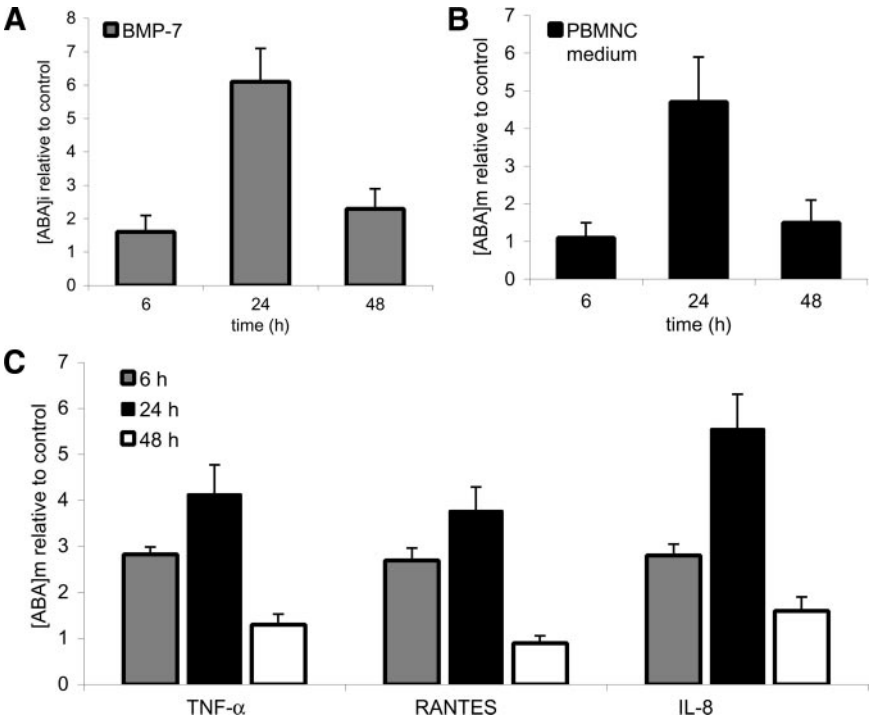

Figure 6. Detection of ABA in human mesenchymal stem cells (MSC). ABA concentrations in MSC culture media and in the cells were measured by high-performance liquid chromatography-coupled mass spectrometry or by ELISA. Results are expressed as means ± SD of three independent experiments performed in quadruplicate. **(A):** Intracellular ABA in BMP-7-stimulated MSC, relative to control cells. **(B):** ABA released in the medium by MSC cultured with PBMNC-conditioned medium, relative to time 0. **(C):** ABA release in TNF-α-, RANTES-, and IL-8-stimulated MSC, relative to control cells. Abbreviations: ABA, abscisic acid; [ABA]m, abscisic acid concentration in the medium; BMP, bone morphogenetic protein; h, hours; IL, interleukin; PBMNC, peripheral blood mononuclear cells; TNF, tumor necrosis factor.

siRNA experiments, led to almost complete abrogation of ADPRC activity (supplemental online Table 1) and to a significant reduction of the $[Ca^{2+}]_i$ rise elicited by ABA (Fig. 3E). Furthermore, we demonstrated that expression of CD38 is upregulated by ABA treatment at 24 hours (Fig. 2A), unlike expression of BST-1, which remains unchanged (Fig. 2C). Upregulation of CD38 expression by ABA also explains the long-term (24-hour) increase of ADPRC activity observed following ABA treatment of MSC (supplemental online Table 1).

The functional responses elicited in human MSC by ABA and by its second messenger cADPR include an increase in the number of CFU-F in freshly isolated MSC (Fig. 1A), an enhanced proliferation rate at subsequent passages (Fig. 1B), stimulation of chemokine/cytokine production, and stimulation of cell migration. The long-term effects of ABA and cADPR on MSC proliferation raise the question of whether these functional effects could be due to metabolites of ABA or cADPR generated during the several days of incubation of the cells. ABA proved to be stable in the cell culture supernatant for several days. Conversely, cADPR was partially hydrolyzed to ADP-ribose (ADPR) in 24 hours by MSC, as verified by HPLC (analysis described in [39]). cADPR hydrolysis under conditions similar to those used in the proliferation assay (i.e., 3×10^3 MSC in 200 μl of medium containing 10 μM cADPR) accounted for an extracellular ADPR concentration of 35 nM after 24 hours. Concentrations of ADPR up to 100 μM did not stimulate MSC proliferation after 3 and 6 days of incubation and did not increase the CFU-F output in the clonogenic assay (not shown).

Incubation with 10 μM ABA or cADPR for 24 hours also stimulated the release of several cytokines from human MSC (supplemental online Table 3): (a) IL-6, IL-8, and oncostatin-M, which stimulates HSC self-renewal [40–42]; (b) IL-6, IL-8, and IL-10, which are responsible for the immunomodulatory activity of MSC [28, 43]; and (c) vascular endothelial growth factor, IL-6, IL-8, RANTES, macrophage inflammatory protein-1-delta (MIP-1-delta) and growth regulated oncogene (GRO), which are involved in cell mobilization [44–46]. The stimulatory effect of ABA and cADPR on MSC chemokinesis and the less pronounced effect on chemotaxis (Fig. 4D, 4E) may be due in part to release by MSC of the above-mentioned cytokines [45]. ABA stimulates chemokinesis more than cADPR (at 100 nM, the chemokinesis index (CKI) was 4.8 for ABA and 1.5 for cADPR; Fig. 4D, 4E). The fact that 8-Br-cADPR only partially inhibits the ABA-induced stimulation of chemokinesis, whereas CD38 silencing abrogates the effect, suggests that other CD38 products (e.g., $NAADP^+$ [47], Ap2A and its isomers [39]) may be responsible, along with cADPR, for stimulation of untargeted cell migration. The marked stimulation of chemokinesis by ABA may reduce the number of cells migrating across the filter in the chemotaxis assay by increasing cell movement in all directions. Conversely, cADPR stimulates chemokinesis to a lesser extent than ABA, and this could be the reason that the chemotactic effect of the cyclic nucleotide is more evident (Fig. 4D, 4E).

Both ABA and cADPR induce a significant overexpression of COX-2 mRNA and protein (Fig. 4A, 4B) and an increase of PGE_2 release, which is prevented by the specific COX-2 inhibitor NS398 and, in ABA-stimulated MSC, also by the cADPR antagonist 8-Br-cADPR and by CD38 mRNA-silencing (Fig. 4C). PGE_2 release by MSC is believed to affect both fundamental functions of MSC in the hemopoietic niche, namely immunomodulation [28] and trophic support of hemopoiesis [5].

CONCLUSION

Together, the present data suggest that ABA may represent a new autocrine and paracrine signal molecule in the BM environment, targeting both MSC and HSC, possibly regulating the response of the hemopoietic niche to alarm inflammatory signals originating from other districts of the organism. Stress signals such as TNF-α, RANTES, and IL-8, released by PBMNC, stimulate MSC to release ABA (Fig. 6C), which in turn induces a spectrum of functional effects in MSC, including (a) the release of cytokines stimulating he-

mopoiesis [5, 40, 41]; (b) the production of immunomodulatory chemokines and cytokines, downregulating lymphocyte activation and its adverse effects on hemopoiesis; and (c) the stimulation of MSC mobilization from the BM. Indeed, MSC migration from the BM into damaged tissues has been widely reported [45, 48, 49], and recruitment of MSC at sites of inflammation is believed to contribute to tissue repair [45, 50]; thus, ABA could be a key signal leading to MSC mobilization and activation.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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