Nitric Oxide and ERK mediates regulation of cellular processes by Ecdysterone

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ABSTRACT

The complex process of wound healing is a major problem associated with diabetes, venous or arterial disease, old age and infection. A wide range of pharmacological effects including anabolic, anti-diabetic and hepato-protective activities have been attributed to Ecdysterone. In earlier studies, Ecdysterone has been shown to modulate eNOS and iNOS expression in diabetic animals and activate osteogenic differentiation through the Extracellular-signal-Regulated Kinase (ERK) pathway in periodontal ligament stem cells. However, in the wound healing process, Ecdysterone has only been shown to enhance granulation tissue formation in rabbits. There have been no studies to date, which elucidate the molecular mechanism underlying the complex cellular process involved in wound healing. The present study, demonstrates a novel interaction between the phytosteroid Ecdysterone and Nitric Oxide Synthase (NOS), in an Epidermal Growth Factor Receptor (EGFR)-dependent manner, thereby promoting cell proliferation, cell spreading and cell migration. These observations were further supported by the 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF FM) fluorescence assay which indicated that Ecdysterone activates NOS resulting in increased Nitric Oxide (NO) production. Additionally, studies with inhibitors of both the EGFR and ERK, demonstrated that Ecdysterone activates NOS through modulation of EGFR and ERK. These results clearly demonstrate, for the first time, that Ecdysterone enhances Nitric Oxide production and modulates complex cellular processes by activating ERK1/2 through the EGF pathway.

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1. Introduction

The complex cellular processes underlying wound healing are intricate and enables skin or tissue to repair itself after injury. In normal skin, the steady state equilibrium which exists between the epidermis, the outermost layer and the dermis or inner layer, provides a barrier against the external environment. The abnormal interruption of this fragile balance leads to problems associated with non-healing chronic wounds. Abrupt wound healing is associated with several disease states like diabetes, venous or arterial disease, infection and old age [1].

The different phases of the wound healing process include hemostasis, inflammation, re-epithelialization, granulation tissue formation and tissue remodeling, all of which though functionally distinct, are seen to be overlapping in several stages and are carefully orchestrated by the signaling of various cell types [2]. Both macrophages and fibroblasts act as stimulators for these different cell types by producing various signaling factors like Platelet Derived Growth Factor (PDGF), Transforming Growth Factor Beta (TGF-β), Fibroblast Growth Factor (FGF), Nitric Oxide (NO), Cytokines and Matrix Metalloproteinases (MMPs) [1,3]. Among the various signaling molecules, NO has several beneficial effects on wound repair since it functions as a key regulator in different phases of the wound healing process including inflammation, angiogenesis, cell proliferation and matrix remodeling.

NO is produced by the enzyme Nitric Oxide Synthase (NOS) which converts L-arginine to L-citrulline. It is a small diffusible intercellular molecule which has an important role in the wound healing process [4]. There are three different known isomers of NOS: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) [5]. iNOS is an inducible enzyme and was initially discovered in macrophages [4] while eNOS and nNOS are constitutive enzymes that are predominantly present in endothelial
cells and neurons respectively. All three isoforms eNOS, iNOS and nNOS are regulated by different signaling pathways. Among them, the Epidermal Growth Factor Receptor (EGFR) signaling cascade plays a significant role in many important functions of wound healing like cell proliferation, survival and migration [6–8], besides playing a major role in the defensive response during wound healing and chronic inflammatory skin disorders [9]. Heparin-binding EGF-like growth factor (HB-EGF) is one of the epidermal growth factor type ligands which interacts with EGF receptors and up-regulates eNOS expression leading to the increase in NO production, which occurs through the activation of Phosphoinositide 3-kinase (PI3K) pathway. The NO released helps in cell migration and angiogenesis [10]. Hepatocyte Growth Factor (HGF) is yet another tyrosine kinase receptor ligand which also up-regulates the eNOS expression and NO synthesis. The phosphorylation of eNOS which occurs as a result of HGF signaling is mediated by crosstalk between Mitogen-activated protein kinase (MAPK) and PI3K pathway [11].

Ecdysterone is an insect molting hormone which regulates molting, metamorphosis and reproduction in Arthropods. Ecdysteroid, (also known as phyoecdysones), are also present in 5–6% of plant species, like Sesuvium portulacastrum, contributing to their protection from invertebrate predators [12]. Ecdysteroids are structurally different from mammalian steroids and has been reported to have cell proliferation activity in mammals. Recently, Ecdysterone has been reported to alter NO production by NOS [13], in addition to having a wide range of pharmacological properties including adaptogenic, anabolic, anti-diabetic, hepatoprotective and wound healing actions [14–18]. Studies have shown that Ecdysterone enhances wound healing in rabbits by inducing faster granulation tissue formation and epithelial cell proliferation [19].

The present study demonstrates that Ecdysterone activates ERK phosphorylation and promotes cell proliferation, cell spreading and cell migration. Our findings also indicate for the first time a novel link between Ecdysterone mediated NO production and EGFR-ERK signaling pathway and provides a better understanding of the various complex cellular processes mediated by Ecdysterone.

2. Materials and methods

2.1. Chemicals and reagents

All cell culture media and supplements were obtained from Sigma (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA). 3T3-L1 mouse fibroblast cells and RAW 264.7 mouse macrophage cells were obtained from the Indian National Center for Cell Sciences, Pune, Maharashtra, India. Primary antibody against ERK, phosphorylated ERK, Actin and secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Enhanced chemiluminescence (ECL) kit was purchased from Thermo Scientific Pierce.

2.2. Isolation, characterization and purification of Ecdysterone from Sesuvium portulacastrum

Ecdysterone was isolated and characterized from Sesuvium portulacastrum. The entire plant was dried, crushed and extracted using soxhlet apparatus with chloroform (CHCl₃) followed by ethyl methyl ketone (MEK). The MEK extract, which was subsequently concentrated to dryness, resulted in a sticky material. Column chromatography (Alumina) was used for further purification of MEK extract. The characterization of the fractions obtained was performed using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and compared with commercially available standard compound.

2.3. High performance liquid chromatography

HPLC was performed with a Shimadzu LC-20 instrument, employing a Phenomenex C-18 reverse-phase Luna column and a Prominence diode array detector for analysis. Methanol: water (1:1) was used as the mobile phase at 1 mL/min and absorbance was monitored at 254 nm. Each analysis was carried out by dissolving 1 mg of sample in 1 mL of methanol, and injecting a 20 μL sample.

2.4. Preparation of Ecdysterone stock solutions

5 mg/mL (~10 mM) solution of Ecdysterone was prepared in Sterile PBS (pH 7.2).

2.5. Cell culture

3T3-L1 mouse fibroblast cells and RAW 264.7 mouse macrophage cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (v/v), 1% penicillin, 1% streptomycin and 0.1% Amphotericin B.

2.6. In vitro scratch assay

In order to study the role of Ecdysterone on cell migration, in vitro scratch assay was used [20]. ~1.5 × 10⁶ 3T3-L1 fibroblast cells were suspended in 1 mL DMEM containing 10% FBS (complete growth medium) were seeded in 12 well plates. Following incubation at 37 °C for 24 h upon reaching ~90% confluence, a linear wound was created by scratching the monolayer with a 200 μL micropipette tip. In order to obtain the same field during the image acquisition, reference points were made by a marker on the exterior of the plate. The cells were washed once with 500 μL of the growth medium or PBS to remove any debris and treated with different concentrations of Ecdysterone in 2% serum containing media. Images were captured under an inverted bright field microscope with 4× magnification by leaving the reference mark outside the capture image field but within the eye-piece field of view (0 h image). Subsequently, the plate was incubated for 24 h at 37 °C, following which, the images were acquired using Image Pro-Plus software (Media Cybernetics).

2.7. Cell spreading assay

To elucidate the role of Ecdysterone on cell migration, cell spreading assay was used [21]. For this, the 3T3L1 cells were pretreated with Ecdysterone for 24 h, collected by centrifugation, and washed with PBS followed by serum-free DMEM. The cells were then treated with Ecdysterone (50, 75 and 100 μM) and incubated in 2% serum media in 15 mL falcon tube for 1 h at 37 °C in an orbital shaker before seeding onto cover slips. The cells were incubated in a CO₂ incubator at 37 °C for 20 min after which they were washed once with PBS and fixed in 4% paraformaldehyde (PFA). The cells were then permeabilized and stained for actin using Alexa Fluor 488 Phalloidin (Molecular Probes). 15 random fields were imaged for each cover slip, with the Zeiss Fluorescence field microscope with 4× magnification by leaving the reference mark outside the capture image field but within the eye-piece field of view (0 h image). Subsequently, the plate was incubated for 24 h at 37 °C, following which, the images were acquired using Image Pro-Plus software (Media Cybernetics).

2.8. Cell migration assay

To further confirm cell spreading, cell culture inserts containing porous translucent polyethylene terephthalate track-etched membranes (8.0 μm pore size; Becton Dickinson Falcon) were utilized [22]. The inserts were placed in a new 24-well plate
containing 700 µL of 2% serum containing media in the presence or absence of 100 µM Ecdysterone. 1 × 10^4 3T3-L1 viable cells were seeded into the upper chamber of the cell culture insert in 100 µL of 2% serum containing media. Cells were then incubated for 24 h at 37 °C to allow for adhesion and migration through the porous membrane on the underside of the cell culture insert. After incubation, the media was aspirated and the inserts were washed twice with PBS. The cells on the upper side of the cell culture insert were removed with a cotton swab. The underside of the membrane containing the migrated cells was washed twice with PBS and fixed with 4% PFA for 10 min at room temperature (RT). Following two washes with PBS, the migrated cells were permeabilized with 100% Methanol for 10 min at RT and stained with 1% Crystal Violet (Sigma, USA) for 10 min at RT. The cell culture inserts were then allowed to air dry upside down for 15 min at RT and the migrated cells on the underside of the membrane were visualized using phase contrast microscopy at 4X magnification.

2.9. MTT assay

Cell viability was assessed by MTT (3-(4,5-dimethyl-2-thiazoly)-2, 5-diphenyl-2H-tetrazolium bromide) assay, which were performed on control as well as Ecdysterone treated cells in serum free DMEM [23]. 3T3-L1 cells were seeded at a density of 7500 cells/well in a 96 well microtiter plate and incubated overnight at 37 °C. Cells were treated with Ecdysterone (at a concentration range of 20–100 µM) in serum-free DMEM in the presence or absence of 2 mM L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) for 24 h following which 20 µL of 5 mg/mL MTT was added to each well and incubated for 3 h at 37 °C. The media was aspirated after incubation and 150 µL of MTT solvent (4 mM HCl, 0.1% NP-40 in isopropanol) was added for solubilization followed by incubation at room temperature with gentle shaking for 5 min. The absorbance was read at 590 nm with a reference filter of 620 nm using Synergy HT Multi-Mode Microplate Reader (BioTek).

2.10. Determination of NO from fibroblast

To confirm the role of Ecdysterone on NO production, DAF FM fluorescence assay was performed [24]. 3T3-L1 fibroblasts were plated in a 96-well microplate (1 x 10^5 cells/well), for 6 h to ensure that the cells adhere to the wells. The cells were subsequently cultured for 16 h in 200 µL of fresh medium and washed twice with PBS, followed by addition of 10 µM DAF-FM, 10 µM PD98059, 1 mM L-arginine, and an appropriate concentration of Ecdysterone dissolved in 200 µL of PBS. After incubation for 2 h, the supernatants were transferred to black microplates and the fluorescence was measured with a fluorescence microplate reader (Synergy HT Multi-Mode Microplate Reader (BioTek)) calibrated for excitation at 485 nm and emission at 538 nm.

2.11. Griess assay

Griess assay was performed in order to study the role of ERK/MEK pathway in Ecdysterone mediated NO production [25]. 3T3-L1 fibroblasts were seeded in serum containing DMEM in 24 well plates and incubated at 37 °C for 24 h. When the cells reached > 90% confluency, scratch assay was performed with different concentrations of Ecdysterone in the presence or absence of Tyrphostin (AG1478, EGFR inhibitor) or PD98059 (MEK inhibitor) as required. The concentration of nitrates in the cell supernatant was taken as a measure of NO production, which was determined by the Griess assay. A nitrite calibration curve was used to convert the absorbance to µM nitrite.

2.12. Protein phosphorylation study

To further confirm the effect of Ecdysterone on ERK pathway, phosphorylation studies were performed. 3T3-L1 fibroblasts or RAW 264.7 macrophage cells were seeded in 6 cm plates. After incubation at 37 °C for 24 h, the cells were treated with Ecdysterone at different concentrations (5–200 µM) for 30 min. The cells were then washed twice with ice-cold PBS and lysed in 1X lysis buffer (20 mMTris pH-7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na3VO4) and 1 µg/mL leupeptin) for 5 min. The cells were scraped, briefly sonicated for 5 s and centrifuged at 14,000 g for 10 min to remove the cell debris. Protein concentration was estimated by Bradford assay and the protein was resolved using 10% SDS polyacrylamide gel, transferred to PVDF membrane, blocked with 5% (w/v) non-fat dried milk in a 10 mM phosphate buffer containing 150 mM NaCl (pH 7.4) and 0.05% Tween 20 for 1 h at room temperature and then incubated with respective antibodies. The bands were visualized using ECL kit from Thermo Scientific Pierce.

2.13. Statistical analysis

Statistical analysis was conducted using Prism (GraphPad Software). Statistical comparisons were performed using student's t-test, one-way analysis of variance followed by Dunnett’s or Tukey’s test. A value of p < 0.05 was considered significant. All the values are expressed as the mean ± S.E.M. of triplicate determinations from three independent experiments.

3. Results

3.1. Isolation of Ecdysterone from Sesuvium portulacastrum

Ecdysterone was isolated from Sesuvium portulacastrum using a sequential extraction process with CHCl3 followed by MEK. The MEK extract was further purified using alumina column chromatography. The different fractions were eluted using varying proportions of CHCl3 and MeOH, resulting in bioactive fraction, F1 [MeOH:CHCl3 (20:80)], which on removal of solvent yielded a white powder (Fig. 1). This was further characterized using TLC and HPLC.

3.2. Characterization and identification of Ecdysterone

Thin-layer chromatography (TLC) analysis of F1 fraction was carried out using a solvent system [MeOH: Ethyl Acetate (15:85)]. The analysis showed a UV sensitive band, which on spraying with vanillin-HCl reagent and heating gave a green spot, similar to standard Ecdysterone. The identity of Ecdysterone was established employing HPLC, with the absorbance being monitored at 254 nm (Fig. 2), which was further confirmed by NMR and Mass spectrometry analysis (data not shown).

**Fig. 1.** Schematic representation of the extraction procedure.
3.3. Effect of Ecdysterone on cell migration

An in vitro scratch assay was employed to study the effect of Ecdysterone on cell migration. A linear scratch was created on a monolayer of 3T3L1 cells and treated with or without Ecdysterone at different concentrations (25, 50 and 100 μM). Ecdysterone significantly enhanced cell migration in a dose dependent manner when compared to the control (Fig. 3).

3.4. Effect of Ecdysterone on cell spreading

Cell spreading assay, which reflects a measure of initiation of cell migration, was performed to determine if Ecdysterone influenced this process during wound healing. The results of the cell spreading assay demonstrate that Ecdysterone enhances the filopodia formation (Green protrusion- Alexa Fluor 488 Phalloidin stain) in a dose dependent manner, leading to enhanced cell spreading. The results of cell spreading assay clearly demonstrated that Ecdysterone enhanced the actin cytoskeletal rearrangement, as seen by the expansion in cell spreading in a dose-dependent manner (Fig. 4) suggesting that Ecdysterone played a role in the initiation of cell migration.

3.5. Effect of Ecdysterone on cell migration using transwell migration assay

The transwell migration assay was used to confirm the role of Ecdysterone in cell migration. The results of cell migration using transwell chamber assay further confirmed that Ecdysterone enhanced cell migration at a concentration of 100 μM, (Fig. 5) implicating the importance of Ecdysterone in cell migration.

3.6. Role of NO in Ecdysterone mediated cell proliferation

The MTT analysis established that Ecdysterone promotes cell proliferation in a dose-dependent manner (Fig. 6) suggesting that in addition to initiation of cell migration, Ecdysterone also plays a critical role in cell proliferation.
NO is known to play a role in enhancing cell migration and proliferation. Sodium Nitroprusside (SNP), a known NO donor was used along with L-NG-Nitroarginine methyl ester (LNAME), a specific inhibitor of NOS in this assay. As shown in Fig. 6, addition of SNP along with Ecdysterone further enhanced cell proliferation implicating NO in this process. This was further confirmed by the addition of L-NAME which resulted in a decrease in cell proliferation because of NOS inhibition at lower concentrations of Ecdysterone (20, 40 and 80 μM). However as observed in Fig. 6, the inhibition by L-NAME was overcome by Ecdysterone (100 and 200 μM), clearly indicating the role of NO in Ecdysterone mediated cell proliferation.

3.7. Effect of Ecdysterone on NO production

DAF-FM diacetate fluorescence assay, a highly sensitive and direct method of NO detection was also employed to confirm the presence of NO. DAF-FM diacetate, a cell-permeable, non-fluorescent compound, reacts with NO intracellularly to form a fluorescent Benzotriazole. 3T3L1 cells were treated with Ecdysterone at increasing concentrations, in the presence of DAF-FM for 2 h and the cell supernatant from treated and control (untreated) cells was collected for these studies. Controls for these studies showed a distinct 5 fold increase in NO (Fig. 7 inset bar B) over basal (Fig. 7 inset bar A), upon incubating DAF-FM diacetate with cells. As shown in Fig. 7, the NO production of Ecdysterone treated cells was significantly enhanced in a dose-dependent manner when compared to untreated control, indicating that Ecdysterone enhanced NO production in a dose-dependent manner. Additionally, the level of NO production in the presence of Ecdysterone at a concentration of 100 μM is equivalent to 1 mM Arginine, which serves as a substrate for NOS suggesting that Ecdysterone results in the enhancement of NO production (Supplementary Fig. 1). Furthermore, DAF FM studies with L-NAME a well-known NOS inhibitor along with Ecdysterone implicated that the decrease in NO production observed in the presence of L-NAME (2 mM) was restored back with Ecdysterone (100 μM) confirming the role of Ecdysterone in NO production via activation of NOS (Supplementary Fig. 2).

3.8. Effect of EGFR signaling in Ecdysterone mediated NO production

Inhibitor studies were performed to determine whether EGFR signaling is involved in Ecdysterone mediated NO production. Griess assay was carried out with or without Tyrophostin AG1478, a known specific pharmacologic inhibitor of EGFR. The nitrite content was significantly reduced in the presence AG1478, implicating an EGFR dependent pathway for Ecdysterone mediated NO production.
3.9. Involvement and activation of ERK in Ecdysterone mediated NO production

To determine downstream signaling events activated by Ecdysterone, the involvement of MEK/ERK in Ecdysterone mediated NO production was characterized. PD98059, which is a specific inhibitor of MEK1 and MEK2 was used for this purpose. As shown in Fig. 9, NO production induced by Ecdysterone was markedly inhibited when cells were treated with PD98059, suggesting the importance of MEK signaling in Ecdysterone mediated NO production.

Additionally, western blot studies also indicated that the level of phosphorylated ERK1/2 was significantly increased after production (Fig. 8).
treatment with Ecdysterone, in a dose dependent manner in both 3T3L1 and RAW 264.1 cell lines (Fig. 10). These results clearly underline the importance of ERK1/2 phosphorylation in the Ecdysterone mediated generation of NO.

4. Discussion

Wounds result from the disruption of the anatomical structure and function of a tissue and the healing of wounds generally occur in an orderly manner, wherein various phases like inflammation, tissue formation and tissue remodeling are maintained and regulated. Although several traditional remedies have indicated that a large number of plant extracts have potential wound healing properties, only a few studies have investigated their exact mechanism of action, identified individual molecules, and provided insight into the mechanisms responsible for their wound healing activity. This study was therefore primarily focused on dissecting the molecular mechanism underlying the wound healing properties exhibited by Ecdysterone, a phytosteroid that was isolated and characterized from a common weed Sesuvium portulacastrum.

In the present study, 3T3L1 fibroblasts treated with purified Ecdysterone, showed enhancement in cell migration, in a time and dose dependent manner. In addition to cell migration, Ecdysterone also enhanced cell spreading, which is representative of initiation of cell migration in a dose dependent manner. Cell migration plays an important role in wound healing in a variety of cell types including endothelial cells, epithelial cells, keratinocytes and fibroblasts [26–31]. The cell migration process is closely linked with the cytoskeletal dynamics including the actin polymerization and actin-myosin interaction. Filopodial membrane protrusions are also essential for cell migration and are formed as a result of actin polymerization and cytosiskeletal rearrangement. Since cytoskeletal modifications are required for cell migration [32], Ecdysterone could be essential for the tissue repair process by enhancing the cytoskeletal rearrangement. In addition to cell migration, cell proliferation also plays a significant role in tissue repair. Cytokines, NO and several growth factors including Vascular endothelial growth factor (VEGF) and EGF are known to enhance cell proliferation and thereby facilitate the wound healing process. Our studies with Ecdysterone demonstrated a dose-dependent increase in cell proliferation (Fig. 6), suggesting that in addition to cell spreading and cell migration, cell proliferation is also clearly influenced by Ecdysterone.

Since NO plays an important role in wound healing by regulating cell proliferation and migration [33], further studies were carried out to understand the role of NO in Ecdysterone mediated cellular processes. The iNOS mediated release of NO that occurs during the early phases of wound healing, regulates various processes like collagen formation and cell proliferation [34]. In chronic ulcers like diabetic wounds, the level of NO is significantly reduced because of lower expression of iNOS and eNOS [4,35]. Studies using different NO activators like L-arginine [36] and vitamin B12 [37] resulted in enhanced wound healing. The decrease in cell viability observed in the presence of L-NAME and SNP at lower concentrations of Ecdysterone was restored back at 100 μM Ecdysterone, confirming the role of Nitric Oxide in Ecdysterone mediated enhanced cell proliferation. These observations were further confirmed by a dose dependent increase in NO production in DAF FM fluorescence assay, indicating an interaction of Ecdysterone with NOS resulting in the enhancement of NO production. Moreover, the level of NO produced by 100 μM Ecdysterone was equivalent to that obtained from Arginine, the natural substrate of NOS. Additionally, the decrease in NO production observed with LNAME, a specific inhibitor of NOS, was reversed by addition of Ecdysterone, thereby establishing an interaction of Ecdysterone with NOS.

Furthermore, a similar pattern was also observed in mRNA expression studies that demonstrated a time dependent increase in iNOS and eNOS expression [Supplementary Fig. 3]. Thus, both the activity as well as the expression studies clearly demonstrated that NO plays a critical role in Ecdysterone mediated regulation of cellular processes.

Among the different signal transduction cascades, the EGFR-ERK pathway has a significant role in wound healing [38]. The present study demonstrated that the Ecdysterone mediated NO production involves the EGFR pathway. Additionally, Ecdysterone was also shown to phosphorylate and activate ERK1/2, resulting in increased NO production, which is crucial for the regulation of various cellular processes like cell proliferation, migration, differentiation and actin remodeling, all of which are essential for the wound healing process.

5. Conclusion

In summary, the present study, demonstrates the role of NO in ecdysterone mediated cellular processes. The Ecdysterone mediated increase in cell spreading could be due to cytoskeletal rearrangement, resulting in improved cell migration. Additionally, the enhancement in cell proliferation was also shown to be NO dependent, as indicated by the inhibitor studies, thereby establishing a novel link between Ecdysterone mediated NO production and EGFR-ERK signaling pathway. The proposed pathway for Ecdysterone mediated regulation of cellular processes is shown in Fig. 11. Furthermore, the present study can also be used as a basis for utilizing Ecdysterone as a suitable template for the design of new drug candidates that target therapeutic strategies focused on cellular processes mediated by EGFR-ERK1/2-NO signaling.

Funding

This work was supported by Institutional Funding (Amrita University Research) grant ASBT/2013/03 (BGN) and the Council of Scientific and Industrial Research – 09/942(0005)/2010-EMR-1 (AO).
Con the wound healing process.

Fig. 11. Proposed pathway for the regulation of cellular processes by Ecstasy in the wound healing process.

Authorship contributions

1. Participated in research design: Nair, Kumar and Omanakuttan.
2. Conducted experiments: Omanakuttan and Bose.
3. Contributed new reagents or analytic tools: Bose, Pandurangan and Baneri.
4. Performed data analysis: Omanakuttan, Kumar and Baneri.
5. Wrote or contributed to the writing of the manuscript: Omanakuttan, Kumar and Nair.
6. Wrote or contributed to the writing of the manuscript: Omanakuttan.
7. Performed data analysis: Omanakuttan, Kumar, Banerji and Nair.
8. Conducted experiments: Omanakuttan and Bose.
9. Contributed new reagents or analytic tools: Bose, Pandurangan and Baneri.
10. Participated in research design: Nair, Kumar and Omanakuttan.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

We acknowledge Mata Amritanandamayi Devi, Chancellor, Amrita Vishwa Vidyapeetham (Amrita University) for her constant support and guidance and for being the inspiration behind this study. We thank Dr. Jyotsna Nambiar (Amrita) for critical reading of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2016.07.019.

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